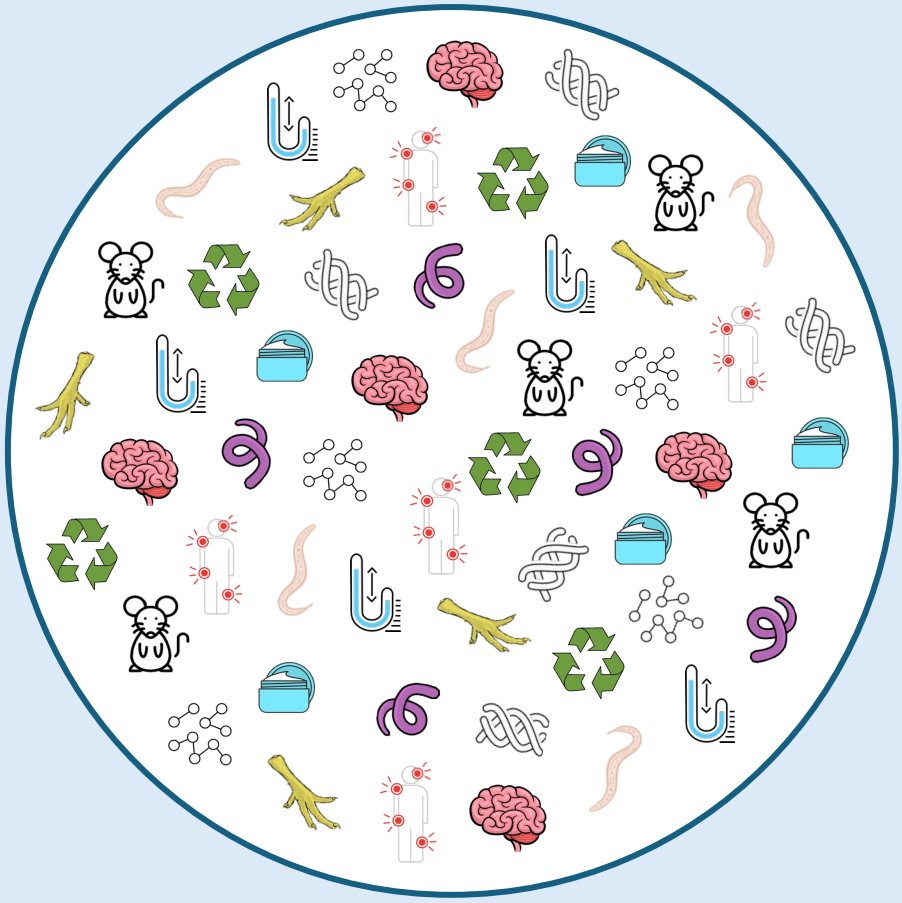
PROTEIN HYDROLYSATES AND PEPTIDES FROM ANIMAL BY-PRODUCTS AS A PREVENTIVE STRATEGY FOR LOCAL INFLAMMATION AND NEUROINFLAMMATION

Néstor Ibarz Blanch

Protein hydrolysates and peptides from animal by-products as a preventive strategy for local inflammation and neuroinflammation



NÉSTOR IBARZ BLANCH

DOCTORAL THESIS

UNIVERSITAT ROVIRA i VIRGILI

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Supervised by: Dr. Francisca Isabel Bravo Vázquez

Departamento de Bioquímica y Biotecnología

Grupo de Investigación en Nutrigenómica

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Departament de Bioquímica
i Biotecnologia

FAIG CONSTAR que aquest treball, titulat "**Protein hydrolysates and peptides from animal by-products as a preventive strategy for local inflammation and neuroinflammation**", que presenta Néstor Ibarz Blanch per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament de Bioquímica i Biotecnologia de la Universitat Rovira i Virgili i que compleix els requisits per a l'obtenció de la Menció Internacional de Doctorat.

HAGO CONSTAR que el presente trabajo, titulado "**Protein hydrolysates and peptides from animal by-products as a preventive strategy for local inflammation and neuroinflammation**", que presenta Néstor Ibarz Blanch para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Departamento de Bioquímica y Biotecnología de la Universitat Rovira i Virgili y que cumple con los requisitos para la obtención de la Mención Internacional de Doctorado.

I STATE that the present study, entitled "**Protein hydrolysates and peptides from animal by-products as a preventive strategy for local inflammation and neuroinflammation**", presented by Néstor Ibarz Blanch for the award of the degree of Doctor, has been carried out under my supervision at the Department of Biochemistry and Biotechnology of the University Rovira i Virgili and that is eligible to apply for the International Doctoral Mention.

Tarragona, 8 de abril de 2024

La directora de la tesis doctoral
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puede ir a mejor. Quizá volvamos a coincidir en la empresa M, procesando las materias primas A, B y C… ¿Quién sabe?

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A mi yo de mañana: perdona, era necesario.

A mi familia;

a los míos;

a tí, que estás leyendo esto.

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"Lo importante es el camino; y en él,
caer, levantarse, insistir, aprender."

Txus di Fellatio (Mägo de Oz)

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ABSTRACT

Inflammation is a common factor in many pathologies such as arthritis and neurodegenerative disorders. Therefore, there is a trend towards the development of selective COX-2 inhibitors. In addition, neuroinflammation promotes the synthesis of enzymes such as prolyl oligopeptidase (POP), and inhibition of this enzyme has been proposed as a therapeutic target for the development of neuroprotective drugs for Alzheimer's disease (AD). In this regard, bioactive peptides obtained from food and agri-food by-products have emerged as good candidates as functional ingredients in the prevention of inflammatory-associated diseases. In this context, this doctoral thesis focused on developing protein hydrolysates from animal by-products with anti-inflammatory or neuroprotective properties and identifying their bioactive peptides. In the first part of the study, hydrolysate H8 from pigskin collagen showed the highest COX-2 inhibitory activity, which was selective for COX-2. The peptide fraction <10 kDa of H8 hydrolysate prevented localized acute inflammation in a carrageenan-induced paw edema model and exhibited antinociceptive capacity in an acute pain model after topical application. This peptide fraction reduced nitric oxide production, COX-2 activity, and COX-2 and inducible nitric oxide synthase protein expression in lipopolysaccharide-stimulated RAW 264.7 cells. Moreover, the anti-inflammatory peptides AGERGEQ and GSKGRPG were identified in the hydrolysate H8. In the second part of this thesis, twelve hydrolysates were obtained from an animal protein-rich by-product using different enzymatic hydrolysis conditions. The hydrolysates CFH3 and CFH5 showed good POP inhibitory activity and their neuroprotective effects were studied in *Caenorhabditis elegans* strain CL4176, a model of AD. CFH3 delayed paralysis induced by amyloid-ß aggregation in *C. elegans* in a dose-dependent manner, demonstrating its neuroprotective effects. Twenty-three novel peptides were identified in CFH3, nine of them with potential activity according to the PeptideRanker score.

The results obtained support the potential of these hydrolysates as preventive tools for localized inflammation and neuroprotection, as well as for promoting sustainability and circular economy in the agri-food sector. The developed hydrolysates and the identified peptides could be useful for food and pharmacological industries to develop anti-inflammatory and neuroprotective products.

RESUMEN

La inflamación es un factor común en muchas patologías. Los principales fármacos antiinflamatorios, los no esteroideos (AINE), actúan inhibiendo las ciclooxigenasas (COX). La inhibición prolongada de COX-1 se asocia con efectos secundarios por lo que se buscan inhibidores selectivos para COX-2. Además, la neuroinflamación promueve la síntesis de la enzima prolil oligopeptidasa (POP), y la inhibición de esta enzima se ha propuesto como una diana terapéutica para el desarrollo de fármacos neuroprotectores para la enfermedad de Alzheimer (EA). En este sentido, los péptidos bioactivos obtenidos de alimentos y subproductos agroalimentarios se han convertido en buenos candidatos para su uso como ingredientes funcionales en la prevención de enfermedades con un componente inflamatorio. En este contexto, esta tesis doctoral se centró en el desarrollo de hidrolizados de proteínas a partir de subproductos animales con propiedades antiinflamatorias o neuroprotectoras, y en la identificación de sus péptidos bioactivos. En la primera parte del estudio, el hidrolizado H8 obtenido de colágeno de piel de cerdo mostró la mayor actividad inhibidora de la COX-2, que fue selectiva para COX-2. La fracción peptídica <10 kDa de H8 previno la inflamación aguda localizada en un modelo de edema de pata inducido por carragenato y mostró capacidad antinociceptiva en un modelo de dolor agudo después de su aplicación tópica. Esta fracción redujo también la producción de óxido nítrico, la actividad COX-2 y la expresión proteica de COX-2 y óxido nítrico sintasa inducible en células RAW 264.7 estimuladas con lipopolisacárido. Además, se identificaron los péptidos antiinflamatorios AGERGEQ y GSKGRPG en H8. En la segunda parte de esta tesis, se obtuvieron doce hidrolizados de un subproducto animal utilizando diferentes condiciones de hidrólisis enzimática. Los hidrolizados CFH3 y CFH5 mostraron una buena actividad POP inhibitoria y sus efectos neuroprotectores se estudiaron en la cepa transgénica CL4176 de *Caenorhabditis elegans*, un modelo de EA. CFH3 retrasó la parálisis inducida por la agregación de ß-amiloide de manera dosis dependiente, demostrando sus efectos neuroprotectores. Se identificaron 23 péptidos nuevos en CFH3, 9 de ellos con potencial actividad según la puntuación PeptideRanker. Los resultados muestran el potencial de estos hidrolizados como herramientas preventivas para inflamación localizada y neuroprotección, así como para promover la sostenibilidad y la economía circular en el sector agroalimentario. Los hidrolizados desarrollados y los péptidos identificados podrían ser útiles para las industrias alimentaria y farmacológica para el desarrollo de productos antiinflamatorios y neuroprotectores.

RESUM

La inflamació és un factor comú en moltes patologies. Els principals fàrmacs antiinflamatoris, els no esteroidals (AINE), actuen inhibint les ciclooxigenases (COX). La inhibició prolongada de COX-1 s'associa amb efectes secundaris pel que es busquen inhibidors selectius per a COX-2. A més, la neuroinflamació promou la síntesi de l'enzim prolil oligopeptidasa (POP), i la inhibició d'aquest enzim s'ha proposat com una diana terapèutica per al desenvolupament de fàrmacs neuroprotectors per a la malaltia d'Alzheimer. En aquest sentit, els pèptids bioactius obtinguts d'aliments i subproductes agroalimentaris s'han convertit en bons candidats per al seu ús com a ingredients funcionals en la prevenció de malalties amb un component inflamatori. En aquest context, aquesta tesi doctoral es va centrar en el desenvolupament d'hidrolitzats de proteïnes a partir de subproductes animals amb propietats antiinflamatòries o neuroprotectores, i en la identificació dels seus pèptids bioactius. En la primera part de l'estudi, l'hidrolitzat H8 obtingut de col·lagen de pell de porc va mostrar la major activitat inhibidora de la COX-2, que va ser selectiva per a COX-2. La fracció peptídica <10 kDa d'H8 va prevenir la inflamació aguda localitzada en un model d'edema de pota induït per carragenat i va mostrar capacitat antinociceptiva en un model de dolor agut després de la seva aplicació tòpica. Aquesta fracció va reduir també la producció d'òxid nítric, l'activitat COX-2 i l'expressió proteica de COX-2 i òxid nítric sintasa induïble en cèl·lules RAW264.7 estimulades amb lipopolisacàrid. A més, es van identificar els pèptids antiinflamatoris AGERGEQ i GSKGRPG en H8. En la segona part d'aquesta tesi, es van obtenir dotze hidrolitzats d'un subproducte animal utilitzant diferents condicions d'hidròlisi enzimàtica. Els hidrolitzats CFH3 i CFH5 van mostrar una bona activitat POP inhibitòria i els seus efectes neuroprotectors es van estudiar un model transgènic de malaltia d'Alzheimer (*Caenorhabditis elegants,* CL4176). CFH3 va retardar la paràlisi induïda per l'agregació de ß-amiloide de manera dosi depenent, demostrant els seus efectes neuroprotectors. Es van identificar 23 pèptids nous en CFH3, 9 d'ells amb potencial activitat segons la puntuació PeptideRanker. Els resultats mostren el potencial d'aquests hidrolitzats com a eines preventives per a inflamació localitzada i neuroprotecció, així com per a promoure la sostenibilitat i l'economia circular en el sector agroalimentari. Els hidrolitzats desenvolupats i els pèptids identificats podrien ser útils per a les indústries alimentària i farmacològica per al desenvolupament de productes antiinflamatoris i neuroprotectors.

UNIVERSITAT ROVIRA I VIRGILI
PROTEIN HYDROLYSATES AND PEPTIDES FROM ANIMAL BY-PRODUCTS AS A PREVENTIVE STRATEGY FOR LOCAL INFLAMMATION AND NEUROINFLAMMATION
Néstor Ibarz Blanch

LIST OF ABBREVIATIONS

aa	Amino acid
ACE	Angiotensin.converter enzyme
AD	Alzheimer's disease
APP	Amyloid precursor protein
AUC	Area uder the curve
Aβ	Amyloid-β
BCA	Bicinchoninic acid
BW	Body weight
COX	Cyclooxygenase
COX-1	Cyclooxygenase isoform 1
COX-2	Cyclooxygenase isoform 2
DAMPs	Damage-associated molecular patterns
DH	Degree of hydrolysis
DMEM	Dulbecco's modified Eagle medium
DPP-IV	Dipeptidyl peptidase IV
E/S	Enzyme/substrate
eNOS	Endothelial nitric oxide synthase
FBS	Fetal bovine serum
HMGB1	High-mobility group box protein 1
Hyp	Hydroxyproline
IFN-γ	Interferon-gamma
IkB	inhibitor of NF-kB
IKK	IkB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
LPS	Lipopolysaccharides
m/z	mass/charge
MAPK	Mitogen activated kinase
MAPKAPK	MAPK-Activated protein kinase
MAPKK	MAPK kinase

MAPKKK	MAPKK kinase
MS	Mass
mtNOS	Mitochondrial nitric oxide synthase
MTT	Methylthiazolyl Tetrazolium
NED	N-(1-Naphthyl)ethylenediamine
NF-kB	Nuclear factor-kappa B
NGM	Nematode growth medium
NIK	NF-kB-inducing kinase
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory drug
PAMPs	Pathogen-associated molecular patterns
PG	Prostaglandin
PGE_2	Prostaglandin E2
PGH_2	Prostaglandin H2
POP	Prolyl oligopeptidase
POPi	Prolyl oligopeptidase inhibitory
Pro	Proline
PRR	Pattern recognition receptor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RP-HPLC	Reversed phase-High performance liquid chromatography
STAT	Signal transducer and activator of transcription
TLR	Toll-like receptor
TMPD	N, N, N', N'-tetramethyl-p-phenylenediamine
TNF-α	Tumor necrosis factor-alpha
UHPLC	Ultra-high performance liquid chromatography

INTRODUCTION

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1. Inflammation

Inflammation is a natural response of an organism to nocuous stimuli or conditions such as infection, tissue damage, and stress [1]. It has long been recognized for its five cardinal signs: *calor* (fever), *rubor* (redness), *tumor* (swelling and edema), *dolor* (pain), and *function laesa* (loss of function) [2]. The physiological role of inflammation is to maintain or restore homeostasis in tissues, and the response depends on the nature of the inducer [3,4]. However, if it gets uncontrolled and remains prolonged in time, it can lead to the development of all kinds of diseases and pathologies. Indeed, inflammation, in a lower or higher grade, is a common factor in a wide range of pathologies such as obesity, arthritis, inflammatory bowel disease, asthma, neurodegenerative diseases, or cancer, among others [1]. Chronic inflammatory diseases are identified by the World Health Organization as the leading cause of mortality globally, with 3 out of every 5 deaths worldwide attributed to them [5]. Moreover, the prolonged use of anti-inflammatory drugs, habitual in chronic pathologies, usually leads to undesired side effects [6]. Thus, the necessity to prevent inflammation-associated diseases, and to obtain less harmful anti-inflammatory therapies is evident [7].

1.1. Phases of inflammatory response

A typical inflammatory response occurs in three phases: induction, activation, and resolution. Figure 1 graphically shows the processes that occur in these inflammatory phases. The inflammatory response can be induced by exogenous or endogenous factors [3]. The most studied inflammatory response is triggered by exogenous inducers, specifically microbial factors (bacteria, viruses, or other microorganisms), not necessarily derived from pathogens [4]. The inflammatory response after infection is triggered by the recognition of pathogen-associated molecular patterns (PAMPs), or virulence factors [8]. However, a sterile inflammatory response may occur under chemical or physical insults such as toxins, allergens, injury, or trauma [9]. In this case, the role of inflammation is to protect the affected zone from opportunistic pathogens and induce the regeneration of damaged tissue [10]. The immune response can be triggered by some endogenous intracellular molecules released from dead, damaged, or stressed cells, known as damage-associated molecular patterns (DAMPs) [11].

PAMPs and DAMPs are both recognized by pattern recognition receptors (PRR) expressed in innate immune cells, such as macrophages, neutrophils, and dendritic cells [12]. When PRRs are activated, intracellular responses occur leading to the expression

of a wide variety of pro-inflammatory genes and finally, the edema formation via the secretion of pro-inflammatory cytokines and chemokines, which attract and recruit circulating immune cells to the affected zone [1,8]. The recruited cells, especially macrophages and neutrophils, are also activated and secrete inflammatory mediators that amplify the response [13]. Moreover, other mechanisms are triggered such as phagocytosis, secretion of different hydrolytic enzymes, or formation of free radicals and reactive species of oxygen (ROS) or nitrogen (RNS) are triggered [14]. There is also an interaction with nociceptors, which are the receptors of pain, to locally protect the zone. Some inflammatory mediators, such as prostaglandins, can reduce the pain threshold by increasing the sensitivity of nociceptors [15,16]. In the brain, the inflammatory response is similarly orchestrated by microglial cells, which are activated by DAMPs and secrete pro-inflammatory mediators [17,18]. Under pathological conditions, microglia may also be activated by peripheral DAMPs entering the brain through a damaged blood-brain barrier [1,19]. During acute inflammation, the inflammatory response disappears when the pathogen is eliminated or the damaged tissue is regenerated [10]. However, progression from acute to chronic inflammation can occur if the resolution process is not well-managed [20]. After a few days, acute inflammation reaches a transition state, the subacute inflammation. This phase lasts from 2 to 6 weeks, and if inflammation persists, is considered as chronic inflammation, which may last for months or years [13].

Chronic inflammation, characterized by prolonged activation of the immune system, plays an important role in the development and progression of various diseases [21]. This persistent immune response can induce tissue damage and dysfunction in various organs and tissues, contributing to the pathogenesis of several diseases such as cardiovascular diseases, autoimmune disorders, and neurodegenerative diseases [22]. In addition, chronic inflammation, closely related to aging, is implicated in the occurrence of inflammaging, which is characterized by low-grade systemic inflammation and is increasingly recognized as a hallmark of aging and a key factor in health [23]. It is associated with several age-related diseases such as osteoarthritis and neurodegenerative disorders. In osteoarthritis, chronic inflammation aggravates the disease [24]. For example, following joint injuries, the resulting inflammation may contribute to the progression of osteoarthritis [25]. In addition, systemic inflammation associated with inflammaging may also play a role in the development and progression of osteoarthritis [26]. Similarly, in neurodegenerative diseases, such as Alzheimer's disease (AD),

inflammaging is thought to contribute to neuroinflammation and neuronal damage, further highlighting the interconnection between chronic inflammation, inflammaging, and diseases [27].

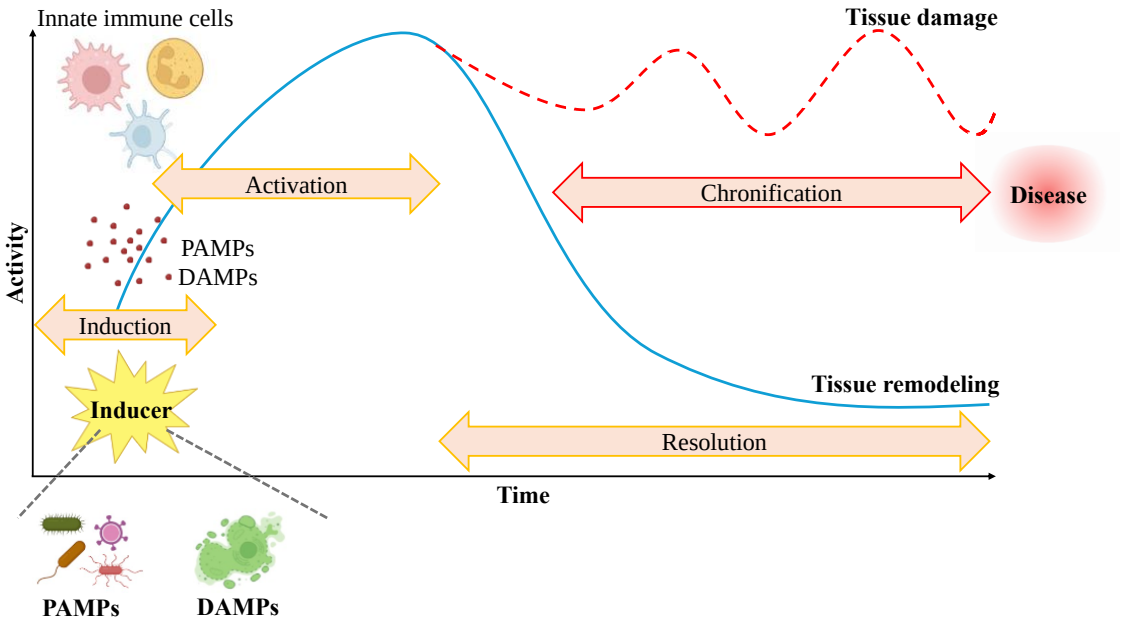

Figure 1: Phases of inflammatory response. Phase 1 (induction): Inflammation is usually induced by exogenous (microbes, toxins, allergens, etc.) or endogenous (cellular components) factors, which are recognized as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). **Phase 2 (activation):** PAMPs and DAMPs activate cells of the innate immune system (mainly macrophages, neutrophils, and dendritic cells) to execute the inflammatory response. **Phase 3 (resolution):** The inflammatory response disappears when the pathogen is eliminated or the damaged tissue is regenerated. **Chronification**: Sustained inflammatory stimuli may lead to progressive tissue damage and various diseases.

1.2. Inflammatory response mechanisms

1.2.1. Inducers and receptors

As mentioned, the inflammatory process starts with the recognition of virulence factors, or PAMPs, and DAMPs. PAMPs refer to a specific and restricted collection of conserved molecular patterns present in all microorganisms belonging to a particular class, regardless of whether they are pathogenic or commensal [4]. DAMPs include a wide set of intracellular molecules, passively released from dying cells or induced by any kind of traumatic cell stress or tissue injury, such as surgical procedures, blows,

thermal traumas, or metabolic traumas [11]. These molecules include high-mobility group box 1 (HMGB1), heat shock proteins, histones, free nucleic acids (nuclear and mitochondrial), and uric acid, among others [9]. All these PAMPs and DAMPs are recognized by specific PRRs. The Toll-like receptors (TLRs) family, are one of the most important PRRs, and each isoform is associated with specific ligands. HMGB1 and lipopolysaccharides (LPS) are recognized by this class of receptors, for example [1]. Virulence factors are a wide variety of inducers, restricted to pathogen microbial. They are not sensed directly by specialized sensors, but the effects of their activity instead, such as the pores formed by exotoxins of certain bacteria [4].

1.2.2. Signaling pathways

PRR activation triggers intracellular responses through several signaling pathways. The most common pro-inflammatory signaling pathways are nuclear factor kappa-B (NF-kB), mitogen-activated protein kinase (MAPK), and Janus kinase (JAK)- signal transducer and activator of transcription (STAT) [12,28,29]. NF-kB is a highly ubiquitous family of five related transcription factors involved in cell survival, proliferation, and differentiation, but their main role is in inflammation and autoimmune responses [30]. In basal conditions, the inhibitor of NF-kB (IkB) is retaining NF-kB in the cytoplasm. Once the inflammation pathway has been started, the IkB kinase (IKK), through phosphorylation, inhibits IkB (canonical pathway) or activates the NF-kB-inducing kinase (NIK) (non-canonical pathway). Both, IkB inhibition or NIK activation, trigger NF-kB nuclear translocation and expression of pro-inflammatory genes [31].

MAPKs are a wide family of serine/threonine kinases that play critical cellular functions such as cell cycle progression, cell adherence, cell metabolism, or inflammatory cytokines expression [32]. Despite a large amount of possible combinations between them, a MAPK signaling pathway consists, of at least, three components: a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK). In subsequential phosphorylations, a MAPKKK activates a MAPKK, which in turn activates a MAPK, and this ends phosphorylating and activating a MAPK-Activated protein kinase (MAPKAPK) [33]. These last are responsible for transmitting the signal by phosphorylating other substrates at different levels. The response can be regulated by activating transcription factors, but also phosphorylation of histones or mRNA stabilization are other possible mechanisms for the MAPKAPK to continue triggering

the inflammatory process [34]. Due to the wide range of stimuli that MAPKKK can integrate, the MAPK signaling pathway is a very versatile response.

JAK/STAT is a highly phylogenetically conserved signaling pathway [29]. The activation of the specific receptor triggers a conformational change that brings the associated JAKs closer together and allows them to phosphorylate each other and the intracellular region of the receptor [35]. These phosphorylations create docking sites for the cytoplasmic latent STATs, which undergo further phosphorylation by the JAKs, dimerization, and DNA binding after nuclear translocation [36]. Different combinations of JAKs and STATs (depending on the stimulus) trigger different responses [35]. Therefore, the JAK/STAT signaling pathway is a mechanism that gives direct and specific responses to a wide range of extracellular factors.

All three signaling pathways lead to the release of pro-inflammatory cytokines and mediators, such as interleukin (IL) -1ß, IL-6, tumor-necrosis factor - α (TNF-α), or interferon-gamma (IFN-γ). The principal cytokines and their functions are well summarized in *Chen et al. (2018)* [1]. NF-kB, MAPK, or JAK/STAT pathways malfunction is associated with chronic inflammatory, autoimmune, and metabolic diseases [31,33,36].

1.3. Key pro-inflammatory enzymes

Many enzymes are implicated in the inflammatory process, and some of them are of special interest because they are responsible for the production of inflammatory mediators. Specifically, cyclooxygenases and nitric oxide synthases are common targets for the development of anti-inflammatory therapies.

1.3.1. Cyclooxygenases

Cyclooxygenase (COX) enzymes, also known as prostaglandin endoperoxidase synthases, are responsible for prostaglandin synthesis. More precisely, COX enzymes catalyze the conversion of arachidonic acid to prostaglandin H2 (PGH_2) through bis-oxygenation [37]. PGH_2 serves as a precursor for various prostaglandins, thromboxanes, and prostacyclins, collectively known as eicosanoids [38]. Additionally, PGH_2 synthesis involves ROS generation, which increases oxidative stress [39]. Moreover, ROS can induce the NF-kB pathway, producing the expression of pro-inflammatory cytokines such as NO, IL-6, and TNF-α [40]. COX isoform 1 (COX-1) is constitutively expressed in many tissues and organs such as the stomach, intestine, or kidney, and is mainly

involved in the regulation of homeostatic functions through the production of thromboxane A2 and various prostaglandins (PGs) (PGD_2, $PGF_2\alpha$, PGI_2, and PGE_2) [6,41]. However, COX isoform 2 (COX-2) is induced in response to inflammatory stimuli and is expressed in fibroblasts, activated macrophages, and other cells [42]. COX-2, in combination with microsomal PGE synthase-1, produces PGE_2, which is implicated in inflammation, fever, and pain [41,43]. Moreover, it intensifies the inflammatory response by strengthening and prolonging the signals generated by other pro-inflammatory substances, such as bradykinin, histamine, and neurokinins [44].

1.3.2. Nitric oxide synthase

Nitric oxide synthase (NOS) is a metalloenzyme that catalyzes the conversion of L-arginine into L-citrulline, liberating NO [45]. The NOS family comprises four isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS), mitochondrial NOS (mtNOS), and inducible NOS (iNOS) [46]. All isoforms have a calmodulin (a calcium-regulating enzyme) binding domain, indicating the necessity of calcium for their activity and regulation [45]. However, the function and structure of mtNOS have not yet been clarified [47]. nNOS is not only expressed in the brain but also in skeletal muscle, vascular smooth muscle, spinal cord, and sympathetic ganglia [48]. eNOS, which is mainly expressed in endothelial cells, is the main regulator of endothelial function and produces vasodilation through NO release. Moreover, eNOS might play a role in inflammatory processes, increasing microvascular hyperpermeability to macromolecules and promoting edema formation [49]. iNOS is not normally expressed in cells and its expression and activity are induced by bacterial lipopolysaccharides (LPS), cytokines, and other factors [50]. Once expressed, iNOS is continuously activated, without calcium regulation, and secretes large amounts of NO that produce cytotoxicity and tissue damage, in combination with ROS and RNS [48]. The reduction of NO secretion has been widely used as an *in vitro* screening tool for anti-inflammatory drugs, in LPS-stimulated macrophages [51,52].

1.4. Treatments

Many classes of anti-inflammatory treatments have been developed. Anti-inflammatory drugs, especially non-steroidal (NSAIDs), are, in fact, the most common class of drugs, utilized for a variety of disorders, including headaches, pain states, fever, and others [53].

COX enzymes are the target enzyme NSAIDs, such as ibuprofen, naproxen, aspirin, or diclofenac [37]. Traditional NSAIDs inhibit both COX isoforms, leading to gastrointestinal and renal side effects owing to the inhibition of COX-1 [6,53]. Consequently, the development of selective COX-2 inhibitors has been pursued in recent decades [40,54,55]. Moreover, some of them have been incorporated into gels, creams, or patches for topical application [56,57].

Other anti-inflammatory drugs include corticosteroids or monoclonal antibodies against specific interleukins such as TNF-α. Some of these, also inhibit the pain, exerting whether nociceptive or analgesic effects [58]. They are commonly used for both acute and chronic inflammation. However, the prolonged use of anti-inflammatory drugs has been related to undesirable side effects, hence, more natural therapies are emerging [59].

2. Neuroinflammation and Alzheimer's disease

It is important to highlight that inflammation occurs as well in the central nervous system. Indeed, neuroinflammation is a common condition in multiple neurodegenerative diseases, such as AD [18]. AD is a progressive neurodegenerative disorder and the most common form of dementia. AD is responsible for 40 million of the 55 million dementia cases worldwide [60]. However, there is not yet a cure or an effective treatment [61], and prevention through lifestyle changes, including diet and bioactive compounds, is a crucial strategy [62,63]. The main risk factor for this disease is aging, and as a consequence of increased life expectancy and an increasingly aging population, it is believed to triple its prevalence by 2050 [63]. AD is irreversible and disabling and is quickly becoming one of the largest socioeconomic burdens of this century [64]. The physiopathology of AD is characterized by two fundamental hallmarks: the extracellular accumulation of amyloid-ß (Aβ) peptides and the hyperphosphorylation of intracellular tau proteins in a neuroinflammatory context [65]. Aβ is a 40-42 amino acids-long peptide cleaved from the amyloid precursor protein (APP) by β- and γ- secretases. According to the Aβ hypothesis, Aβ accumulation occurs when there is an imbalance between Aβ production and clearance [66]. The clearance mechanisms include the ubiquitin-proteasome system, autophagic processes, proteolytic enzymes, transportation across the blood-brain barrier, cellular uptake, and heat shock proteins [67]. When these mechanisms are insufficient, Aβ aggregation impairs cell-to-

cell communication and produces neurotoxicity [68]. Moreover, it is believed that there is a synergistic effect between Aβ aggregation and tau hyperphosphorylation [69]. Under non-pathological conditions, tau proteins support neuronal structure and function. Under pathological conditions, this protein accumulates phosphorylations and aggregates as neurofibrillary tangles (hyperphosphorylated tau aggregates) [68,70]. Additionally, the phosphorylation of tau impairs its binding to the microtubules, affecting neuronal activities and leading to neuronal cell death [71,72].

Both, Aβ aggregation and cell death triggered by tau hyperphosphorylation, induce a neuroinflammatory state that aggravates protein aggregation, thereby generating a complex feedback scenario (Figure 2) [18].

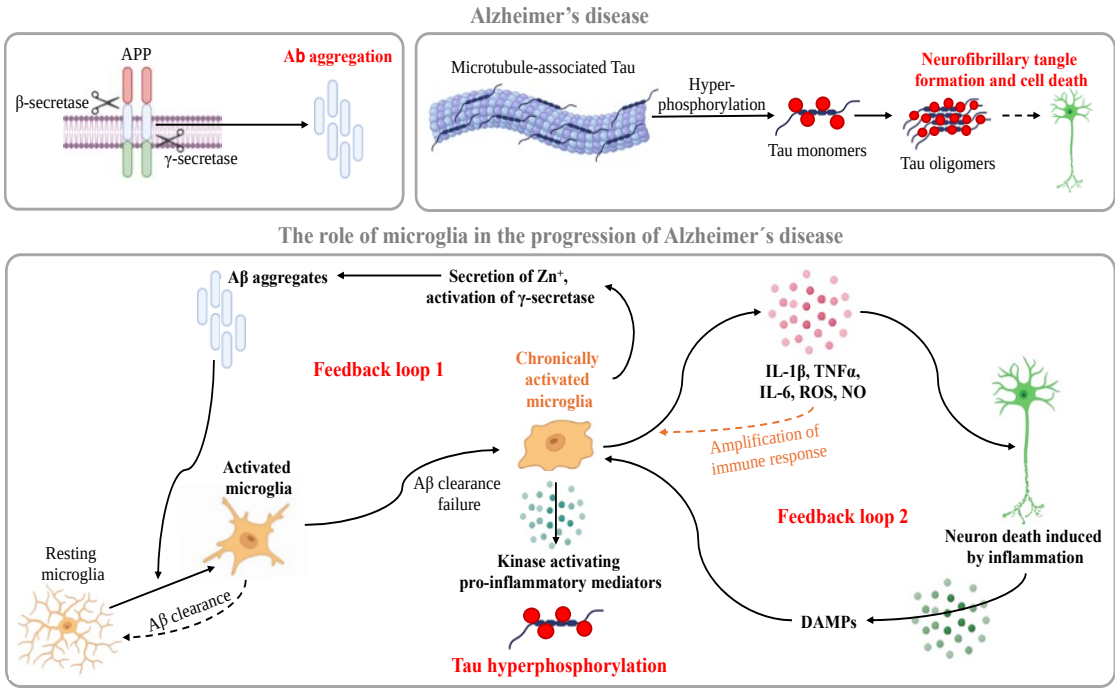

Figure 2: Role of microglia in Alzheimer's disease (AD). AD is characterized by Aβ aggregation and neurofibrillary tangle formation owing to tau hyperphosphorylation. These phenomena lead to neuronal toxicity and cell death, thereby favoring disease progression. Microglia play an essential role in the progression of AD mainly through three different mechanisms: i) chronically activated microglia activates γ-secretases and secrete metals that facilitate Aβ aggregation (feedback loop 1), ii) activates kinases leading to tau hyperphosphorylation due to pro-inflammatory signaling mediators, and iii) secretes an array of pro-inflammatory cytokines that, as a chronic stimulus, promote neuronal death (feedback loop 2). APP: amyloid-precursor protein; Aβ: amyloid-β; DAMPs: damage-associated molecular patterns.

After an inflammatory insult, or Aβ-aggregation-derived activation, activated microglia secrete a wide set of pro-inflammatory mediators, enzymes, and cytokines, including ROS and NO [27]. This secretion amplifies the immune response, leading to neurotoxicity and cell death after chronic activation, which liberates DAMPs and produces positive feedback in the inflammatory process [73]. Secreted pro-inflammatory mediators also activate kinases, which will phosphorylate tau proteins [68]. Activated microglia might induce γ-secretase activity increasing the cleavage of Aβ fragments from the APP and also enhancing its solubilization and aggregation by secreting metals [18]. Moreover, neuroinflammation can be triggered by low-grade chronic inflammation associated with aging (inflammaging), suggesting an initiating role in AD [74,75].

2.1. Prolyl oligopeptidase and Alzheimer's disease

During the neuroinflammatory context, microglia cells also secrete inflammation-related enzymes, such as Prolyl oligopeptidase (POP). POP (EC:3.4.21.26), also known as prolyl endopeptidase, PREP, PO, or PEP, is a conserved intracellular serine protease [76]. It has both catalytic and non-catalytic domains, where the enzymatic activity hydrolyzes the bonds at the carboxyl side of the Pro amino acid, and the non-catalytic domain limits access to the active site to peptides larger than approximately 30 amino acids [77]. POP, which is distributed throughout the body and highly expressed in the brain, has been associated with multiple biological implications (Figure 3).

For instance, it is involved in the development and breakdown of several neuropeptides and hormones (thyrotropin-releasing hormone, substance P, vasopressin, thymosin β4, angiotensin I, and angiotensin II) that contain Pro in their amino acid sequence [78].

POP has demonstrated *in vitro* a role in the modulation of neuronal plasticity via the degradation of neural cell adhesion molecules [79]. Thus, the aberrant activity of this enzyme could trigger poor cellular adhesion and loss of neuronal functionality. In addition, the action of this enzyme appears to be linked to an inflammatory process [19,80,81]. POP knock-out mice are resistant to microglial activation by lipopolysaccharides (LPS), and LPS-stimulated microglial cells have a reduced inflammatory response when treated with a POP inhibitor [80]. Under neuroinflammatory conditions, POP is secreted by microglia into the extracellular

matrix, and systemic inflammation has been demonstrated to increase POP protein expression in AD models and patients [19,81]. Moreover, the POP enzyme has been co-localized with Aβ and tau aggregates in the postmortem brains of patients with AD, suggesting a role in the generation of neurotoxic peptides [82]. Finally, it has been elucidated that POP might be a negative regulator of autophagy and the inhibition of this enzyme might enhance the clearance of protein aggregates [83].

Considering all this, the inhibition of this enzyme emerges as a promising therapeutic target, particularly considering the lack of effective treatments for neurodegenerative diseases [61]. Moreover, given the increasingly aging population, the risk of developing AD or other neurodegenerative disorders is a growing concern [65], hence the importance of exploring the therapeutic potential of POP is highlighted [84,85].

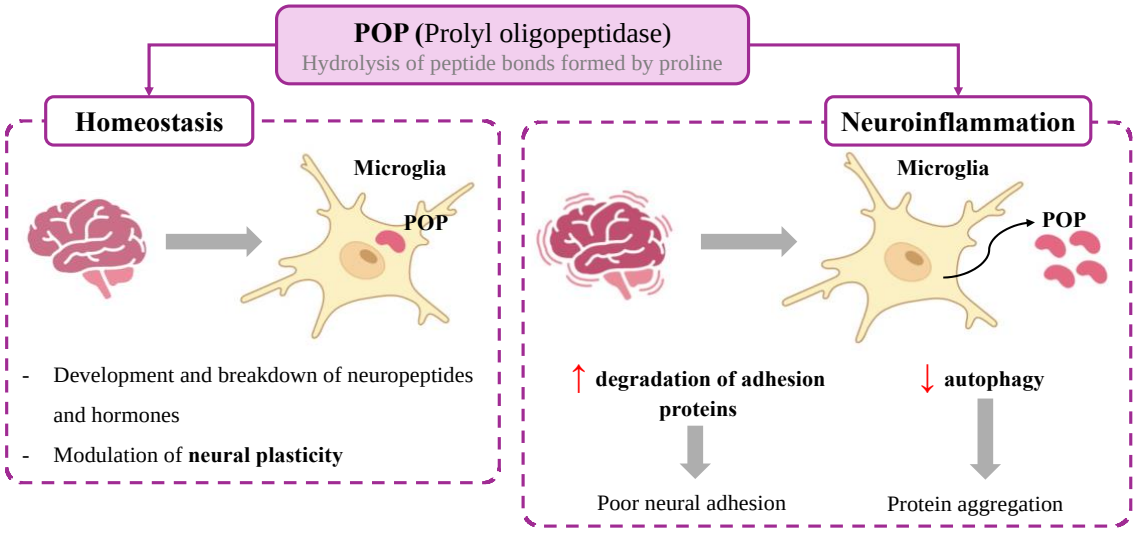

Figure 3: Prolyl oligopeptidase (POP) implications. The role of POP in homeostasis and under neuroinflammatory conditions.

3. Bioactive peptides in preventive healthcare

Due to the lack of effective treatments or the side effects of conventional drugs, medicine is shifting to a more preventive approach; from “sick care” to “health care”. Natural compounds from the diet have emerged as potential tools to reduce the risk of developing a wide range of diseases [86–88]. A nutrient or a bioactive compound administered as a preventive tool for a disease is known as nutraceutical, while its use in cosmetics is known as cosmeceutical [89,90]. Many different groups of molecules have been studied for these purposes, such as fatty acids, fiber, polyphenols, or other

secondary metabolites from plants [87,91]. Bioactive peptides are a group of bioactive compounds of increasing interest because they have shown a wide range of bioactivities such as cardioprotective, neuroprotective, antidiabetic, or anti-inflammatory effects [92,93].

Bioactive peptides are short amino acidic sequences that exhibit beneficial bioactivities for the health of organisms once released from the native protein [94]. The bioactivity of the peptides is determined by their amino acid sequence, which usually consists of 2 - 20 amino acid residues in length, and their intrinsic characteristics such as molecular weight, hydrophobicity, or presence of certain amino acids such as Pro [95].

3.1. Animal by-products as a source of bioactive peptides

Traditionally, the most common protein sources have been eggs, milk (and dairy products), and legumes such as soybeans [96–98], but currently, bioactive peptides are obtained from all kinds of biological sources, including animal, vegetal, or fungi [99–101]. Moreover, there is a trend to obtain bioactive peptides and protein hydrolysates more sustainably [102]. In this sense, the use of by-products from the agri-food industry plays a major role. In line with the circular economy principles, it reduces waste and contributes to waste management, adding value to a by-product that otherwise would be discarded, or used to produce compost, bio-gas, and low-value products [103]. By-products from many agri-food industries, including fish, dairy, cocoa, or winery, have been valorized through the generation of bioactive hydrolysates [104–106]. However, meat industry by-products are gaining importance as a source of bioactive peptides [107]. Although it might seem contrary to the increasing vegan and vegetarian currents in the last decades, the fact is that the meat industry generates every year over 300 million metric tons of meat products [108]. Thus, tons of by-products are generated exerting a relevant impact on the environment, remarking the necessity of improving their management [109]. Moreover, most of these by-products are rich in protein, especially collagen [107].

Collagen is the major structural and one of the most ubiquitous proteins found in vertebrates, especially in bones, joints, and skin [110]. It is one of the most abundant components of the extracellular matrix and accounts for more than one-third of the protein weight of the body [24]. The collagen structure is shown in Figure 4. At the most basic level of the molecule, its amino acid sequence is usually defined as the

repetition of the sequence Gly-X-Y, where X and Y are often Hyp and Pro, forming a left-handed helix [111]. A single collagen helix polymerizes to form a right-handed triple helix (tropocollagen), which can be homotrimeric or heterotrimeric. At the same time, tropocollagen molecules assemble to form microfibrils and lastly, macroscopic fibers and networks, creating a complex supramolecular structure [112]. Specific macromolecular combinations of tropocollagen lead to different types of fibers with different localizations and functions. There are at least 28 types of collagen but types I and III, with a widespread location, represent 80 % of the total collagen in the body [113].

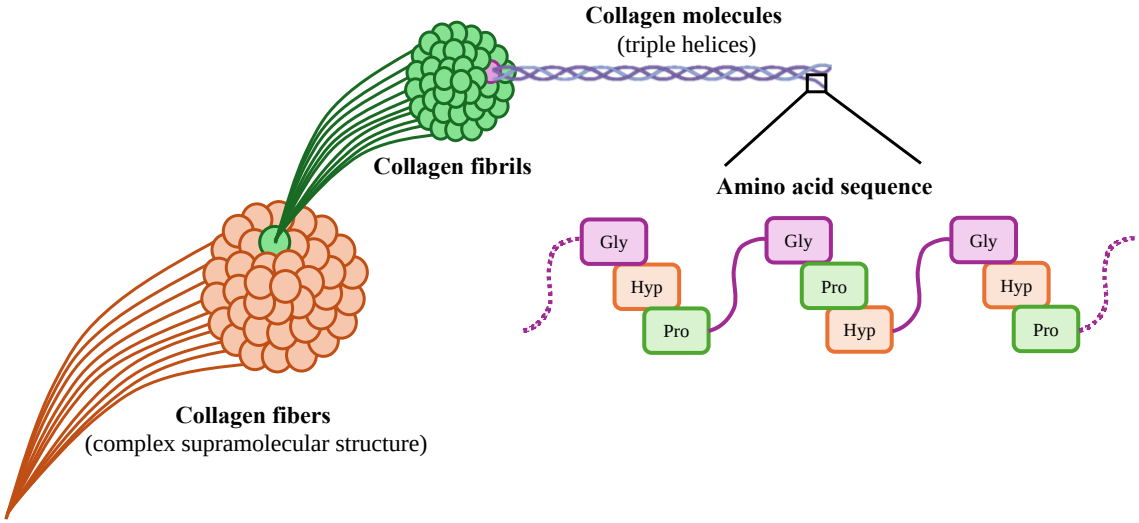

Figure 4: Collagen structure. Collagen is a complex supramolecular structure composed of collagen fibrils. Collagen fibrils are formed by a right-handed triple helix of the collagen molecules. Single collagen molecules are formed by a sequence of the following amino acids: Gly (glycine), Hyp (Hydroxyproline), Pro (Proline).

As an aging natural consequence, collagen is gradually lost although the process is accelerated by various external factors such as inflammation derived from intense physical exercise, trauma, or obesity [114]. This depletion of collagen can lead to several pathologies, including osteoarthritis, owing to an imbalance between the synthesis and degradation of this molecule [24]. In the skin, which is the principal barrier against the external environment, collagen plays a crucial role in maintaining its structural integrity and function. The inflammatory response after exposure to environmental factors such as solar radiation or pollution can accelerate collagen loss, accelerating skin aging and reducing the protective function of the skin [114]. Collagen supplementation has been widely used in the cosmetic and pharmaceutical industries,

and different studies have shown that collagen consumption can mitigate collagen loss [115–118]. Recent studies suggest promising outcomes with collagen supplementation in reducing pain and stiffness of the joints [86,117,118]. Supplementation with collagen provides the necessary amino acids, particularly Pro and Hyp, for the synthesis of endogenous collagen and stimulates its production [119]. Moreover, other mechanisms might be considered, such as the suggested immunomodulatory effect of the collagen peptides [115,120].

3.2. Obtainment of protein hydrolysates and bioactive peptides

Although there are many protocols for obtaining bioactive peptides, a consensus can be reached on the working methodology to obtain peptides with the desired bioactivity. A schematic explanation of this process is shown in Figure 5.

The first step is to select the protein source. Some factors, including the protein content or the amino acid profile, are relevant to consider. In this sense, a higher protein amount will facilitate the obtention of bioactive peptides. Additionally, some bioactivities, like angiotensin-converting enzyme (ACE) or POP inhibition, are enhanced with hydrophobic sequences or the presence of certain amino acids such as Pro or branched-chain amino acids, for example [121]. Thus, a protein source rich in these specific amino acids may facilitate the obtention of the desired bioactivity [122]. Moreover, some processing methods for the raw material can be carried out depending on their characteristics to facilitate protein release. Physicochemical processing is usually necessary and may include anything from milling or ultrasound application to temperature or pH changes to facilitate protein solubilization [123]. Solid protein sources, such as soybeans, meat, and other animal by-products, might require a grinding process, while liquid sources, such as milk, can be directly hydrolyzed. However, additional steps such as defatting or protein extraction have been reported to facilitate protein solubilization [124,125].

Multiple strategies have been utilized to generate bioactive peptides, such as chemical (acid or alkali) hydrolysis, or enzymatic hydrolysis using microbial fermentation, gastrointestinal digestion, or commercial enzymes [122]. Regarding this last process, numerous enzymes have been used to produce bioactive peptides from food. The most commonly used proteases for obtaining bioactive peptides include trypsin, pepsin, papain, Alcalase, Protamex, and Flavourzyme [126]. Temperature (usually from 40 to 60 ºC), time, pH (6.0 - 8.0), and substrate/enzyme ratio are

hydrolysis conditions that determine the generated peptides, and thus, they might remain controlled to minimize the variability [123]. Hydrolysis ends with heat inactivation of enzymes or bacteria at temperatures between 80 and 95 ◦C for 15 – 30 min. Finally, it is common to include a filtering or centrifugation step to remove insoluble solids [127]. In some cases, an ultrafiltration step is also included to obtain the 3 – 10 kDa fraction to purify and concentrate the bioactive peptides [128].

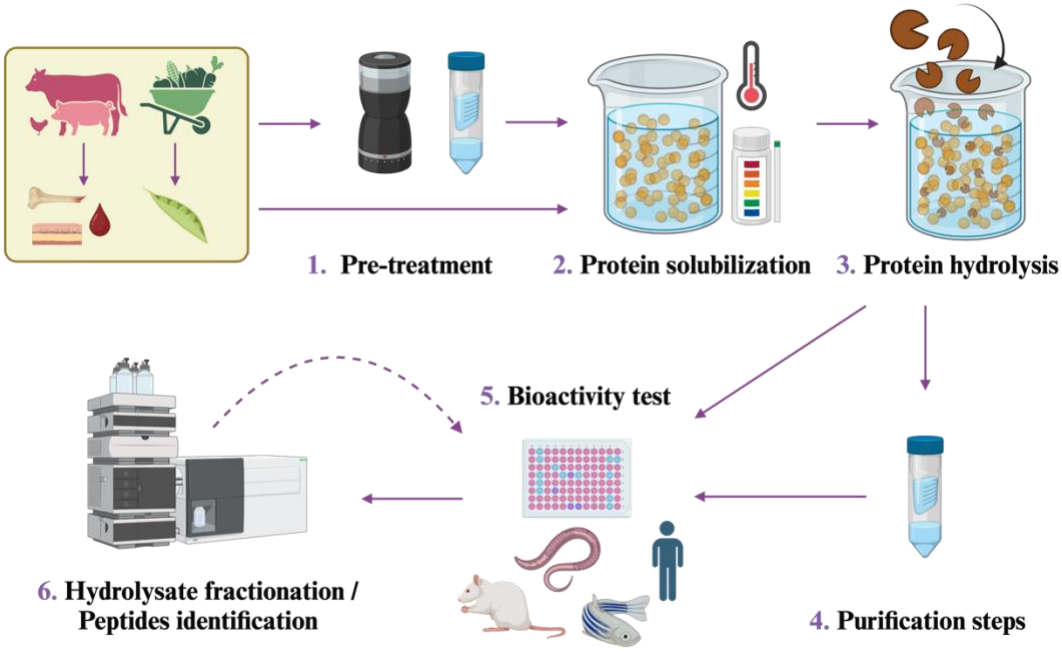

Figure 5: Bioactive peptides obtention. After the identification of target bioactivity and the protein source, obtainment of bioactive peptides can be schematized in the following steps: 1) Raw material pre-treatments, 2) protein solubilization, 3) hydrolysis process, 4) optional purification steps, 5) screening for bioactivity *in vitro* or *in vivo*, as well as in clinical trials, and 6) peptide identification.

Once protein hydrolysates are obtained, their bioactivity is usually evaluated *via in vitro* techniques [129–132], which are faster and more economical than *in vivo* studies. Most of these methodologies of bioactivity screening are based on the potential of these hydrolysates to inhibit enzymes involved in the progression of deleterious conditions, such as ACE for hypertension or dipeptidyl peptidase IV for type-II diabetes mellitus [94]. However, other mechanisms, such as radical scavenging activity or ion chelation activity are also evaluated to identify antioxidant bioactive peptides [133]. After demonstrating the effects *in vitro*, these protein hydrolysates can be tested in more

complex *in vitro* systems, such as cell cultures, *in silico* approaches, *ex vivo* assays, or *in vivo* models with fewer ethical considerations (e.g. *D. melanogaster, C. elegans*, or *D. rerio*) than mammalian models [134–137]. Murine models are the most extended *in vivo* models for pre-clinical trials of hydrolysates and bioactive peptides [134]. Finally, clinical trials are performed with these hydrolysates and peptides with satisfying pre-clinical trial results [138,139].

Complementary, once the bioactivity is determined, subsequent purification steps, such as ultrafiltration with membranes with different molecular weight cutoffs followed by reverse-phase HPLC fractionation, are used for the isolation and identification of the bioactive peptides potentially responsible for the observed effects [101].

3.3.Anti-inflammatory hydrolysates derived from animal agri-food by-products

Bioactive peptides derived from agri-food by-products of animal origin as nutraceuticals or cosmeceuticals are good candidates for the prevention of inflammation-associated diseases or pathologies [140–142]. Known peptides with anti-inflammatory activities do not share clear common characteristics. However, length (2-20 amino acids), low molecular weight (<1 kDa), the presence of hydrophobic amino acids (Val, Ile, Pro), or positively charged amino acids (His, Arg, Lys) could enhance the anti-inflammatory effect [131,143]. Thus, the high abundance of Pro and Hyp in collagen molecules makes animal by-products good candidates for the obtainment of anti-inflammatory bioactive peptides and hydrolysates. Some representative examples of both *in vitro* and *in vivo* studies are collected in Table 1 and Table 2, respectively.

As stated in Table 1, the majority of anti-inflammatory peptides and hydrolysates derived from collagen-rich by-products have been obtained from fish by-products, especially skin and scales. Their anti-inflammatory bioactivity was assayed *in vitro* using many cell models of inflammation, which was induced with either TNF-α or LPS. Treatment with collagen-rich by-product hydrolysates reduced the expression and secretion of several pro-inflammatory cytokines and mediators, including TNF-α, NO, IL-1β, IL-6, or PGE_2. In addition, some components of the MAPK and NF-kB signaling pathways have been reported to be downregulated by treatment with collagen-rich by-product hydrolysates [144,145]. Additionally, some hydrolysates reduced the expression of iNOS and the protein levels of COX-2 [144,146]. Liu and Li 2022 identified the amino acid sequences APD, QA, KA, and WG in a Salmo salar skin collagen hydrolysate, which reduced the secretion of NO, IL-6, IL-1β, and TNF-α in

inflammatory macrophages. QA was the peptide showing the highest bioactivity with an IC_{50} value of 849.3 μM in reduction of NO production [147]. In terms of neuroinflammation, the inhibition of POP has been demonstrated by hydrolysates elaborated from bovine lung [148], pigskin collagen, and many fish skin hydrolysates [149,150].

Table 1: *In vitro* studies of collagen-rich by-products anti-inflammatory hydrolysates.

By-product	Model	Effect	Ref.
Oreochromis genus scales	TNF-α-stimulated HaCaT cells	↓MAPK and NF-kB pathways ↓*Tnf-α,* ↓*iNos,* ↓*Il-1β*	[144]
Salmo salar skin	LPS-induced RAW 264.7 cells	↓NO, ↓Tnf-α, ↓ IL-6, ↓ IL-1β	[147]
Gallus gallus feet	TNF-α - activation HUVECs	↓*iNos*, ↓ IL-8	[151]
Clarias batrachus, and *Pangasius pangasius* skin	LPS-induced RAW 264.7 cells	↓*Tnf-α*, ↓ *Il-6,* ↓ *Il-1β* ↓ NO secretion ↓ TNF-α, ↓ IL-6, ↓ NF-kB, ↓ p-IkB	[145]
Oreochromis genus scales	LPS-stimulated chondrocytes	↓ TNF-α, ↓ IL-6, ↓ IL-1β , ↓ NO, ↓ PGE_2 ↓ COX-2, ↓ p-IkB	[146]
Bovine lung	POP inhibition	-	[148]
Oreochromis genus, Chanos chanos, Hippoglossus hippoglossus, and *Salmo salar* skin Pigskin collagen	POP inhibition	-	[149]
Barbus barbus skin	POP inhibition	-	[150]

Abbreviations: COX-2: cyclooxygenase 2; HUVEC: Human umbilical vein endothelial cell; IL: Interleukin; iNOS: inducible nitric oxide synthase; LPS: Lipopolysaccharide; MAPK: mitogen-activated protein kinase; NF-kB: nuclear factor kappa b; NO: nitric oxide; PGE_2: Prostaglandin E_2; p-IkB: phosphorylated inhibitor of NF-kB; POP: Prolyl oligopeptidase; TNF-α: tumor necrosis factor-α.

The anti-inflammatory effects of several hydrolysates were also evaluated *in* vivo (Table 2). Most of the anti-inflammatory bioactive peptides and hydrolysates derived from collagen-rich by-products tested *in vivo* were obtained from fish by-products, especially skin. Similar to the *in vitro* studies, the expression and secretion of several pro-inflammatory cytokines, and mediators, including IFN-γ, TNF-α, IL-6, NO, IL-1β, and PGE_2 were reduced after the treatment with the collagen-rich by-product hydrolysates. It was also observed a reduction in the chemoattractant chemokine Cxcl1 expression [152], and protein [139]. This chemokine is one of the most important molecules in cell recruitment [153]. In addition, an increase in both the expression and protein levels of the anti-inflammatory interleukin IL-10 has been reported after the consumption of fish by-product hydrolysates [139,152]. Moreover, some components of the NF-kB and MAPK signaling pathways were downregulated after treatment with several collagen-rich by-product hydrolysates [146,154,155]. Additionally, some of the by-products were able to reduce the expression of *iNos* and *Cox2* [156] and the protein levels of COX-2 [146].

It is worth mentioning that all hydrolysates were orally administered. Thus, there is no evidence as to whether these collagen-rich by-product hydrolysates could have anti-inflammatory effects after topical administration. This might be an interesting anti-inflammatory approach, given that topical application of NSAIDs for localized inflammation is associated with fewer side effects [57,157], and small peptides are known to penetrate skin [140].

Finally, the reported studies evaluated the anti-inflammatory effects of the hydrolysates in different diseases or conditions with inflammatory components, including atherosclerosis [158], low-grade inflammation in obesity [156], colitis [152], skin photoaging [154,155], osteoarthritis [146], and intestinal bowel disease in human patients [139]. However, neuroinflammation has not been targeted by collagen-rich by-products in *in vivo* models.

Table 2: *In vivo* studies of collagen-rich by-products anti-inflammatory hydrolysates.

By-product	Model	Administration	Effect	Ref.
Gallus gallus legs	C57BL/6 KOR-ApoEshI Mice	Chow	↓TNF-α, ↓ IL-6 in plasma	[158]
Raja kenojei skin	HFD - C57BL/6 Mice	Oral	↓ *iNos,* ↓ *Cox2,* ↓ *Il-6*	[156]
Salmo salar and *Scomber scombrus* head and backbone. *Platichthys flesus* skin collagen	DSS-induced colitis in C57BL/6 mice	Chow	↓ *Tnf-α,* ↓ *Il-6,* ↓ *Cxcl1,* ↑ *Il-10* in intestine	[152]
Katsuwonus pelamis skin	UV-irradiated SD rats	Drinking water	↓MAPK and NF-kB patwhways ↓ IL-1β, ↓ IL-6 in skin	[154]
Theragra chalcogramma skin	UV-irradiated SD rats	Drinking water	↓MAPK and NF-kB patwhways ↓ IL-1ß, ↓ IL-6 in skin	[155]
Oreochromis genus scales	MIA-induced osteoarthritis in SD rats	Oral	↓ TNF-α, ↓ IL-6, ↓ IL-1β, ↓ NO, ↓ PGE_2, ↓ COX-2,↓ p-IkB	[146]
Fish skin (undetermined)	Intestinal bowel diseases patients	Oral	↓ IFN-γ, ↓ TNF-α, ↓ IL-1β,↓ IL-6, ↓ Cxcl1, ↑ IL-10 in monocytes	[139]

Abbreviations: COX-2: cyclooxygenase 2; Cxcl1: CXC motif chemokine ligand 1; DSS: Dextran sulfate sodium; HDF: high-fat diet; IFN-γ: Interferon-γ; IL: Interleukin; iNOS: inducible nitric oxide synthase; MAPK: mitogen-activated protein kinase; MIA: mono iodoacetate; NF-kB: nuclear factor kappa b; PGE_2: Prostaglandin E_2; NO: nitric oxide; p-IkB: phosphorylated inhibitor of NF-kB; SD: Sprague-Dawley; TNF-α: tumor necrosis factor-α; UV: ultra-violet.

In summary, all the findings mentioned show the potential of collagen-rich animal by-product hydrolysates and their derived peptides as functional ingredients for the prevention of inflammation-related diseases, highlighting the importance of more effective and safer preventive anti-inflammatory strategies.

REFERENCES

1. Chen, L.; Deng, H.; Cui, H.; Fang, J.; Zuo, Z.; Deng, J.; Li, Y.; Wang, X.; Zhao, L. Inflammatory Responses and Inflammation-Associated Diseases in Organs. *Oncotarget* **2018**, *9*, 7204–7218.
2. Majno, G. *The Healing Hand. Man and Wound in the Ancient World*; Cambridge University Press: Cambridge, MA, **1976**; Vol. 20; ISBN 0-674-38331-1.
3. Medzhitov, R. The Spectrum of Inflammatory Responses. *Science (1979)* **2021**, *374*, 1070–1075.
4. Medzhitov, R. Origin and Physiological Roles of Inflammation. *Nature* **2008**, *454*, 428–435.
5. Pahwa, R.; Goyal, A.; Jialal, I. Chronic Inflammation. *Pathobiology of Human Disease: A Dynamic Encyclopedia of Disease Mechanisms* **2018**, 300–314.
6. Süleyman, H.; Demircan, B.; Karagöz, Y. Anti-Inflammatory and Side Effects of Cyclooxygenase Inhibitors. *Pharmacological Reports* **2007**, *59*, 247–258.
7. Nisar, A.; Jagtap, S.; Vyavahare, S.; Deshpande, M.; Harsulkar, A.; Ranjekar, P.; Prakash, O. Phytochemicals in the Treatment of Inflammation-Associated Diseases: The Journey from Preclinical Trials to Clinical Practice. *Front Pharmacol* **2023**, *14*, 1177050.
8. Medrano-Bosch, M.; Simón-Codina, B.; Jiménez, W.; Edelman, E.R.; Melgar-Lesmes, P. Monocyte-Endothelial Cell Interactions in Vascular and Tissue Remodeling. *Front Immunol* **2023**, *14*, 1196033.
9. Shen, H.; Kreisel, D.; Goldstein, D.Robert. Processes of Sterile Inflammation. *J Immunol* **2013**, *191*, 2857.
10. Medzhitov, R. Inflammation 2010: New Adventures of an Old Flame. *Cell* **2010**, *140*, 771–776.
11. Relja, B.; Land, W.G. Damage-Associated Molecular Patterns in Trauma. *European Journal of Trauma and Emergency Surgery* **2020**, *46*, 751–775.
12. Guo, Q.; Jin, Y.; Chen, X.; Ye, X.; Shen, X.; Lin, M.; Zeng, C.; Zhou, T.; Zhang, J. NF-KB in Biology and Targeted Therapy: New Insights and Translational Implications. *Signal Transduction and Targeted Therapy 2024 9:1* **2024**, *9*, 1–37.
13. Hannoodee, S.; Nasuruddin, D.N. *Acute Inflammatory Response*; StatPearls Publishing, Treasure Island (FL), **2023**.
14. Mantovani, A.; Garlanda, C. Humoral Innate Immunity and Acute-Phase Proteins. *New England Journal of Medicine* **2023**, *388*, 439–452.
15. Dubin, A.E.; Patapoutian, A. Nociceptors: The Sensors of the Pain Pathway. *J Clin Invest* **2010**, *120*, 3760.
16. Jang, Y.; Kim, M.; Hwang, S.W. Molecular Mechanisms Underlying the Actions of Arachidonic Acid-Derived Prostaglandins on Peripheral Nociception. *Journal of Neuroinflammation 2020 17:1* **2020**, *17*, 1–27.
17. Liew, Y.; Retinasamy, T.; Arulsamy, A.; Ali, I.; Jones, N.C.; O'Brien, T.J.; Shaikh, M.F. Neuroinflammation: A Common Pathway in Alzheimer's Disease and Epilepsy. *J Alzheimers Dis* **2023**, *94*, S253–S265.
18. Zhang, W.; Xiao, D.; Mao, Q.; Xia, H. Role of Neuroinflammation in Neurodegeneration Development. *Signal Transduction and Targeted Therapy 2023 8:1* **2023**, *8*, 1–32.
19. Tenorio-Laranga, J.; Montoliu, C.; Urios, A.; Hernandez-Rabaza, V.; Ahabrach, H.; García-Horsman, J.A.; Felipo, V. The Expression Levels of Prolyl Oligopeptidase Responds Not Only to Neuroinflammation but Also to Systemic Inflammation upon Liver Failure in Rat Models and Cirrhotic Patients. *J Neuroinflammation* **2015**, *12*, 183.

20. Panigrahy, D.; Gilligan, M.M.; Serhan, C.N.; Kashfi, K. Resolution of Inflammation: An Organizing Principle in Biology and Medicine. *Pharmacol Ther* **2021**, *227*, 107879.
21. Wang, R.; Zhou, M.; Ma, H.; Qiao, Y.; Li, Q. The Role of Chronic Inflammation in Various Diseases and Anti-inflammatory Therapies Containing Natural Products. *ChemMedChem* **2021**, *16*, 1576–1592.
22. Nathan, C.; Ding, A. Leading Edge Review Nonresolving Inflammation. **2003**.
23. Li, X.; Li, C.; Zhang, W.; Wang, Y.; Qian, P.; Huang, H. Inflammation and Aging: Signaling Pathways and Intervention Therapies. *Signal Transduction and Targeted Therapy 2023 8:1* **2023**, *8*, 1–29.
24. Ouyang, Z.; Dong, L.; Yao, F.; Wang, K.; Chen, Y.; Li, S.; Zhou, R.; Zhao, Y.; Hu, W. Cartilage-Related Collagens in Osteoarthritis and Rheumatoid Arthritis: From Pathogenesis to Therapeutics. *Int J Mol Sci* **2023**, *24*.
25. Leite, C.B.G.; Merkely, G.; Charles, J.F.; Lattermann, C. From Inflammation to Resolution: Specialized Pro-Resolving Mediators in Posttraumatic Osteoarthritis. *Curr Osteoporos Rep* **2023**, 1–13.
26. Umoh, I.O.; dos Reis, H.J.; de Oliveira, A.C.P. Molecular Mechanisms Linking Osteoarthritis and Alzheimer's Disease: Shared Pathways, Mechanisms and Breakthrough Prospects. *Int J Mol Sci* **2024**, *25*, 3044.
27. Heneka, M.T.; Carson, M.J.; Khoury, J. El; Landreth, G.E.; Brosseron, F.; Feinstein, D.L.; Jacobs, A.H.; Wyss-Coray, T.; Vitorica, J.; Ransohoff, R.M.; et al. Neuroinflammation in Alzheimer's Disease. *Lancet Neurol* **2015**, *14*, 388–405.
28. Hu, X.; li, J.; Fu, M.; Zhao, X.; Wang, W. The JAK/STAT Signaling Pathway: From Bench to Clinic. *Signal Transduction and Targeted Therapy 2021 6:1* **2021**, *6*, 1–33.
29. Gadina, M.; Gazaniga, N.; Vian, L.; Furumoto, Y. Small Molecules to the Rescue: Inhibition of Cytokine Signaling in Immune-Mediated Diseases. *J Autoimmun* **2017**, *85*, 20–31.
30. Mitchell, S.; Vargas, J.; Hoffmann, A. Signaling via the NFκB System. *Wiley Interdiscip Rev Syst Biol Med* **2016**, *8*, 227–241.
31. Sun, S.-C. The Non-Canonical NF-KB Pathway in Immunity and Inflammation. *Nat Rev Immunol* **2017**, *17*, 545–558.
32. Moens, U.; Kostenko, S.; Sveinbjørnsson, B. The Role of Mitogen-Activated Protein Kinase-Activated Protein Kinases (MAPKAPKs) in Inflammation. *Genes (Basel)* **2013**, *4*, 101–133.
33. Cuevas, B.D.; Abell, A.N.; Johnson, G.L. Role of Mitogen-Activated Protein Kinase Kinase Kinases in Signal Integration. *Oncogene* **2007**, *26*, 3159–3171.
34. Canovas, B.; Nebreda, A.R. Diversity and Versatility of P38 Kinase Signalling in Health and Disease. *Nat Rev Mol Cell Biol* **2021**, *22*, 346–366.
35. Villarino, A. V.; Kanno, Y.; Ferdinand, J.R.; O'Shea, J.J. Mechanisms of Jak/STAT Signaling in Immunity and Disease. *J Immunol* **2015**, *194*, 21–27.
36. Welsch, K.; Holstein, J.; Laurence, A.; Ghoreschi, K. Targeting JAK/STAT Signalling in Inflammatory Skin Diseases with Small Molecule Inhibitors. *Eur J Immunol* **2017**, *47*, 1096–1107.
37. Turini, M.E.; DuBois, R.N. Cyclooxygenase-2: A Therapeutic Target. *Annu Rev Med* **2002**, *53*, 35–57.
38. Tsatsanis, C.; Androulidaki, A.; Venihaki, M.; Margioris, A.N. Signalling Networks in Focus Signalling Networks Regulating Cyclooxygenase-2. *Int J Biochem Cell Biol* **2006**, *38*, 1654–1661.

39. Rea, I.M.; Gibson, D.S.; McGilligan, V.; McNerlan, S.E.; Denis Alexander, H.; Ross, O.A. Age and Age-Related Diseases: Role of Inflammation Triggers and Cytokines. *Front Immunol* **2018**, *9*.
40. Ju, Z.; Li, M.; Xu, J.; Howell, D.C.; Li, Z.; Chen, F.E. Recent Development on COX-2 Inhibitors as Promising Anti-Inflammatory Agents: The Past 10 Years. *Acta Pharm Sin B* **2022**, *12*, 2790–2807.
41. Lee, Y.; Rodriguez, C.; Dionne, R. The Role of COX-2 in Acute Pain and the Use of Selective COX-2 Inhibitors for Acute Pain Relief. *Curr Pharm Des* **2005**, *11*, 1737–1755.
42. Williams, J.A.; Shacter, E. Regulation of Macrophage Cytokine Production by Prostaglandin E2. *Journal of Biological Chemistry* **1997**, *272*, 25693–25699.
43. Guay, J.; Bateman, K.; Gordon, R.; Mancini, J.; Riendeau, D. Carrageenan-Induced Paw Edema in Rat Elicits a Predominant Prostaglandin E2 (PGE2) Response in the Central Nervous System Associated with the Induction of Microsomal PGE2 Synthase-1. *Journal of Biological Chemistry* **2004**, *279*, 24866–24872.
44. Kidd, B.L.; Urban, L.A. Mechanisms of Inflammatory Pain. *Br J Anaesth* **2001**, *87*, 3–11.
45. Groves, J.T.; Wang, C.C.Y. Nitric Oxide Synthase: Models and Mechanisms. *Curr Opin Chem Biol* **2000**, *4*, 687–695.
46. Förstermann, U.; Schmidt, H.H.; Pollock, J.S.; Sheng, H.; Mitchell, J.A.; Warner, T.D.; Nakane, M.; Murad, F. Isoforms of Nitric Oxide Synthase. Characterization and Purification from Different Cell Types. *Biochem Pharmacol* **1991**, *42*, 1849–1857.
47. Rodkin, S. V.; Nwosu, C.D. Role of Nitric Oxide and Hydrogen Sulfide in Neuronal and Glial Cell Death in Neurodegenerative Processes. *Biochemistry (Moscow), Supplement Series A: Membrane and Cell Biology 2023 17:4* **2023**, *17*, 223–242.
48. Huang, J.B.; Chen, Z.R.; Yang, S.L.; Hong, F.F. Nitric Oxide Synthases in Rheumatoid Arthritis. *Molecules 2023, Vol. 28, Page 4414* **2023**, *28*, 4414.
49. Durán, W.N.; Breslin, J.W.; Sánchez, F.A. The NO Cascade, ENOS Location, and Microvascular Permeability. *Cardiovasc Res* **2010**, *87*, 254–261.
50. Cinelli, M.A.; Do, H.T.; Miley, G.P.; Silverman, R.B. Inducible Nitric Oxide Synthase: Regulation, Structure, and Inhibition. *Med Res Rev* **2020**, *40*, 158–189.
51. Chanput, W.; Mes, J.J.; Wichers, H.J. THP-1 Cell Line: An in Vitro Cell Model for Immune Modulation Approach. **2014**.
52. Facchin, B.M.; dos Reis, G.O.; Vieira, G.N.; Mohr, E.T.B.; da Rosa, J.S.; Kretzer, I.F.; Demarchi, I.G.; Dalmarco, E.M. Inflammatory Biomarkers on an LPS-Induced RAW 264.7 Cell Model: A Systematic Review and Meta-Analysis. *Inflammation Research* **2022**, *71*, 741–758.
53. LaForge, J.M.; Urso, K.; Day, J.M.; Bourgeois, C.W.; Ross, M.M.; Ahmadzadeh, S.; Shekoohi, S.; Cornett, E.M.; Kaye, A.M.; Kaye, A.D. Non-Steroidal Anti-Inflammatory Drugs: Clinical Implications, Renal Impairment Risks, and AKI. *Adv Ther* **2023**, *40*, 2082–2096.
54. Everts, B.; Währborg, P.; Hedner, T. COX-2-Specific Inhibitors--the Emergence of a New Class of Analgesic and Anti-Inflammatory Drugs. *Clin Rheumatol* **2000**, *19*, 331–343.
55. Stiller, C.O.; Hjemdahl, P. Lessons from 20 Years with COX-2 Inhibitors: Importance of Dose–Response Considerations and Fair Play in Comparative Trials. *J Intern Med* **2022**, *292*, 557–574.
56. Bhat, C.; Rosenberg, H.; James, D. Topical Nonsteroidal Anti-Inflammatory Drugs. *Can Med Assoc J* **2023**, *195*, E1231–E1231.

57. Lee, J.; Abbas, A.A.; Cheah, T.; Simanjuntak, R.N.; Sockalingam, S.; Roohi, S. Topical Nonsteroidal Anti-inflammatory Drugs for Management of Osteoarthritis Pain: A Consensus Recommendation. *Journal of Orthopaedic Research* **2023**, *41*, 1916–1924.
58. Simmons, D.L. What Makes a Good Anti-Inflammatory Drug Target? *Drug Discov Today* **2006**, *11*, 210–219.
59. De Vita, D.; Jamtsho, T.; Yeshi, K.; Perry, M.J.; Loukas, A.; Wangchuk, P. Approaches, Strategies and Procedures for Identifying Anti-Inflammatory Drug Lead Molecules from Natural Products. *Pharmaceuticals 2024, Vol. 17, Page 283* **2024**, *17*, 283.
60. Dubois, B.; Hampel, H.; Feldman, H.H.; Scheltens, P.; Aisen, P.; Andrieu, S.; Bakardjian, H.; Benali, H.; Bertram, L.; Blennow, K.; et al. Preclinical Alzheimer's Disease: Definition, Natural History, and Diagnostic Criteria. *Alzheimers Dement* **2016**, *12*, 292–323.
61. Cummings, J.; Lee, G.; Nahed, P.; Kambar, M.E.Z.N.; Zhong, K.; Fonseca, J.; Taghva, K. Alzheimer's Disease Drug Development Pipeline: 2022. *Alzheimer's and Dementia: Translational Research and Clinical Interventions* **2022**, *8*.
62. Arora, S.; Santiago, J.A.; Bernstein, M.; Potashkin, J.A. Diet and Lifestyle Impact the Development and Progression of Alzheimer's Dementia. *Front Nutr* **2023**, *10*.
63. Crous-Bou, M.; Minguillón, C.; Gramunt, N.; Molinuevo, J.L. Alzheimer's Disease Prevention: From Risk Factors to Early Intervention. *Alzheimers Res Ther* **2017**, *9*, 1–9.
64. Scheltens, P.; De Strooper, B.; Kivipelto, M.; Holstege, H.; Chételat, G.; Teunissen, C.E.; Cummings, J.; van der Flier, W.M. Alzheimer's Disease. *Lancet* **2021**, *397*, 1577–1590.
65. The Alzheimer's Association *2023 Alzheimer's Disease Facts and Figures*; John Wiley and Sons Inc, **2023**; Vol. 19.
66. Hardy, J.; Selkoe, D.J. The Amyloid Hypothesis of Alzheimer's Disease: Progress and Problems on the Road to Therapeutics. *Science (1979)* **2002**, *297*, 353–356.
67. Madadi, S.; Schwarzenbach, H.; Saidijam, M.; Mahjub, R.; Soleimani, M. Potential MicroRNA-Related Targets in Clearance Pathways of Amyloid-β: Novel Therapeutic Approach for the Treatment of Alzheimer's Disease. *Cell & Bioscience 2019 9:1* **2019**, *9*, 1–19.
68. Iliyasu, M.O.; Musa, S.A.; Oladele, S.B.; Iliya, A.I. Amyloid-Beta Aggregation Implicates Multiple Pathways in Alzheimer's Disease: Understanding the Mechanisms. *Front Neurosci* **2023**, *17*, 1081938.
69. Busche, M.A.; Hyman, B.T. Synergy between Amyloid-β and Tau in Alzheimer's Disease. *Nature Neuroscience 2020 23:10* **2020**, *23*, 1183–1193.
70. De Felice, F.G.; Wu, D.; Lambert, M.P.; Fernandez, S.J.; Velasco, P.T.; Lacor, P.N.; Bigio, E.H.; Jerecic, J.; Acton, P.J.; Shughrue, P.J.; et al. Alzheimer's Disease-Type Neuronal Tau Hyperphosphorylation Induced by Aβ Oligomers. *Neurobiol Aging* **2008**, *29*, 1334–1347.
71. Del C. Alonso, A.; Grundke-Iqbal, I.; Iqbal, K. Alzheimer's Disease Hyperphosphorylated Tau Sequesters Normal Tau into Tangles of Filaments and Disassembles Microtubules. *Nat Med* **1996**, *2*, 783–787.
72. Dong, Y.; Yu, H.; Li, X.; Bian, K.; Zheng, Y.; Dai, M.; Feng, X.; Sun, Y.; He, Y.; Yu, B.; et al. Hyperphosphorylated Tau Mediates Neuronal Death by Inducing Necroptosis and Inflammation in Alzheimer's Disease. *J Neuroinflammation* **2022**, *19*, 1–18.
73. Onyango, I.G.; Jauregui, G. V.; Čarná, M.; Bennett, J.P.; Stokin, G.B. Neuroinflammation in Alzheimer's Disease. *Biomedicines* **2021**, *9*.
74. Wareham, L.K.; Liddelow, S.A.; Temple, S.; Benowitz, L.I.; Di Polo, A.; Wellington, C.; Goldberg, J.L.; He, Z.; Duan, X.; Bu, G.; et al. Solving Neurodegeneration: Common

Mechanisms and Strategies for New Treatments. *Molecular Neurodegeneration 2022 17:1* **2022**, *17*, 1–29.

75. Zheng, Y.; Zhang, X.; Zhang, R.; Wang, Z.; Gan, J.; Gao, Q.; Yang, L.; Xu, P.; Jiang, X. Inflammatory Signaling Pathways in the Treatment of Alzheimer's Disease with Inhibitors, Natural Products and Metabolites (Review). *Int J Mol Med* **2023**, *52*.
76. García-Horsman, J.A. The Role of Prolyl Oligopeptidase, Understanding the Puzzle. **2020**, *8*.
77. Taraszkiewicz, A.; Sinkiewicz, I.; Sommer, A.; Staroszczyk, H. The Biological Role of Prolyl Oligopeptidase and the Procognitive Potential of Its Peptidic Inhibitors from Food Proteins. *Crit Rev Food Sci Nutr* **2023**, 1–14.
78. García-Horsman, J.A.; Männistö, P.T.; Venäläinen, J.I. On the Role of Prolyl Oligopeptidase in Health and Disease. **2006**.
79. Jaako, K.; Waniek, A.; Parik, K.; Klimaviciusa, L.; Aonurm-Helm, A.; Noortoots, A.; Anier, K.; Van Elzen, R.; Gérard, M.; Lambeir, A.M.; et al. Prolyl Endopeptidase Is Involved in the Degradation of Neural Cell Adhesion Molecules in Vitro. *J Cell Sci* **2016**, *129*, 3792–3802.
80. Natunen, T.A.; Gynther, M.; Rostalski, H.; Jaako, K.; Jalkanen, A.J. Extracellular Prolyl Oligopeptidase Derived from Activated Microglia Is a Potential Neuroprotection Target. *Basic Clin Pharmacol Toxicol* **2019**, *124*, 40–49.
81. Puris, E.; Jalkanen, A.; Auriola, S.; Loppi, S.; Korhonen, P.; Kanninen, K.M.; Malm, T.; Koistinaho, J.; Gynther, M. Systemic Inflammation Elevates Cytosolic Prolyl Oligopeptidase Protein Expression but Not Peptidase Activity in the Cerebral Cortices of Familial Alzheimer`s Disease Modeling Mice. *Brain Disord* **2022**, *6*, 100035.
82. Hannula, M.J.; Myöhänen, T.T.; Tenorio-Laranga, J.; Männistö, P.T.; Garcia-Horsman, J.A. Prolyl Oligopeptidase Colocalizes with α-Synuclein, β-Amyloid, Tau Protein and Astroglia in the Post-Mortem Brain Samples with Parkinson's and Alzheimer's Diseases. *Neuroscience* **2013**, *242*, 140–150.
83. Svarcbahs, R.; Jäntti, M.; Kilpeläinen, T.; Julku, U.H.; Urvas, L.; Kivioja, S.; Norrbacka, S.; Myöhänen, T.T. Prolyl Oligopeptidase Inhibition Activates Autophagy via Protein Phosphatase 2A. *Pharmacol Res* **2020**, *151*, 104558.
84. Svarcbahs, R.; Julku, U.; Kilpeläinen, T.; Kyyrö, M.; Jäntti, M.; Myöhänen, T.T. New Tricks of Prolyl Oligopeptidase Inhibitors – A Common Drug Therapy for Several Neurodegenerative Diseases. *Biochem Pharmacol* **2019**, *161*, 113–120.
85. Lawandi, J.; Gerber-Lemaire, S.; Juillerat-Jeanneret, L.; Moitessier, N. Inhibitors of Prolyl Oligopeptidases for the Therapy of Human Diseases: Defining Diseases and Inhibitors. *J Med Chem* **2010**, *53*, 3423–3438.
86. Colletti, A.; Cicero, A.F.G. Nutraceutical Approach to Chronic Osteoarthritis: From Molecular Research to Clinical Evidence. *International Journal of Molecular Sciences 2021, Vol. 22, Page 12920* **2021**, *22*, 12920.
87. Rivellese, A.A.; Ciciola, P.; Costabile, G.; Vetrani, C.; Vitale, M. The Possible Role of Nutraceuticals in the Prevention of Cardiovascular Disease. *High Blood Pressure & Cardiovascular Prevention* **2019**, *26*, 101–111.
88. Rochlani, Y.; Pothineni, N.V.; Kovelamudi, S.; Mehta, J.L. Metabolic Syndrome: Pathophysiology, Management, and Modulation by Natural Compounds. *Ther Adv Cardiovasc Dis* **2017**, *11*, 215–225.
89. Alali, M.; Alqubaisy, M.; Aljaafari, M.N.; Alali, A.O.; Baqais, L.; Molouki, A.; Abushelaibi, A.; Lai, K.S.; Lim, S.H.E. Nutraceuticals: Transformation of Conventional

Foods into Health Promoters/Disease Preventers and Safety Considerations. *Molecules* **2021**, *26*.

90. Dini, I.; Laneri, S. Nutricosmetics: A Brief Overview. *Phytotherapy Research* **2019**, *33*, 3054–3063.
91. Pons, Z.; Margalef, M.; Bravo, F.I.; Arola-Arnal, A.; Muguerza, B. Chronic Administration of Grape-Seed Polyphenols Attenuates the Development of Hypertension and Improves Other Cardiometabolic Risk Factors Associated with the Metabolic Syndrome in Cafeteria Diet-Fed Rats. *British Journal of Nutrition* **2017**, *117*, 200–208.
92. Katayama, S.; Nakamura, S. Emerging Roles of Bioactive Peptides on Brain Health Promotion. *Int J Food Sci Technol* **2019**, *54*, 1949–1955.
93. Hayes, M.; Aluko, R.E.; Aurino, E.; Mora, L. Generation of Bioactive Peptides from Porphyridium Sp. and Assessment of Their Potential for Use in the Prevention of Hypertension, Inflammation and Pain. *Mar Drugs* **2023**, *21*, 422.
94. Chakrabarti, S.; Guha, S.; Majumder, K. Food-Derived Bioactive Peptides in Human Health: Challenges and Opportunities. *Nutrients* **2018**, *10*.
95. Ulug, S.K.; Jahandideh, F.; Wu, J. Novel Technologies for the Production of Bioactive Peptides. *Trends Food Sci Technol* **2021**, *108*, 27–39.
96. Chatterjee, C.; Gleddie, S.; Xiao, C.-W. Soybean Bioactive Peptides and Their Functional Properties. *Nutrients* **2018**, *10*, 1211.
97. Liu, Y.-F.; Oey, I.; Bremer, P.; Carne, A.; Silcock, P. Bioactive Peptides Derived from Egg Proteins: A Review. *Crit Rev Food Sci Nutr* **2018**, *58*, 2508–2530.
98. Muro Urista, C.; Álvarez Fernández, R.; Riera Rodriguez, F.; Arana Cuenca, A.; Téllez Jurado, A. Review: Production and Functionality of Active Peptides from Milk. *Food Sci Technol Int* **2011**, *17*, 293–317.
99. Admassu, H.; Gasmalla, M.Abdalbasit.A.; Yang, R.; Zhao, W. Bioactive Peptides Derived from Seaweed Protein and Their Health Benefits: Antihypertensive, Antioxidant, and Antidiabetic Properties. *J Food Sci* **2018**, *83*, 6–16.
100. Li, M.; Ge, Q.; Du, H.; Lin, S. Tricholoma Matsutake-Derived Peptides Ameliorate Inflammation and Mitochondrial Dysfunction in RAW264.7 Macrophages by Modulating the NF-KB/COX-2 Pathway. *Foods* **2021**, *10*.
101. Toldrá, F.; Gallego, M.; Reig, M.; Aristoy, M.-C.; Mora, L. Recent Progress in Enzymatic Release of Peptides in Foods of Animal Origin and Assessment of Bioactivity. *J Agric Food Chem* **2020**, *68*, 12842–12855.
102. Ribeiro, T.B.; Voss, G.B.; Coelho, M.C.; Pintado, M.E. Food Waste and By-Product Valorization as an Integrated Approach with Zero Waste: Future Challenges. *Future Foods: Global Trends, Opportunities, and Sustainability Challenges* **2022**, 569–596.
103. Etemadian, Y.; Ghaemi, V.; Shaviklo, A.R.; Pourashouri, P.; Sadeghi Mahoonak, A.R.; Rafipour, F. Development of Animal/ Plant-Based Protein Hydrolysate and Its Application in Food, Feed and Nutraceutical Industries: State of the Art. *J Clean Prod* **2021**, *278*, 123219.
104. Ramakrishnan, S.R.; Jeong, C.-R.; Park, J.-W.; Cho, S.-S.; Kim, S.-J. A Review on the Processing of Functional Proteins or Peptides Derived from Fish By-Products and Their Industrial Applications. *Heliyon* **2023**, *9*, e14188.
105. Martorell, P.; Bataller, E.; Llopis, S.; Gonzalez, N.; Alvarez, B.; Montón, F.; Ortiz, P.; Ramón, D.; Genovés, S. A Cocoa Peptide Protects Caenorhabditis Elegans from Oxidative Stress and β-Amyloid Peptide Toxicity. *PLoS One* **2013**, *8*, e63283.

106. Bravo, F.I.; Mas-Capdevila, A.; López-Fernández-Sobrino, R.; Torres-Fuentes, C.; Mulero, M.; Alcaide-Hidalgo, J.M.; Muguerza, B. Identification of Novel Antihypertensive Peptides from Wine Lees Hydrolysate. *Food Chem* **2022**, *366*, 130690.
107. Mora, L.; Reig, M.; Toldrá, F. Bioactive Peptides Generated from Meat Industry By-Products. *Food Research International* **2014**, *65*, 344–349.
108. OECD-FAO Agricultural Outlook **2021-2030**.
109. Li, Y.; Arulnathan, V.; Heidari, M.D.; Pelletier, N. Design Considerations for Net Zero Energy Buildings for Intensive, Confined Poultry Production: A Review of Current Insights, Knowledge Gaps, and Future Directions. *Renewable and Sustainable Energy Reviews* **2022**, *154*, 111874.
110. Siadat, S.M.; Ruberti, J.W. Mechanochemistry of Collagen. *Acta Biomater* **2023**, *163*, 50–62.
111. Kirkness, M.W.; Lehmann, K.; Forde, N.R. Mechanics and Structural Stability of the Collagen Triple Helix. *Curr Opin Chem Biol* **2019**, *53*, 98-105.
112. Shoulders, M.D.; Raines, R.T. Collagen Structure and Stability. *Annu Rev Biochem* **2009**, *78*, 929–958.
113. Espinales, C.; Romero-Peña, M.; Calderón, G.; Vergara, K.; Cáceres, P.J.; Castillo, P. Collagen, Protein Hydrolysates and Chitin from by-Products of Fish and Shellfish: An Overview. *Heliyon* **2023**, *9*, e14937.
114. Dias Campos, L.; De Almeida, V.; Junior, S.; Demuner Pimentel, J.; Lusi, G.; Carregã, F.; Baú, C.; Cazarin, B. Collagen Supplementation in Skin and Orthopedic Diseases: A Review of the Literature. *Heliyon* **2023**, *9*, 2405–8440.
115. Barati, M.; Jabbari, M.; Navekar, R.; Farahmand, F.; Zeinalian, R.; Salehi-Sahlabadi, A.; Abbaszadeh, N.; Mokari-Yamchi, A.; Davoodi, S.H. Collagen Supplementation for Skin Health: A Mechanistic Systematic Review. *J Cosmet Dermatol* **2020**, *19*, 2820–2829.
116. de Miranda, R.B.; Weimer, P.; Rossi, R.C. Effects of Hydrolyzed Collagen Supplementation on Skin Aging: A Systematic Review and Meta-Analysis. *Int J Dermatol* **2021**, *60*, 1449–1461.
117. Lugo, J.P.; Saiyed, Z.M.; Lane, N.E. Efficacy and Tolerability of an Undenatured Type II Collagen Supplement in Modulating Knee Osteoarthritis Symptoms: A Multicenter Randomized, Double-Blind, Placebo-Controlled Study. *Nutr J* **2016**, *15*, 14.
118. Schauss, A.G.; Stenehjem, J.; Park, J.; Endres, J.R.; Clewell, A. Effect of the Novel Low Molecular Weight Hydrolyzed Chicken Sternal Cartilage Extract, BioCell Collagen, on Improving Osteoarthritis-Related Symptoms: A Randomized, Double-Blind, Placebo-Controlled Trial. *J Agric Food Chem* **2012**, *60*, 4096–4101.
119. Pu, S.Y.; Huang, Y.L.; Pu, C.M.; Kang, Y.N.; Hoang, K.D.; Chen, K.H.; Chen, C. Effects of Oral Collagen for Skin Anti-Aging: A Systematic Review and Meta-Analysis. *Nutrients* **2023**, *15*.
120. Brandao-Rangel, M.A.R.; Oliveira, C.R.; da Silva Olímpio, F.R.; Aimbire, F.; Mateus-Silva, J.R.; Chaluppe, F.A.; Vieira, R.P. Hydrolyzed Collagen Induces an Anti-Inflammatory Response That Induces Proliferation of Skin Fibroblast and Keratinocytes. *Nutrients* **2022**, *14*, 4975.
121. Olalere, O.A.; Yap, P.-G.; Gan, C.-Y. Comprehensive Review on Some Food-Derived Bioactive Peptides with Anti-Hypertension Therapeutic Potential for Angiotensin-Converting Enzyme (ACE) Inhibition. *Journal of Proteins and Proteomics 2023 14:2* **2023**, *14*, 129–161.

122. Nasri, M. Protein Hydrolysates and Biopeptides: Production, Biological Activities, and Applications in Foods and Health Benefits. A Review. *Adv Food Nutr Res* **2017**, *81*, 109–159.
123. Mora, L.; Toldrá, F. Advanced Enzymatic Hydrolysis of Food Proteins for the Production of Bioactive Peptides. *Curr Opin Food Sci* **2023**, *49*, 100973.
124. Lin, K.; Ma, Z.; Ramachandran, M.; De Souza, C.; Han, X.; Zhang, L. wei ACE Inhibitory Peptide KYIPIQ Derived from Yak Milk Casein Induces Nitric Oxide Production in HUVECs and Diffuses via a Transcellular Mechanism in Caco-2 Monolayers. *Process Biochemistry* **2020**, *99*, 103–111.
125. Kim, S.E.; Kim, H.H.; Kim, J.Y.; Kang, Y.I.; Woo, H.J.; Lee, H.J. Anticancer Activity of Hydrophobic Peptides from Soy Proteins. *Biofactors* **2000**, *12*, 151–155.
126. Toldrá, F.; Reig, M.; Aristoy, M.-C.; Mora, L. Generation of Bioactive Peptides during Food Processing. **2017**.
127. Le Maux, S.; Nongonierma, A.B.; Lardeux, C.; FitzGerald, R.J. Impact of Enzyme Inactivation Conditions during the Generation of Whey Protein Hydrolysates on Their Physicochemical and Bioactive Properties. *Int J Food Sci Technol* **2018**, *53*, 219–227.
128. Hong, H.; Fan, H.; Chalamaiah, M.; Wu, J. Preparation of Low-Molecular-Weight, Collagen Hydrolysates (Peptides): Current Progress, Challenges, and Future Perspectives. *Food Chem* **2019**, *301*, 125222.
129. Esfandi, R.; Walters, M.E.; Tsopmo, A. Antioxidant Properties and Potential Mechanisms of Hydrolyzed Proteins and Peptides from Cereals. *Heliyon* **2019**, *5*, e01538.
130. Casanova-Martí, À.; Bravo, F.I.; Serrano, J.; Ardévol, A.; Pinent, M.; Muguerza, B. Antihyperglycemic Effect of a Chicken Feet Hydrolysate via the Incretin System: DPP-IV-Inhibitory Activity and GLP-1 Release Stimulation. *Food Funct* **2019**, *10*, 4062–4070.
131. Tawalbeh, D.; Al-U'datt, M.H.; Wan Ahmad, W.A.N.; Ahmad, F.; Sarbon, N.M. Recent Advances in In Vitro and In Vivo Studies of Antioxidant, ACE-Inhibitory and Anti-Inflammatory Peptides from Legume Protein Hydrolysates. *Molecules* **2023**, *28*, 2423.
132. Mansinhbhai, C.H.; Sakure, A.; Liu, Z.; Maurya, R.; Das, S.; Basaiawmoit, B.; Bishnoi, M.; Kondepudi, K.K.; Padhi, S.; Rai, A.K.; et al. Anti-Inflammatory, ACE Inhibitory, Antioxidative Activities and Release of Novel Antihypertensive and Antioxidative Peptides from Whey Protein Hydrolysate with Molecular Interactions. *Journal of the American Nutrition Association* **2022**, 1–15.
133. Inkanuwat, A.; Sukaboon, R.; Reamtong, O.; Asawanonda, P.; Pattaratanakun, A.; Saisavoey, T.; Sangtanoo, P.; Karnchanatat, A. Nitric Oxide Synthesis Inhibition and Anti-Inflammatory Effect of Polypeptide Isolated from Chicken Feather Meal in Lipopolysaccharide-Stimulated RAW 264.7 Macrophages. *Food Technol Biotechnol* **2019**, *57*, 200–212.
134. Manzoor, M.; Singh, J.; Gani, A. Exploration of Bioactive Peptides from Various Origin as Promising Nutraceutical Treasures: In Vitro, in Silico and in Vivo Studies. *Food Chem* **2022**, *373*, 131395.
135. Álvarez-Olguín, M.A.; Beltrán-Barrientos, L.M.; Hernandez-Mendoza, A.; González-Córdova, A.F.; Vallejo-Cordoba, B. Current Trends and Perspectives on Bioaccessibility and Bioavailability of Food Bioactive Peptides: In Vitro and Ex Vivo Studies. *J Sci Food Agric* **2022**, *102*, 6824–6834.
136. Giraldo, N.D.; Sánchez, J.D.; López, A.; González, N.; Balaguer, F.; Redondo, J.; Llopis, S.; Barrena, M.; Klotz, B. Functional Assessment of a Whey Protein Hydrolysate: In Vivo Responses in Lipid Regulation, Oxidant Defense, and Neural Protection in Caenorhabditis Elegans. *Int Dairy J* **2024**, *154*, 105921.

137. Wang, S.; Su, G.; Fan, J.; Xiao, Z.; Zheng, L.; Zhao, M.; Wu, J. Arginine-Containing Peptides Derived from Walnut Protein Against Cognitive and Memory Impairment in Scopolamine-Induced Zebrafish: Design, Release, and Neuroprotection. *J Agric Food Chem* **2022**, *70*, 11579–11590.
138. Recio, I.; del Mar Contreras, M.; Gómez-Sala, B. Effect of a Casein Hydrolysate Containing Novel Peptides in Hypertensive Subjects. *Ann Nutr Metab* **2011**, *58*, 1–444.
139. Rahabi, M.; Salon, M.; Bruno-Bonnet, C.; Prat, M.; Jacquemin, G.; Benmoussa, K.; Alaeddine, M.; Parny, M.; Bernad, J.; Bertrand, B.; et al. Bioactive Fish Collagen Peptides Weaken Intestinal Inflammation by Orienting Colonic Macrophages Phenotype through Mannose Receptor Activation. *Eur J Nutr* **2022**, *61*, 2051–2066.
140. Pai, V.V.; Bhandari, P.; Shukla, P. Topical Peptides as Cosmeceuticals. *Indian J Dermatol Venereol Leprol* **2017**, *83*, 9–18.
141. Dini, I.; Mancusi, A. Food Peptides for the Nutricosmetic Industry. *Antioxidants* **2023**, *12*, 788.
142. Aguirre-Cruz, G.; León-López, A.; Cruz-Gómez, V.; Jiménez-Alvarado, R.; Aguirre-Álvarez, G. Collagen Hydrolysates for Skin Protection: Oral Administration and Topical Formulation. *Antioxidants 2020, Vol. 9, Page 181* **2020**, *9*, 181.
143. Rivera-Jiménez, J.; Berraquero-García, C.; Pérez-Gálvez, R.; García-Moreno, P.J.; Espejo-Carpio, F.J.; Guadix, A.; Guadix, E.M. Peptides and Protein Hydrolysates Exhibiting Anti-Inflammatory Activity: Sources, Structural Features and Modulation Mechanisms. *Food Funct* **2022**, *13*, 12510–12540.
144. Subhan, F.; Kang, H.Y.; Lim, Y.; Ikram, M.; Baek, S.-Y.; Jin, S.; Jeong, Y.H.; Kwak, J.Y.; Yoon, S. Fish Scale Collagen Peptides Protect against $CoCl_2$/TNF-α-Induced Cytotoxicity and Inflammation via Inhibition of ROS, MAPK, and NF-KB Pathways in HaCaT Cells. *Oxid Med Cell Longev* **2017**, *2017*.
145. Sivaraman, K.; Shanthi, C. Role of Fish Collagen Hydrolysate in Attenuating Inflammation—An in Vitro Study. *J Food Biochem* **2021**, *45*, e13876.
146. Cho, W.; Park, J.; Kim, J.; Lee, M.; Park, S.J.; Kim, K.S.; Jun, W.; Kim, O.-K.; Lee, J. Low-Molecular-Weight Fish Collagen Peptide (Valine-Glycine-Proline-Hydroxyproline-Glycine-Proline-Alanine-Glycine) Prevents Osteoarthritis Symptoms in Chondrocytes and Monoiodoacetate-Injected Rats. *Mar Drugs* **2023**, *21*.
147. Liu, H.; Li, B. Separation and Identification of Collagen Peptides Derived from Enzymatic Hydrolysate of Salmo Salar Skin and Their Anti-inflammatory Activity in Lipopolysaccharide (LPS)-induced RAW264.7 Inflammatory Model. *J Food Biochem* **2022**, *46*.
148. Lafarga, T.; Hayes, M. Effect of Pre-Treatment on the Generation of Dipeptidyl Peptidase-IV- and Prolyl Endopeptidase-Inhibitory Hydrolysates from Bovine Lung. *Journal of Agricultural and Food Research* **2017**, *56*, 12–24.
149. Hsieh, C.-H.; Wang, T.-Y.; Hung, C.-C.; Hsieh, Y.-L.; Hsu, K.-C. Isolation of Prolyl Endopeptidase Inhibitory Peptides from a Sodium Caseinate Hydrolysate. *Food Funct* **2016**, *7*, 565–573.
150. Sila, A.; Martinez-Alvarez, O.; Haddar, A.; Gómez-Guillén, M.C.; Nasri, M.; Montero, M.P.; Bougatef, A. Recovery, Viscoelastic and Functional Properties of Barbel Skin Gelatine: Investigation of Anti-DPP-IV and Anti-Prolyl Endopeptidase Activities of Generated Gelatine Polypeptides. *Food Chem* **2015**, *168*, 478–486.
151. Kouguchi, T.; Ito, A.; Iwai, K.; Shimizu, M.; Takahata, Y.; Suzuki, T.; Morimatsu, F.; Tanabe, S. Chicken Collagen Hydrolysate-Derived Peptides Inhibit Tumor Necrosis

Factor-α-Induced Inflammatory Response in Endothelial Cells. *Food Sci Technol Res* **2012**, *18*, 667–671.

152. Daskalaki, M.G.; Axarlis, K.; Aspevik, T.; Orfanakis, M.; Kolliniati, O.; Lapi, I.; Tzardi, M.; Dermitzaki, E.; Venihaki, M.; Kousoulaki, K.; et al. Fish Sidestream-Derived Protein Hydrolysates Suppress DSS-Induced Colitis by Modulating Intestinal Inflammation in Mice. *Mar Drugs* **2021**, *19*.
153. Zhou, C.; Gao, Y.; Ding, P.; Wu, T.; Ji, G. The Role of CXCL Family Members in Different Diseases. *Cell Death Discovery 2023 9:1* **2023**, *9*, 1–12.
154. Xu, D.; Zhao, M. Photoaging Induced by Long-Term Exposure to UV Irradiation and Amelioration by *Skipjack Tuna Skin* Hydrolysates: Targeting Inhibition of MAPK and NF-KB Signaling Hyperactivation. *J Funct Foods* **2022**, *98*.
155. Xu, D.; Zhao, M.; Lin, H.; Li, C. *Theragra Chalcogramma* Hydrolysates, Rich in Gly-Leu-Pro-Ser-Tyr-Thr, Exerts Anti-Photoaging Potential via Targeting MAPK and NF-KB Pathways in SD Rats. *Mar Drugs* **2022**, *20*.
156. Woo, M.; Seol, B.G.; Kang, K.-H.; Choi, Y.H.; Cho, E.J.; Noh, J.S. Effects of Collagen Peptides from Skate (*Raja Kenojei*) Skin on Improvements of the Insulin Signaling Pathway *via* Attenuation of Oxidative Stress and Inflammation. *Food Funct* **2020**, *11*, 2017–2025.
157. Toda, K. Are Topical Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) Safer than Oral NSAIDs? Or Are the Adverse Effects of Topical NSAIDs Comparable to Those of Oral NSAIDs? *Osteoarthritis Cartilage* **2021**, *29*, 1624–1625.
158. Zhang, Y.; Kouguchi, T.; Shimizu, K.; Sato, M.; Takahata, Y.; Mormatsu, F. Chicken Collagen Hydrolysate Reduces Proinflammatory Cytokine Production in C57BL/6.KOR-ApoE*shI* Mice. *J Nutr Sci Vitaminol (Tokyo)* **2010**, *56*, 208–210.

HYPOTHESIS AND OBJECTIVES

UNIVERSITAT ROVIRA I VIRGILI
PROTEIN HYDROLYSATES AND PEPTIDES FROM ANIMAL BY-PRODUCTS AS A PREVENTIVE STRATEGY FOR LOCAL INFLAMMATION AND NEUROINFLAMMATION
Néstor Ibarz Blanch

Inflammation is a common factor in multiple pathologies including metabolic syndrome-associated diseases and arthritis. NSAIDs, which target COX, are commonly used to alleviate inflammation, pain, and fever. However, many of these drugs do not selectively inhibit COX-2, which is involved in the inflammatory processes. In contrast, COX-1 inhibition is associated with different side effects. Therefore, there is a trend towards the development of new selective COX-2 inhibitors. Moreover, inflammation is present in neurodegenerative diseases, where it enhances the synthesis of different enzymes such as POP. Inhibition of this enzyme, which is associated with AD, has been targeted in many anti-neurodegenerative therapies.

In the search for new anti-inflammatory compounds, those obtained from natural sources, such as bioactive peptides, are preferred. These compounds have shown a plethora of biological activities, including anti-inflammatory and neuroprotective effects. Some have already been used as functional ingredients in food, nutraceuticals, and cosmetic creams. These bioactive peptides can be obtained from different sources, including food and agri-food by-products, which contribute to their valorization. In this regard, collagen, an important protein in animal agri-food by-products, is an interesting source of bioactive peptides because of its high hydrophobic amino acid content. Peptides containing these amino acids, such as Pro, have been associated with an increase in the potential to inhibit enzymes involved in the development of different non-communicable diseases, such as POP. Moreover, hydrolyzed collagen is widely consumed in skin and joint care. However, the potential of collagen-derived hydrolysates to exhibit acute anti-inflammatory effects after topical application and their neuroprotective effects *in vivo* remain unexplored. Furthermore, although peptides are used to enhance the skin penetration of compounds, the bioactivity of protein hydrolysates upon topical application is uncertain.

Considering this evidence, the hypothesis of this thesis was that **collagen-rich by-products are a good source for the elaboration of protein hydrolysates containing peptides, which can prevent inflammation and inflammation-related diseases such as muscle inflammation, swelling, arthritis, or AD.**

To test this hypothesis, the main objective proposed in this doctoral thesis was to obtain protein hydrolysates from several collagen-rich sources with focal anti-inflammatory properties after topical application or neuroprotective effects *in vivo* and to identify their bioactive peptides.

In order to achieve this aim, the following specific objectives were proposed:

1. **To develop a pigskin-collagen hydrolysate that can prevent focal acute inflammation after topical application and to identify the underlying mechanisms and its bioactive peptides (*Chapter 1*).**

Protein hydrolysis is required for the release of bioactive peptides from the native proteins. Hydrolysis conditions, such as enzyme type, enzyme/substrate ratio, temperature, and hydrolysis time, determine the release of different amino acid sequences, which can exert different bioactivities and grades of bioactivity. Thus, the establishment of optimal hydrolysis conditions is important to obtain COX-2 inhibitory peptides. It must be highlighted that the evaluation of the anti-inflammatory and antinociceptive effects of hydrolysates or peptides after topical application cannot be tested *in vitro*, and *in vivo* models are needed. Carrageenan-induced paw edema and the warm-water tail withdrawal test are some of the most commonly used models to evaluate the mentioned bioactivities. Moreover, the mechanisms underlying the anti-inflammatory effects of protein hydrolysates may be diverse because of the high number of peptides that they contain and its evaluation may be not possible *in vivo*. Thus, a useful *in vitro* model of inflammation is the LPS-stimulated RAW 264.7 cells. Finally, considering that protein hydrolysates contain high amounts of bioactive peptides, the identification of these peptides is very interesting to characterize the hydrolysate and use these peptides as functional compounds in the pharmacological industry. Considering these facts, the following aims were proposed:

a. To determine the optimal hydrolysis conditions for obtaining a pigskin-collagen hydrolysate with selective COX-2 inhibitory activity and the ability to prevent or ameliorate carrageenan-induced acute inflammation and response to acute painful stimuli. [Manuscript 1]
b. To establish the mechanisms underlying the anti-inflammatory effects of the most active pigskin-collagen hydrolysate in LPS-stimulated RAW 264.7 cells. [Manuscript 1]
c. To identify the peptides responsible for the anti-inflammatory effects of the most active pigskin-collagen hydrolysate, evaluating their bioactivity in LPS-stimulated RAW 264.7 cells. [Manuscript 2]

2. **To develop a hydrolysate from a chicken by-product that can prevent the development of Alzheimer's disease and identify the peptides that it contains (*Chapter 2)*.**

Tons of chicken by-products are generated every year. Some of these by-products are rich in proteins, specifically collagen. Different studies have shown that they can be sources of bioactive peptides. Known bioactive peptides with POP and neuroprotective activities contain at least one proline residue in their sequence. The high content of this amino acid in some chicken by-products makes them a potential source of POP inhibitors. However, the selection of the raw material and optimal hydrolysis conditions should be explored to obtain POPi peptides. AD pathophysiology is characterized by the accumulation of Aβ plaques in a scenario of neuroinflammation, where the POP enzyme is highly expressed. Given the connection between POP and Aβ aggregates, a good *in vivo* model to evaluate the neuroprotective effects of POP inhibitors is the Aβ-induced paralysis in the CL4176 mutant strain of *Caenorhabditis elegans*. Finally, considering that protein hydrolysates contain high amounts of bioactive peptides, the identification of these peptides is very interesting to characterize the hydrolysate and to use these peptides as functional compounds in the pharmacological industry. Therefore, the following objectives were proposed:

a. To review the potential of chicken by-products as sources of bioactive peptides and select the best candidate for generating POPi peptides. [Manuscript 3]
b. To obtain a hydrolysate from chicken feet for the prevention of Alzheimer's disease. [Manuscript 4]
    (i) To determine the optimal hydrolysis conditions to obtain chicken-foot hydrolysates with POPi activity and select the most active ones.
    (ii) To assess the effect of the selected chicken-foot hydrolysates on Aß-induced paralysis in the *Caenorhabditis elegans* CL4176 mutant strain.
    (iii) To identify the peptides responsible for the neuroprotective effects of the selected chicken-foot hydrolysate.

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EXPERIMENTAL DESIGNS

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Néstor Ibarz Blanch

Different experimental methodologies were used to assess the hypothesis and achieve the objectives proposed in this doctoral thesis.

1. Obtainment of selective COX-2 inhibitory hydrolysates from pigskin collagen with local acute anti-inflammatory effects after topical application and evaluation of the mechanisms underlying its bioactivity.

The experimental design for the elaboration of pigskin collagen-derived peptides with selective COX-2 inhibitory activity, *in vivo* anti-inflammatory and antinociceptive effects, and the evaluation of the anti-inflammatory mechanism is represented in Figure 6. Pigskin collagen dissolved in distilled water was subjected to enzymatic hydrolysis under different conditions. Then, the *in vitro* COX-2 inhibitory activity of the hydrolysates was evaluated. The hydrolysate with the highest COX-2 inhibitory activity (H8) was produced at a pilot scale (H8p) and the peptide fraction of <10 kDa was obtained. The inhibitory effects of these two samples on *in vitro* COX enzymes were assessed. Their acute anti-inflammatory effects were further evaluated in a carrageenan-induced paw edema model using a plethysmometer. Moreover, the mechanisms underlying the anti-inflammatory effects of the peptide fraction were studied in LPS-stimulated RAW 264.7 macrophages, by measuring nitrite production, COX-1 and COX-2 activities, and iNOS and COX-2 protein expression. Additionally, the antinociceptive effect of this peptide fraction was evaluated *in vivo* using an acute pain model (warm-water tail withdrawal).

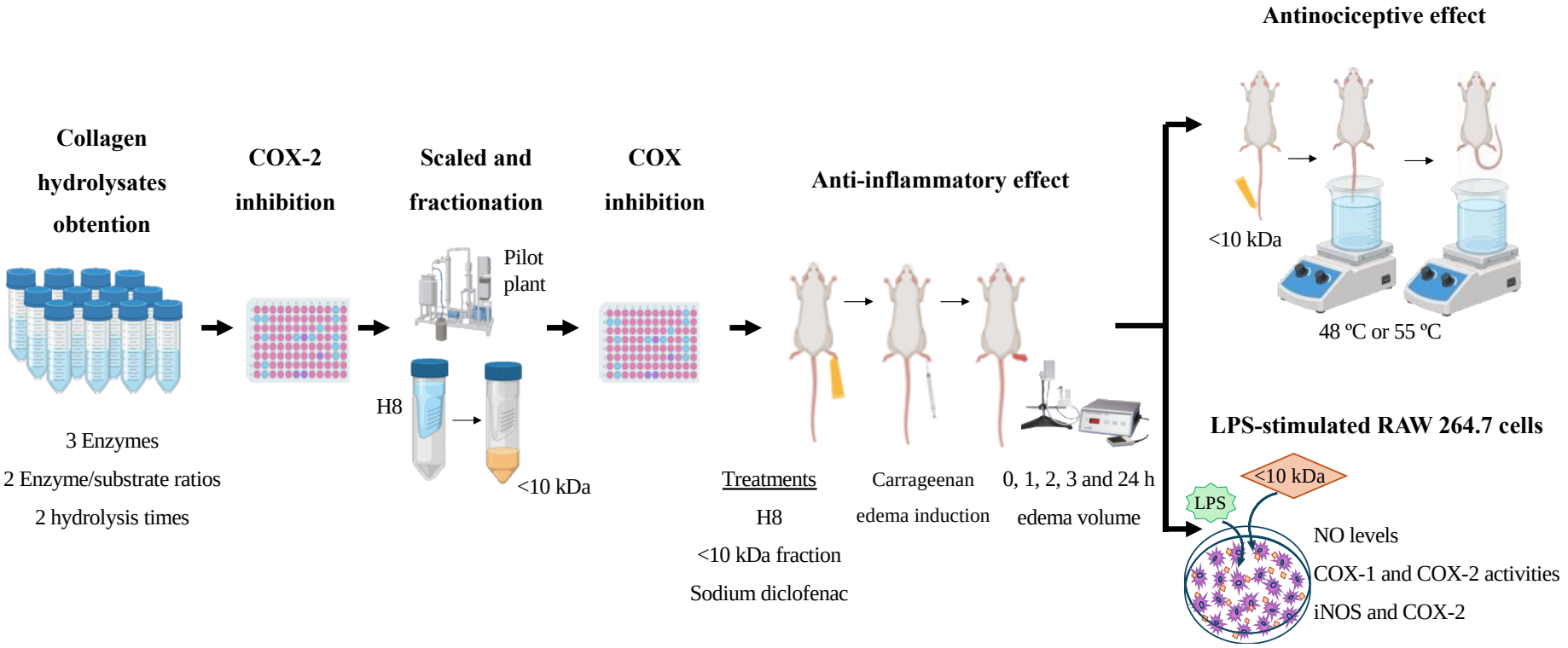

Figure 6: Experimental design to obtain a collagen hydrolysate with *in vitro* selective cyclooxygenase-2 (COX-2) inhibition, anti-inflammatory and antinociceptive effects after topical application and the mechanisms involved. Abbreviations: iNOS (inducible nitric oxide synthase), NO: nitric oxide; LPS (lipopolysaccharide).

2. Identification of anti-inflammatory peptides present in the <10 kDa fraction of H8p collagen hydrolysate.

Peptide identification of the <10 kDa fraction of the H8p collagen hydrolysate was performed to identify the peptides potentially responsible for its anti-inflammatory effects. The experimental procedure is illustrated in Figure 7. Briefly, the <3 kDa fraction of the H8p hydrolysate was fractionated using RP-HPLC, and 22 fractions were obtained. The COX-2 inhibitory activity of the fractions was measured, and those showing the highest inhibition (F3 and F20) were further analyzed using UHPLC-ORBITRAP (MS/MS) to identify their peptides. The identified sequences were chemically synthesized and their *in vitro* COX-2 and COX-1 inhibitory activities were tested. Moreover, its anti-inflammatory effects were evaluated in LPS-stimulated RAW 264.7 macrophages incubated with the peptides by measuring the nitric oxide production and the protein expression of iNOS and COX-2.

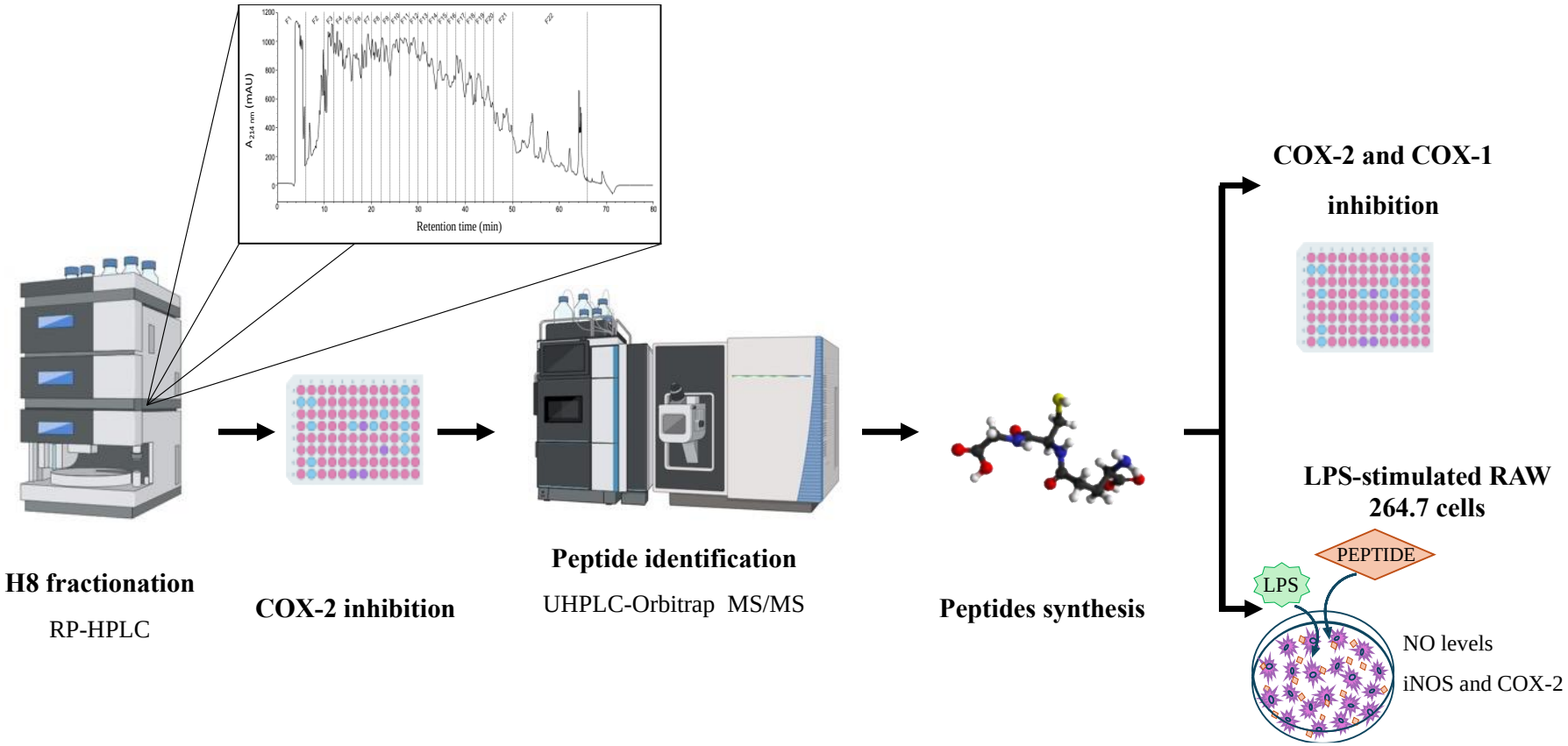

Figure 7: Experimental design for the identification of anti-inflammatory peptides from H8p collagen hydrolysate. Abbreviations: COX (cyclooxygenase), LPS (lipopolysaccharide), iNOS (inducible nitric oxide synthase).

3. Obtainment of chicken-foot hydrolysates with prolyl oligopeptidase inhibitory activity and neuroprotective effects.

The experimental design for the elaboration of chicken foot hydrolysates with POPi activity and neuroprotective effects is shown in **Figure 8**. Chicken feet were minced, boiled, and subjected to different hydrolysis conditions. The POPi activity of the hydrolysates was determined. The hydrolysates with the highest bioactivity (CFH3 and CFH5) were selected to evaluate their potential neuroprotective effects in the *C. elegans* CL4176 mutant strain, by measuring paralyzed worms for 12 h. Three concentrations were tested (0.5, 1, and 5 mg/mL) and unhydrolyzed chicken-foot proteins were used as control. The chicken-foot hydrolysate CFH3 was fractionated by RP-HPLC, and POPi activity was evaluated for all 16 fractions obtained. The peptides in the fraction with the greatest bioactivity, F15, were identified by UHPLC-ORBITRAP (MS/MS). The potential bioactivity of the identified peptides was predicted using the PeptideRanker.

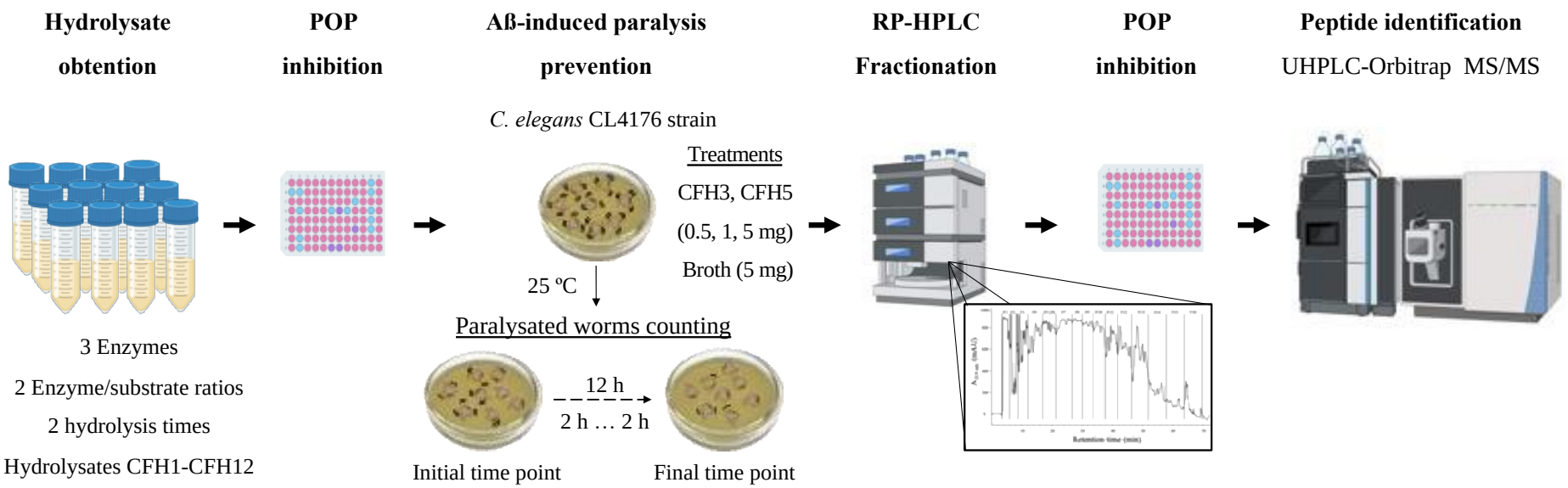

Figure 8: Experimental design for the obtainment of chicken-foot hydrolysates with prolyl oligopeptidase (POP) inhibitory activity and neuroprotective effects in *Caenorhabditis elegans* CL4176 strain.

RESULTS

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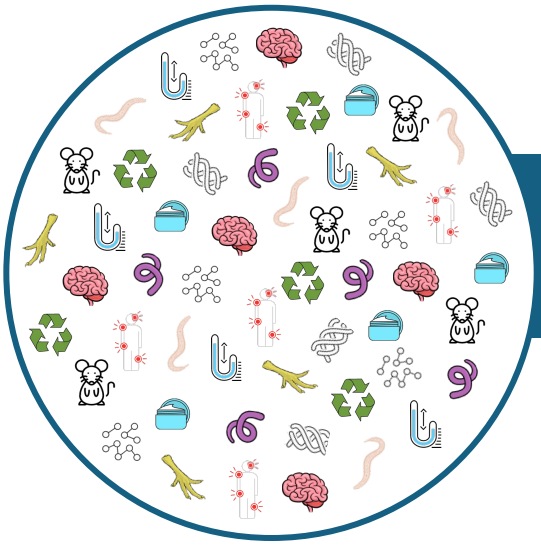

Chapter 1

OBJECTIVE:

To develop a pigskin-collagen hydrolysate that can prevent focal acute inflammation after topical application and to identify the underlying mechanisms and its bioactive peptides.

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Manuscript 1

Objective: To determine the optimal hydrolysis conditions for obtaining a pigskin-collagen hydrolysate with selective COX-2 inhibitory activity able to prevent local inflammation and acute pain, and to establish the mechanisms underlying the anti-inflammatory effects in LPS-stimulated RAW 264.7 cells.

Anti-inflammatory and anti-nociceptive effects of a selective COX-2 inhibitory collagen hydrolysate fraction

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Abstract

The side effects of current anti-inflammatory treatments have led to the development of novel therapies, including selective cyclooxygenase 2 (COX-2) inhibitors. Dietary bioactive peptides are considered a new and less noxious anti-inflammatory approach. However, there is no evidence that these peptides exert local anti-inflammatory effects following topical application. This study aimed to obtain a collagen hydrolysate with anti-inflammatory effects after topical application and evaluate the mechanisms involved in its effect. Twelve hydrolysates were obtained from pigskin collagen under several hydrolysis conditions. The hydrolysate H8 was selected for its *in vitro* cyclooxygenase 2 (COX-2) inhibitory activity. At a pilot scale, hydrolysate H8 (H8p) and its <10 kDa fraction showed selective COX-2 inhibition. Moreover, topical application of the fraction (15 mg) reduced the carrageenan-induced paw edema, mainly at 1 and 2 h post-carrageenan injection (53.7 and 38.1%, respectively). This effect was quicker and more pronounced than that of sodium diclofenac. In LPS-stimulated RAW 264.7, the fraction exerted selective COX-2 inhibitory activity and reduced the protein levels of this enzyme. Inducible nitric oxide synthase protein expression was also reduced, and this was reflected in the NO levels in the supernatant. Finally, the <10 kDa fraction, exhibited an anti-nociceptive effect in rats, retarding the tail-withdrawal time by 1.4-fold in an acute pain model (55 ºC). The results highlight the potential use of this collagen hydrolysate fraction as a novel anti-inflammatory and analgesic therapy.

Keywords: bioactive peptides, carrageenan-induced paw edema, cyclooxygenase-2 inhibition, LPS-stimulated RAW 264.7 cells, tail-withdrawal

1. Introduction

Inflammation is a natural response of an organism to noxious stimuli or conditions such as infection, tissue damage, or stress [1]. Inflammatory responses include vasodilation and vascular permeabilization to facilitate immune cell migration and recruitment (edema formation) and secretion of inflammatory mediators, such as nitric oxide (NO), cytokines, and prostaglandins [2]. This process is tightly regulated by several mechanisms, pathways, and key molecules. One of the most important enzymes involved in this process is the cyclooxygenase (COX) enzyme, mainly isoform 2 (COX-2). COX enzymes catalyze the conversion of arachidonic acid into prostaglandin H_2 (PGH_2) through bis-oxygenation of arachidonic acid. PGH_2 serves as a precursor for various prostaglandins, thromboxanes, and prostacyclins, collectively known as eicosanoids [3]. Isoform 1 (COX-1) is constitutively expressed in many tissues and is mainly involved in the regulation of homeostatic functions through the production of thromboxane A2 and various prostaglandins (PGs) (PGD_2, $PGF_2\alpha$, PGI_2, and PGE_2) [3]. However, COX-2 is induced in response to inflammatory stimuli and is expressed in fibroblasts, activated macrophages, and other cells [4]. COX-2, in combination with microsomal PGE synthase 1, produces PGE_2, which is implicated in inflammation, fever, and pain. Moreover, it intensifies the inflammatory response by strengthening and prolonging the signals generated by other pro-inflammatory substances, such as bradykinin, histamine, or neurokinins [5].

Inflammation is commonly observed in numerous diseases and can lead to chronic inflammatory conditions when dysregulated [6,7]. For this purpose, some of the most used drugs are non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, naproxen, or diclofenac [8], that alleviate inflammation and pain by inhibiting COX activity [9]. However, long-term treatment with some of these drugs might produce different side effects in the gastrointestinal tract (ulcers, obstructions, perforations), cardiovascular events, and renal dysfunction [10]. Some of these effects have been associated with COX-1 inhibition, given that most of the NSAIDs inhibit both COX isoforms [11]. This has prompted the exploration of new selective inhibitors targeting COX-2, especially those of natural origin [9,12]. These compounds are more acceptable to consumers, usually linked to fewer side effects than synthetic drugs, and could prevent inflammation by acting in different pathways.

Bioactive peptides are small amino acid sequences naturally present on a native protein that have a beneficial effect on the organism when cleaved [13]. Many

bioactivities have been reported, including antihypertensive, antioxidant, antidiabetic, or anti-inflammatory activities [14]. The bioactivity of the peptides is linked to their intrinsic characteristics, such as specific amino acid composition and sequence, and the length [15]. Anti-inflammatory peptides included in protein hydrolysates have been obtained from different animal and vegetal sources, such as bee pollen, milk, lupine, soybean, walnut, rice, eggs, and collagen-enriched products among others [16–21]. These peptides can act on various inflammatory targets, such as reducing NO production and several interleukins levels, inhibiting COX-2 and COX-1, or downregulating the gene expression of COX-2 and inducible nitric oxide synthase (iNOS), the key enzyme in NO production [20–22]. However, only a few have been evaluated in an *in vivo* inflammatory model after oral administration [20]. Among the different sources of anti-inflammatory peptides, it is worthy to highlight collagen [23,24]. Collagen composition is unique because of its high content in Pro and Hyp [14]. Peptides containing hydrophobic amino acids such as Pro in their sequences have been associated with greater bioactivities [25]. Although collagen-derived hydrolysates containing peptides are topically applied for different uses, such as for joint or skin health [24,26], to the best of our knowledge, there is no evidence that dietary-derived peptides or hydrolysates could exert anti-inflammatory and analgesic effects after topical application. Topical NSAIDs are indicated for the management of musculoskeletal pain, such as the pain from chronic osteoarthritis, and provide local anti-inflammatory and analgesic effects [27]. In addition, it is known that peptides up to 1,500 kDa can cross the skin barrier [28] and are used as vehicles for other molecules to enhance their skin permeation [29].

Considering this evidence, the objective of this study was to obtain a selective COX-2 inhibitory hydrolysate from collagen with acute and local anti-inflammatory effects after topical administration. A carrageenan-induced paw edema model was used to evaluate the *in vivo* effects of a selected hydrolysate. Moreover, the mechanisms underlying the anti-inflammatory effect of the selected collagen-derived hydrolysate were also elucidated in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Anti-nociceptive effects were also studied in the selected hydrolysate fraction using the tail-withdrawal test.

2. Materials and methods

2.1. Obtaining of the collagen hydrolysates

Collagen was kindly provided by Juncà Gelatines S.L. (Girona, Spain). For the lab scale production, collagen was dissolved in Milli-Q water (0.43 g/mL, w/v), and protein hydrolysis was performed at 50 ºC in a shaker water bath, for two different times (t1 < t2), two enzyme/substrate concentration ratios (C1 < C2) and three proteases E1, E2, or E3. The enzymes were inactivated by heating the hydrolysates at 90 ºC for 20 min. The pilot scale hydrolysate and ultrafiltration were carried out in Juncà Gelatines S.L. (Girona, Spain). A total of 20 liters were produced from which 10 were ultrafiltered to obtain the <10 kDa fraction.

2.2. Characterization of H8 and its peptide fraction

Total nitrogen was determined using the Kjeldahl method [30], and protein content was estimated by multiplying by the collagen protein factor, 5.55. Moisture content was determined by gravimetry, using the AOAC official method [31]. For the degree of hydrolysis (DH), α-amino groups were determined with the TNBS method [32]. All analyses were carried out in triplicate and data are presented as mean ± standard deviation. The amino acid profile and hydroxyproline content were analyzed in an external laboratory certified for this analysis, following the official methodologies of BOE-A-1991-18408 and LFBG §64 06.00-8 [33,34]. The collagen content was determined by multiplying the hydroxyproline content by 8. Data are shown with the uncertainty grade.

2.3. In vitro COX inhibitory activity assay

COX-2 and COX-1 inhibitory activities were evaluated using a colorimetric inhibitor screening assay kit (ref. 701050 - COX Colorimetric inhibitor screening assay kit Cayman Chemical, Michigan, USA), following the procedure given by the manufacturer. Hydrolysates H1 - H12 were assayed at a final concentration of 1 mg/mL. The hydrolysate H8 produced in a pilot scale and its fraction <10 kDa were dissolved in MilliQ water for the assay and were tested at a final concentration of 45 µg/mL. All samples were tested in triplicate. Results are expressed as the percentage of inhibition ± standard deviation, obtained as follows:

$$\%\, Inhibition = \left(1 - \frac{A_S - A_{BS}}{A_{C+} - A_B}\right) * 100$$

where C^+ is the enzyme without inhibitors, B is the background of the reagents without enzyme, S is the enzyme incubated with the sample, and BS is the background of the reagents and the sample without the enzyme.

2.4. Experiments in animals

2.4.1. Gel preparation

Gel containing the treatments (hydrolysate H8p and <10 kDa fraction) were prepared as follows: 0.2 g Dermofeel pa (Evonik Industries AG, Essen, Germany) and 26.0 g glycerin were dissolved in 152.4 mL of water. Then, 10 g of the samples were added to the solution and dissolved by continuing stirring for 30 min. Once solubilized, 4 g Sepimax zentm (Seppic, Castres, France) was added to the solution. The solution was converted to transparent gel using a Silverson mixer emulsifier (East Longmeadow, MA, United States) at 3,750 rpm for 5 min. Then, 5 g pentylenglicol (Lanxess, Cologne, Germany) and 2 g Dermasoft Pea (Evonik Industries AG, Essen, Germany) were incorporated into the gel in two consecutive steps using a Silverson mixer emulsifier at 3,700 rpm until total solubilization.

Control gel was prepared without adding the hydrolysates to check the activity of the vehicles. Diclofenac is the most commonly utilized topical NSAID and is available in commercially prepared formulations in patch, gel, and solution form.

2.4.2. Animal care

12-week-old male Wistar rats were purchased in Envigo RMS Spain (Sant Feliu de Codines, Spain) and housed in pairs in cages with 12 h light/dark cycles at 24 ºC ± 1 and humidity (55 % ± 10), with *ad libitum* access to water and food. All experimental protocols were approved by the Animal Ethics Committee of the Technological Unit of Nutrition and Health of Eurecat (Reus, Spain), and the Generalitat de Catalunya approved all the procedures (11056). Following the 3 Rs principles, animals were reused for carrageenan-induced paw edema and anti-nociceptive activity with two weeks of washout.

2.4.3. Carrageenan-induced paw edema

Right rear paws were cleaned and disinfected, before topical application of 300 mg of a cream containing 5 % hydrolysate H8 (hydrolysate group), 5 % of <10 kDa fraction (<10 kDa group), or a commercial anti-inflammatory cream containing 11.6 mg/g of

sodium diclofenac (sodium diclofenac group) (n = 8 per group). After 30 min, paw edema was induced by injecting 100 µL of 1 % λ-carrageenan, (Sigma-Aldrich, Madrid, Spain) in 0.9 % NaCl in the hind of the treated paw. The paw volume was measured with a plethysmometer (Panlab, Barcelona, Spain) at least in triplicate at 0, 1, 2, 3, and 24 h after the induction of the inflammation. Rats were anesthetized by inhalation of 2 % of isoflurane (RWD Life Sciences, Mainz, Germany) before every measurement. The control group consisted of the left rear paw in each treated group after the application of a base cream and paw edema induction. The volume was normalized to the initial paw volume. The differences in volume were obtained as follows:

$$\Delta Normalized\ Volume = \frac{V_m - V_i}{V_i}$$

where V_i is the initial volume measured for the injected paw (t = 0) and V_m is the volume of the injected paw for the different time points.

2.4.4. *Anti-nociceptive activity*

The analgesic effect on acute-grade pain was determined using the tail-withdrawal method, measuring the latency of the tail-flick response to heated water as the nociceptive stimulus. Rats were randomly distributed into four treatment groups (n = 6 per group). Rat tails were cleaned and disinfected, and the last 3 cm of the distal end was marked. Then, 300 mg of base cream (control group), base cream containing 5 % of H8 <10 kDa fraction (<10 kDa group, application of 15 µg of peptides), or commercial cream containing 11.6 mg/g of sodium diclofenac (sodium diclofenac group, application of 3.48 mg of drug) were applied with soft massages in the marked region of the tail. After 30 min, the animals were gently restrained, and the marked area of the tails was immersed in warm water maintained at 48 ºC ± 0.5 or 55 ºC ± 0.5. The time until the rats purposefully removed their tails from the warm water in response to a painful stimulus (reaction time) was recorded with a stopwatch. The final reaction time was the average of five consecutive measurements, interleaving measurements in warm water for 1 min in lukewarm water (25 ºC ± 0.5). To avoid tissue damage, 10 and 20 seconds were fixed as the maximum latency values at 55 ºC and 48 °C, respectively[35]. The 48 °C and 55 °C warm-water tail-withdrawal tests were carried out on different days. Data are expressed as mean ± standard error of the mean.

2.5. Experiments in RAW 264.7

2.5.1. Cell culture

Murine macrophage cell line RAW 264.7 (American type Culture Collection; ATCC, Rockville, Maryland, USA), was cultured at 37° C in Dulbecco's modified Eagle medium (DMEM, Corning) supplemented with 10 % fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and L-glutamine in a humidified 5 % CO_2 incubator. For maintenance, a cell scraper was used to dislodge cells from the flask. All reagents were provided by Merck Millipore (Madrid, Spain) and filtered by a 0.22 µm filter before adding to the culture. The sample (<10 kDa fraction) was reconstituted in DMEM and also filtered for all the experiments.

2.5.2. Cell viability

Cell viability was determined using the MTT assay adapted from Inkanuwat *et al*. (2019) [36]. RAW 264.7 cells, seeded at a concentration of 1 x 10^5 cells/mL in a 96-well plate, were cultured overnight with supplemented DMEM, and then treated with the <10 kDa fraction (0.25, 0.5, 1, 2, and 5 mg/mL) for 48 h or the same volume of DMEM (control). Subsequently, the medium was replaced with fresh medium containing 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Merck Millipore). After 2 h of incubation in the dark at 37 ºC, the 96-well plates were centrifuged at 700 *x g* for 3 min, and the supernatants were discarded. Dimethyl sulfoxide and 0.1 M glycine buffer were added to the wells to dissolve the formed crystals. Absorbance was read at 570 nm. Results were expressed as a percentage of viability, considering untreated control cells as 100 % viability. The treatments were assayed in triplicate.

2.5.3. Activity of COX-2 and COX-1

Cells were grown in six T-75 flasks per condition. When confluency was about 80 %, cells were preincubated for 24 h with the <10 kDa fraction of the hydrolysate H8 (0.25 mg/mL) or DMEM (control). Subsequently, cells were activated with 1 µg/mL of *E. coli* lipopolysaccharide (LPS; Merck Millipore). 24 h after stimulation, cells were collected with a scrapper, pulled, centrifuged (1,000 x *g*, 10 minutes at 4 ºC), and lysed with cold lysis buffer (1 mM EDTA-0.1 M Tris-HCl; pH 7.8). The COX-1 and COX-2 activity of the sample was determined using a commercial kit (ref. 760151 - COX Activity Assay Kit, Cayman Chemical)following the manufacturer's instructions. A

selective COX-2 inhibitor (DuP-697) was used to test the specific COX-2 activity of the samples. The COX-2 activity of each sample was determined by subtracting the activity calculated for the respective DuP-697-treated wells (*i.e.* COX-1 activity of the sample). COX-2 and COX-1 activities were determined in triplicate and expressed as a percentage ± standard deviation, as follows:

$$\%\,Activity\ of\ COX_n = \left(\frac{COX_{n\ sample}}{COX_{total\ sample}} \Big/ \frac{COX_{n\ 100\,\%}}{COX_{total\ 100\%}}\right) * 100$$

where *COX n* refers either to COX isoform 1 or 2, and *COX total* is the COX activity of the sample, without the specific inhibitor DuP-697. The non-treated LPS-stimulated COX-2 activity was considered as COX-2 100 %. The COX-1 100 % activity was considered for the non-stimulated cells (basal activity).

2.5.4. *Determination of nitrite production*

RAW 264.7 cells at a concentration of 1 x 10^5 cells/mL were seeded in 24-well plates and cultured overnight with supplemented DMEM (as above) at 37 ºC. Cells were pre-incubated for 24 h with the <10 kDa fraction (0.25, 0.5, 1, 2, and 5 mg/mL). Cells were then stimulated with LPS (1 µg/mL) and after 24 h of incubation, NO concentration in the supernatant was determined using the Griess method [37]. Sodium nitrate at different concentrations was used as standard. The NO content concentration was standardized using the amount of protein in the cells, which was determined using the BCA™ Protein Assay Kit. Cells were collected in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl; pH 7.4, 1% Tween 20, 0.25% sodium-deoxycholate) containing protease and phosphatase inhibitors cocktails and phenylmethylsulfonyl fluoride (PMSF) (MilliporeSigma, Madrid, Spain) for protein extraction and quantification. The percentage of reduction of NO production was obtained considering LPS control samples as 100 % of NO production. Results are expressed as the mean of the triplicate ± standard deviation.

2.5.5. *Immunoblot analysis*

Twenty µg of protein from RAW 264.7 cells incubated with different concentrations of the <10 kDa fraction (0.5, 1, 2, and 5 mg/mL) and stimulated with LPS was analyzed for inducible nitric oxide synthase (iNOS) and COX-2 protein expression by Western Blot. . Immunoblot was carried out by using polyclonal

antibodies against iNOS, COX-2, and β-actin which was used as loading control. Proteins were detected with the chemiluminescent reagent ECL Select Western Blotting Detection Reagent (GE Healthcare, Barcelona, Spain). Images were captured using a G:BOX Chemi XL1.4 (Syngene, Cambridge, UK), and densitometry quantification was performed using ImageJ Software 1.54 (NIH, Bethesda, MD, USA). The assay was performed in duplicate. Polyclonal antibodies goat-anti-COX-2, rabbit-anti-iNOS, mouse-anti-β-Actin, and horseradish peroxidase-conjugated donkey-antirabbit, donkey-anti-goat, and goat-anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

2.6. Statistical analysis

Outliers were detected using Grubb's test. Normality and homoscedasticity of the data were determined using the Shapiro-Wilk and Levene's tests. Statistical analyses and graphics were performed with Prism v. 10 software (GraphPad Software, San Diego, CA, USA). Significant differences in COX-2 and COX-1 inhibition between sample/treatment and control were analyzed using Student's t-test ($p < 0.05$). Significant differences in paw edema volume over time between the groups were analyzed using RM two-way ANOVA with Bonferroni post-hoc test ($p < 0.05$). Significant differences in paw edema volume (AUC), nitrite production, COX-2 and COX-1 protein expression, and tail withdrawal time between the groups were analyzed using one-way ANOVA with a Bonferroni post-hoc test ($p < 0.05$).

3. Results and discussion

NSAIDs are used to relieve inflammation and pain by mainly inhibiting the activity of COX activity. However, prolonged use of certain NSAIDs may lead to diverse side effects, which have been associated with inhibition of the COX-1 isoform [11]. Thus, the search for new selective COX-2 inhibitors with fewer side effects, particularly those derived from natural sources, has been prompted [12,38]. Among them, it is worth mentioning the bioactive peptides, which can be obtained from different protein sources and exert different bioactivities, including anti-inflammatory properties [14]. An interesting source of anti-inflammatory peptides is collagen due to the high presence of hydrophobic amino acids such as Pro or Hyp, which has been associated with improved bioactivity. Although peptides are used to enhance the skin delivery of different compounds [28], there is no evidence that anti-inflammatory protein hydrolysates could

exert anti-inflammatory and analgesic effects after topical application. Topical use of anti-inflammatory peptides might be useful to alleviate inflammation and pain linked to arthritis, muscle damage, sprains, and strains with fewer side effects than oral administration. Therefore, this study aimed to obtain a selective COX-2 inhibitory hydrolysate from collagen with acute anti-inflammatory and analgesic (anti-nociceptive) effects after topical administration.

3.1. *Preparation of collagen hydrolysates with COX-2 inhibitory activity*

Twelve hydrolysates were obtained from isolated pigskin collagen using three types of commercial proteases (E1, E2, and E3), two hydrolysis times (t1 and t2), and two enzyme concentrations (C1 and C2) (Table 1).

Table 1. Cyclooxygenase-2 (COX-2) inhibitory activity of the pigskin collagen hydrolysates produced using different hydrolysis conditions.

Hydrolysis conditions		**COX-2 inhibition (%)**			
Time	**Enzyme**				
t1	**E1**	H1	n.e.	H2	4.9 ± 1.7
	E2	H3	n.e.	H4	n.e.
	E3	H5	n.e.	H6	n.e.
t2	**E1**	H7	27.7 ± 1.0	H8	50.5 ± 7.8
	E2	H9	n.e.	H10	n.e.
	E3	H11	33.7 ± 1.5	H12	37.6 ± 9.1
Enzyme/substrate ratio		**C1**		**C2**	

COX-2 inhibitory activity was tested at a final concentration of 1 mg/mL. Results are expressed as the mean of triplicates (%) ± standard deviation. Abbreviations: C: enzyme/substrate ratio (C1 < C2), H: Hydrolysates from H1 to H12, n.e.: no effect. t: hydrolysis time (t1 < t2).

Table 1 shows the *in vitro* COX-2 inhibitory activity of the hydrolysates, which ranged between 0 and 51 % when tested at 1 mg/mL. COX-2 inhibitory activity was only observed in E1 and E3-derived hydrolysates, which depended on the hydrolysis time and enzyme concentration. In addition to enzyme type, hydrolysis time was critical for producing COX-2 inhibition given that at the lowest hydrolysis time (t1), only E1 enzyme solution (at the highest enzyme concentration (C2)) was able to slightly inhibit COX-2 (5 %). At t2, both enzymes produced hydrolysates with COX-2 inhibitory

activity, ranging between 28 and 51 %. The enzyme concentration also affected the COX-2 inhibitory activity of E1-derived hydrolysates, increasing the bioactivity from 28 % to 51 % when the enzyme concentration was increased from C1 to C2. The most active was the hydrolysate H8 (51 % inhibition), which was obtained using E1 at the highest enzyme concentration (C2) and hydrolysis time (t2). Accordingly, several studies have shown that protein hydrolysates can inhibit COX-2 activity. In this context, *in vitro* gastrointestinal digestion of raw and heat-treated proteins isolated from three edible insects produced peptides with COX-2 inhibitory activity (IC_{50}= 10.91-208.24 µg/mL) [21]. The degree of activity depended on the protein origin and thermal treatments applied to the proteins prior to *in vitro* digestion. Moreover, Millán-Linares *et al*. (2014) observed that lupine proteins exerted COX-2 inhibitory activity, which increased (~15-20 %) when proteins were hydrolyzed using Alcalase (pH 8, 1 h, 50 °C) and hydrolysis times ≥ 15 min up to 60 min. However, this activity remained unaltered when proteins were hydrolyzed with Izyme (pH 10, 1 h, 50 °C) with or without a second hydrolysis step using Alcalase (pH 8, 1h, 50 °C) [21]. These results are in concordance with those obtained in the present study, as hydrolysis factors affected COX-2 inhibitory activity. The COX-2 inhibitory activity shown by H8 (51 % at 1 mg/mL) was slightly lower than the bioactivity shown by isolated proteins of edible insects subjected to gastrointestinal digestion [39] but might be similar to that shown by lupine hydrolysates (70 % inhibition at 1.75 mg/mL) [21].

3.2. *Characterization of H8p hydrolysate and its <10 kDa fraction*

Given the COX-2 inhibitory activity of hydrolysate H8, it was elaborated at a pilot-scale level to evaluate whether its production and bioactivity could be reproduced at this scale level. From now on, "H8p" will refer to the pilot-produced hydrolysate. Moreover, the peptide fraction <10 kDa of the H8p was also obtained and tested. Different studies have shown that low-molecular-weight peptides can exert greater anti-inflammatory activity than larger peptides or whole-protein hydrolysates [40,41].

Both samples were then characterized by determining the total protein content, the hydrolysis degree, and the amino acid profile. The protein content of both samples was high (91.7 ± 0.3 and 91.9 ± 0.7 g/100 g dried product for the H8p and the <10 kDa fraction, respectively). This result was expected, given the hydrolysate was elaborated with a protein-rich source as pigskin-isolated collagen. Note that the ultrafiltration process did not affect the protein content. However, the hydrolysis degree was increased

in the <10 kDa fraction compared to H8p (from 63.2 ± 1.7 and 50.2 ± 2.5 %, respectively). This phenomenon is commonly observed as the concentration of small peptides (<10 kDa) and free amino acids per gram of product increases in the permeate during the ultrafiltration step. No significant changes were observed in the amino acid profiles of either sample (Table 2).

Table 2. The amino acid profile of the hydrolysate H8p and the <10 kDa fraction.

Amino acids	Amino acid content (%)	
	H8p	**<10 kDa fraction**
Asp	5.77 ± 0.58	5.77 ± 0.58
Glu	10.71 ± 1.07	10.51 ± 1.05
Ala	9.50 ± 0.95	9.31 ± 0.93
Arg	8.11 ± 0.81	8.07 ± 0.81
Cys	0.07 ± 0.00	0.07 ± 0.00
Phe	1.95 ± 0.25	2.21 ± 0.29
Gly	23.85 ± 2.39	23.60 ± 2.36
His	0.74 ± 0.10	0.72 ± 0.09
Ile	1.17 ± 0.15	1.24 ± 0.16
Leu	2.86 ± 0.37	2.91 ± 0.38
Lys	3.91 ± 1.45	3.81 ± 1.41
Met	0.80 ± 0.10	0.86 ± 0.11
Pro	13.85 ± 1.39	14.12 ± 1.41
Ser	3.46 ± 1.28	3.40 ± 1.26
Tyr	0.58 ± 0.08	0.62 ± 0.08
Thr	1.87 ± 0.24	1.89 ± 0.25
Trp	<0.02	<0.02
Val	2.43 ± 0.32	2.44 ± 0.32
Hyp	12.31	12.37
Collagen	98.49	98.93

Results are presented as g/100 g dry weight. Results are expressed as the mean of triplicates (%) ± standard deviation.

The main amino acid in both the H8p and its peptide fraction was Gly (23.9 and 23.6 %), followed by Pro (13.9 and 14.1 %), Hyp (12.3 and 12.4 %), Ala (9.5 and 9.3 %), and Arg (8.1 and 8.1 %). Other amino acids, such as Trp or Cys were barely detectable. This amino acid profile was expected given that the major amino acids in

collagen are Gly, Pro, and Hyp, while aromatic amino acids such as Trp are poorly found in collagen because strongly destabilize the triple helix structure [42].

Figure 1 shows the COX-2 inhibitory activity of H8p and the <10 kDa fraction. Despite being tested at a significantly lower concentration, COX-2 inhibition of H8p was higher than that exhibited by the hydrolysate at the laboratory scale (70.2 % and 50.5 %, respectively). The <10 kDa fraction inhibited COX-2 activity by 74.0 %. However, the <10 kDa fraction did not improve the COX-2 inhibitory activity of the H8p (p = 0.99). These results differ from those of studies on edible insect proteins subjected to *in vitro* gastrointestinal conditions [39]. In the same study, increased COX-2 inhibitory activity was observed when the <3.5 kDa fraction was obtained with respect to the whole hydrolysate, especially those from *Gryllodes sigillatus* boiled proteins. Specifically, IC_{50} values were 146.8 µg/mL and 18.71 µg/mL for the hydrolysate and the fraction, respectively.

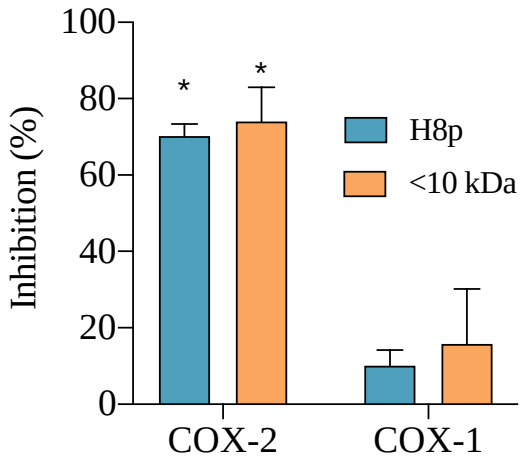

Figure 1. *In vitro* inhibitory activity of cyclooxygenase isoforms 2 and 1 (COX-2 and COX-1, respectively) of the collagen hydrolysate H8 produced in a pilot scale (H8p) and its <10 kDa fraction at final concentration of 45 µg/mL. Data are expressed as the mean of triplicates (%, w/w) ± standard deviation. * indicates significant differences between samples and 100 % activity (data not shown) (p < 0.05; One-way ANOVA).

In addition to COX-2 inhibition, we evaluated the inhibitory activity of both samples against COX-1. COX-1 inhibition has been associated with the intestinal side effects of NSAIDs [11]. No significant differences were found between the treatment groups and the control (p = 0.33) (Figure 1), indicating that the COX inhibition exerted by both samples was selective for COX isoform 2.

Considering this evidence, H8p and its <10 kDa fraction were further evaluated for their *in vivo* anti-inflammatory and analgesic effects.

3.3. Acute anti-inflammatory effect of H8p and its fraction <10 kDa in the carrageenan-induced paw edema model

The *in vivo* anti-inflammatory effects of both samples were tested in a carrageenan-induced paw edema model. It is a well-known model of acute and local inflammation and is commonly used to evaluate new anti-inflammatory compounds [43,44]. Treatments included in a base cream were topically applied to the rat paw 30 min before subplantar carrageenan injection. Paw volume was measured at different time points (Figure 2A).

Non-treated paw edema in each treated group was considered the control group (maximum inflammation). The results indicated that carrageenan injection produced an increase in paw volume (control group), reaching a maximum value 1 h after injection. At 2 h post-injection, inflammation was reduced (40 %) and maintained until 24 h post-administration (Figure 2A). As expected, the application of sodium diclofenac (3.48 mg) reduced carrageenan-induced paw edema compared with the control group ($p < 0.05$) (Figure 2A). The area under the curve showed that this anti-inflammatory effect was significant 3 h after edema induction, and it was still notorious 24 h post-edema induction ($p < 0.05$) (Figure 2B). This drug is widely used to relieve inflammation and pain associated with different diseases [45] and mainly acts on the prostaglandin pathway (inhibiting COX-2 and COX-1 and reducing arachidonic acid availability) [46]. Moreover, its effects have been demonstrated in carrageenan-induced paw edema following oral, intraperitoneal, and topical administration [44,47]. Regarding the peptide samples, no differences in paw edema volume were observed between the group treated with hydrolysate H8p (15 mg) and the control group at any time point ($p > 0.05$) (Figure 2A and B). However, the application of the <10 kDa fraction (15 mg) reduced edema volume by 54 % in the first hour after carrageenan injection, which was gradually attenuated over time until 3 h ($p < 0.05$) (Figure 2A). The area under the curve showed an anti-inflammatory effect of the fraction <10 kDa until 3 h after inflammation induction when compared to the control group ($p < 0,01$, $p < 0.001$, for 2 and 3 h post-carrageenan injection, respectively) (Figure 2B). This anti-inflammatory effect disappeared 24 h after edema formation (Figure 2A). Therefore, the peptides contained in the <10 kDa fraction produced a faster anti-inflammatory response than

diclofenac, but it was shorter in time. This fact could indicate that the mechanisms of action of the two treatments could be different. Carrageenan-induced paw edema is known to have two inflammatory phases. The initial phase (0 – 1 h) is linked to bradykinin, histamine, and serotonin levels (Crunkhorn and Meacock 1971a), although an increase in PGE_2 and thromboxane B_2 levels and upregulation of *Ptgs2* expression (COX-2 gene) have also been reported [49]. However, the second inflammatory phase is associated with an increase in prostaglandins and several cytokine levels [48]. Another factor involved in the differential effect of the <10 kDa fraction and sodium diclofenac could be their potential differences in skin permeability. It is known that only compounds with specific characteristics, such as high hydrophobicity, sizes lower than 500 kDa, and adequate solubility, can be passively transported through the stratum corneum (the outermost skin layer) [28,29]. However, there is evidence that higher-sized peptides between 1,000 and 1,500 kDa can also cross this barrier [28]. Remarkably, some peptides, called skin-penetrating peptides, consisting of up to 30 amino acids, are used as vehicles for other molecules [29]. This fact could also explain the different effects shown by H8p and the <10 kDa fraction, due to the higher proportion of small peptides in the <10 kDa fraction than in the whole hydrolysate.

Little evidence exists regarding the *in vivo* acute anti-inflammatory effects of protein hydrolysates [40], and all of them have been tested after oral administration. In this regard, consumption (five days) of the peptic fraction (1-3 kDa) obtained from a hydrolysate of *Salvia hispanica L.* seeds reduced in 83.9 % the 12-O-tetradecanoylphorbol 13-acetate-induced ear edema 2 h after induction in Balb/c male mice [50]. Tavares *et al.* (2013) demonstrated in Swiss mice a dose-dependent reduction of carrageenan-induced paw edema between 45 and 67 %, 4 h after the oral administration of concentrated peptide obtained from hydrolyzed whey proteins (100-1,000 mg/kg of body weight, respectively) [51]. In addition, the consumption of other whey protein hydrolysates obtained using Alcalase (30, 100, and 300 mg/kg bw) also reduced carrageenan-induced paw edema (3 h post-carrageenan injection) by 60, 40, and 69 %, respectively [52]. The anti-inflammatory reduction shown by the <10 kDa fraction is comparable with these mentioned hydrolysates or peptide concentrates; however, it is worth mentioning that the anti-inflammatory effect was produced at a lower dose and faster than these reported hydrolysates. In topical administration, bioactive compounds bypass the digestive and metabolic processes associated with oral administration, enhancing bioavailability and efficacy and reducing the onset time [53].

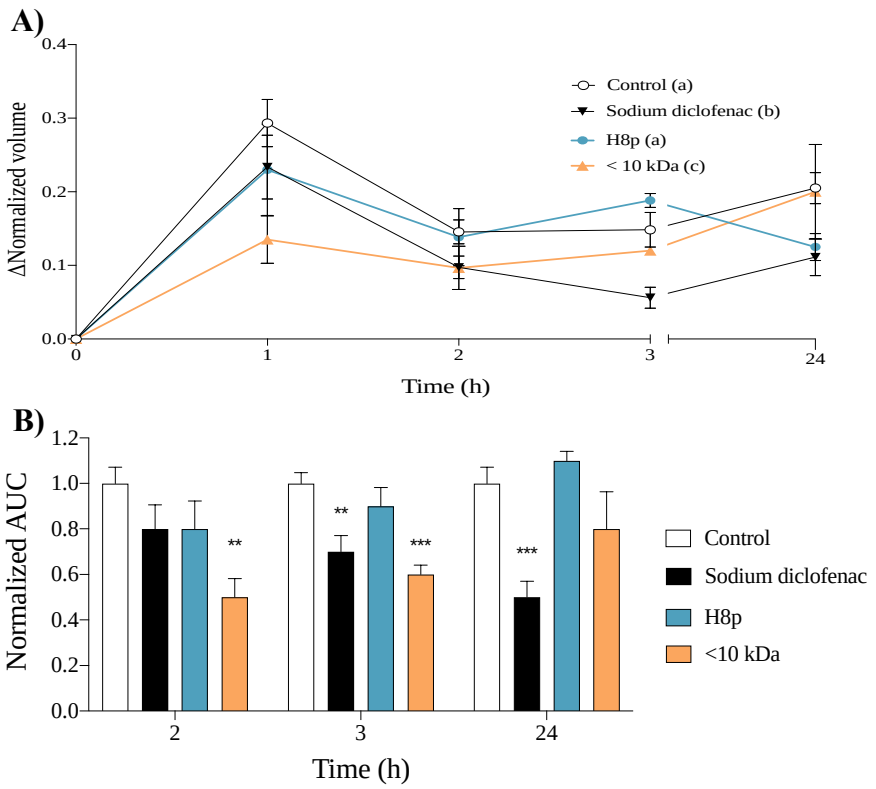

Figure 2. Effects of hydrolysate H8p and the <10 kDa fraction in carrageenan-induced paw edema. **A)** Evolution of the paw edema volume at 1, 2, 3, and 24 h after carrageenan injection (100 µL of 1 % λ-carrageenan in 0.9 % NaCl) and previous topical treatment with 300 mg of a base cream (control group), a commercial cream containing 11.6 mg/g of sodium diclofenac (sodium diclofenac group), a base cream containing 5 % of H8p (H8p group), or a base cream containing 5 % of the H8p <10 kDa fraction (<10 kDa group). Results are expressed as the mean (n = 8 per group) ± standard error of the mean. Different letters represent differences between groups (p < 0.05; RM two-way ANOVA; Bonferroni post-hoc test). **B)** Area under the curve (AUC) of paw edema volume at 2, 3, and 24 h after carrageenan injection for the different treatments, normalized by the control group. Results are expressed as the mean (n = 8 per group) ± standard error of the mean. ** (p < 0.01) and *** (p < 0.001) indicate significant differences with the control group (one-way ANOVA; Bonferroni post-hoc test).

3.4. Anti-inflammatory effect of the <10 kDa fraction in LPS-stimulated RAW 264.7 cells

The LPS-induced inflammation model was used to investigate some of the potential mechanisms underlying the anti-inflammatory effects of the <10 kDa fraction. LPS, when applied to macrophages, initiates an inflammatory response via toll-like receptor 4, which triggers intracellular signaling cascades, activating nuclear transcription factors such as nuclear factor-kappa B (NF-kB). This activation ends with the synthesis of inflammatory mediators, including nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1-beta, IL-6, IL8, and prostaglandins, and also regulates the gene expression of COX-2 and iNOS [54,55]. Different studies have shown that food-derived peptides can exert their anti-inflammatory effects by inhibiting NO production and secretion, the expression of pro-inflammatory cytokines, and the activity and expression of enzymes, such as COX-2 and iNOS, among others (see [20,40,56] for more details).

3.4.1. Collagen-derived peptides inhibited selectively COX-2 activity in LPS-stimulated RAW 264.7 cells

Initially, the viability of cells incubated with different doses (0.25 - 5 mg/mL) of the <10 kDa fraction for 48 h was assessed using the MTT assay. No significant changes were observed in cell viability at any dose of peptide fraction compared to non-treated cells (Figure 3A).

Given the *in vitro* COX-2 inhibitory activity of the <10 kDa fraction, COX-2 activity in LPS-stimulated RAW 264.7 cells was determined after incubation with the peptide fraction at a low dose (0.25 mg/mL) (Figure 3B). As expected, COX-2 activity was increased after exposure to LPS. Both the expression and activity of this isozyme have been reported to increase when RAW 264.7 cells are LPS-stimulated [8]. The <10 kDa fraction-treated cells showed lower COX-2 activity (62.2 % inhibition) than untreated LPS-stimulated cells ($p < 0.05$, Figure 3B). Activity values were significantly similar to those of control cells (cells without LPS stimulation) ($p = 0.68$). To the best of our knowledge, there is no evidence that other protein hydrolysates can inhibit COX-2 activity in this inflammatory model.

The effects of the <10 kDa fraction on COX-1 activity were also evaluated in LPS-stimulated cells. Stimulation with LPS did not produce changes in COX-1 activity of macrophages in comparison with control cells (Figure 3C, $p = 0.06$). COX-1 is a

constitutive isozyme; thus, its activity may remain stable despite LPS stimulation [9]. No differences were also observed between LPS-stimulated macrophages treated with the <10 kDa fraction and control cells (p = 0.79). These results are in concordance with the results obtained in the enzymatic *in vitro* study and show that food-derived peptides can exert selective COX-2 inhibition in macrophages.

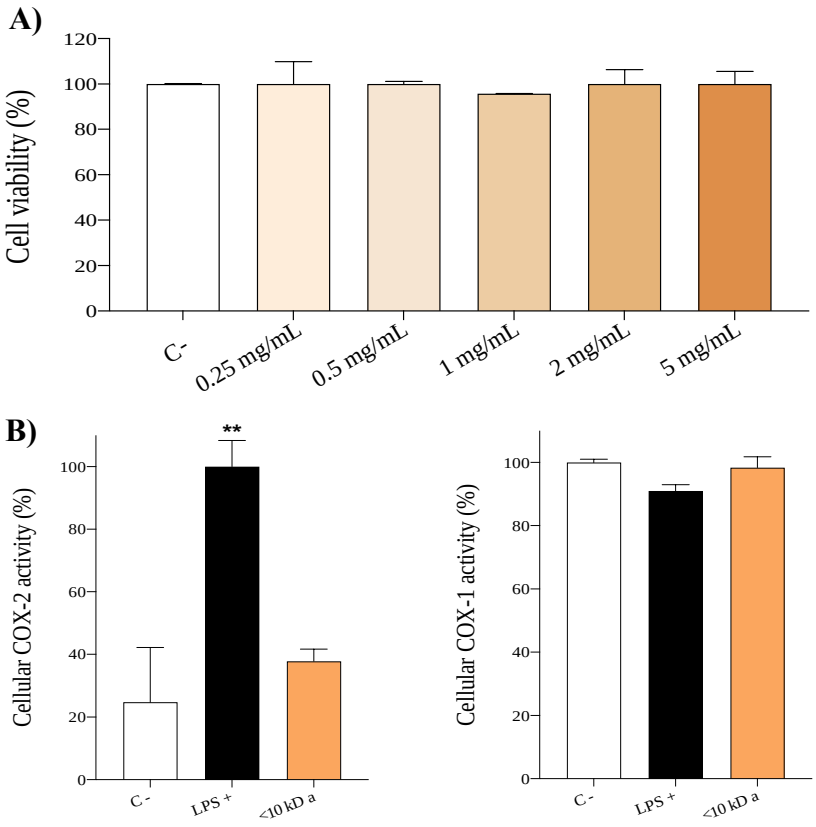

Figure 3. A) Cellular viability for RAW 264.7 cells incubated for 48 h with the H8p <10 kDa fraction at different doses. No significant differences were found between groups (p = 0.89, one-way ANOVA; post-hoc: Bonferroni). **B)** Activity of cyclooxygenase isoforms 2 and 1 (COX-2 and COX-1, respectively) of the H8p <10 kDa fraction-incubated RAW 264.7 cells 24 h before *E. coli* lipopolysaccharide (LPS) stimulation (1 µg/mL). ** indicates significant differences with C- group (p < 0.01 (One-way ANOVA, post-hoc; Bonferroni). Results are expressed as the mean (n=3) ± standard deviation.

3.4.2. *Collagen-derived peptides reduced NO production in LPS-stimulated RAW 264.7 cells at high doses*

In addition to COX-2 activity, the effect of the <10 kDa fraction on LPS-induced NO production was also analyzed in RAW 264.7 cells. NO is produced in response to

iNOS induction, during the conversion of L-arginine into L-citrulline [57] thus being a common inflammatory biomarker used to assess the anti-inflammatory potential of compounds, such as food-derived peptides [14,55].[57] We did not observe inhibition of LPS-induced NO secretion when LPS-stimulated cells were preincubated with concentrations ≤ 1 mg/mL of the <10 kDa fraction (Figure 4). However, when cells were treated with 2 and 5 mg/mL of the <10 kDa fraction, NO production was inhibited by 8 and 15 %, respectively, when compared with non-treated LPS-stimulated macrophages ($p < 0.05$). A significant trend in NO production inhibitory activity was observed between treatments at these two concentrations ($p = 0.06$). Previous research also showed that ovalbumin hydrolysates obtained using different enzymes inhibited NO production in LPS-stimulated RAW 264.7 cells in a concentration-dependent manner (11.3 - 18 % at 0.5 mg/mL, 25.5 - 31.5 % at 1 mg/mL, and 40.1 - 50.7 % at 2 mg/mL) [58]. Additionally, hydrolysates obtained from egg yolk livetins using pepsin or Alcalase inhibited NO production by 32.9 and 22.7 % respectively at a dose of 0.4 mg/mL [59]. In the present study, the reduction in secreted NO was subtle compared to the aforementioned studies. These results indicate that the anti-inflammatory effect of collagen-derived peptides is not mainly mediated by a reduction in NO production, although it can contribute.

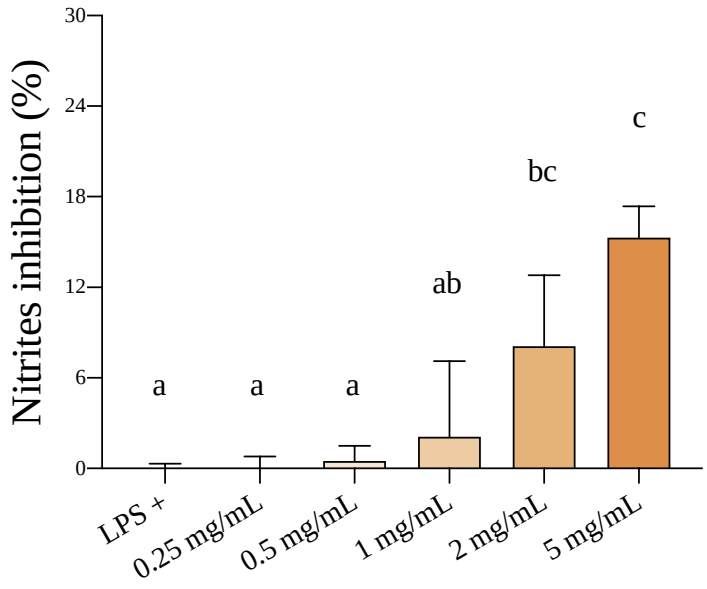

Figure 4. Inhibition of nitrite (NO) production in *E. coli* lipopolysaccharide (LPS)-stimulated cells treated with different concentrations (0.25 - 5 mg/mL) of the H8p <10 kDa fraction. Different letters represent differences between groups ($p < 0.05$, one-way ANOVA; Bonferroni post-hoc test). Abbreviations: LPS+: non-treated LPS-stimulated group. Results are expressed as the mean (n=3) ± standard deviation.

3.4.3. Collagen-derived peptides reduced the amount of iNOS and COX-2 proteins in LPS-stimulated RAW 264.7 cells

The effect of the fraction <10 kDa on iNOS and COX-2 protein expression in LPS-stimulated RAW 264.7 cells was analyzed by western blot (Figure 5). iNOS and COX-2 protein expression was increased by 90 and 40 %, respectively, by LPS stimulation (Figure 5B and C). This was expected because the levels of both enzymes are increased in macrophages after NF-kB pathway activation in response to LPS exposure [60]. The <10 kDa fraction significantly reduced iNOS protein expression in LPS-stimulated macrophages at the highest dose ($p < 0.05$) (Figure 5 A and B).

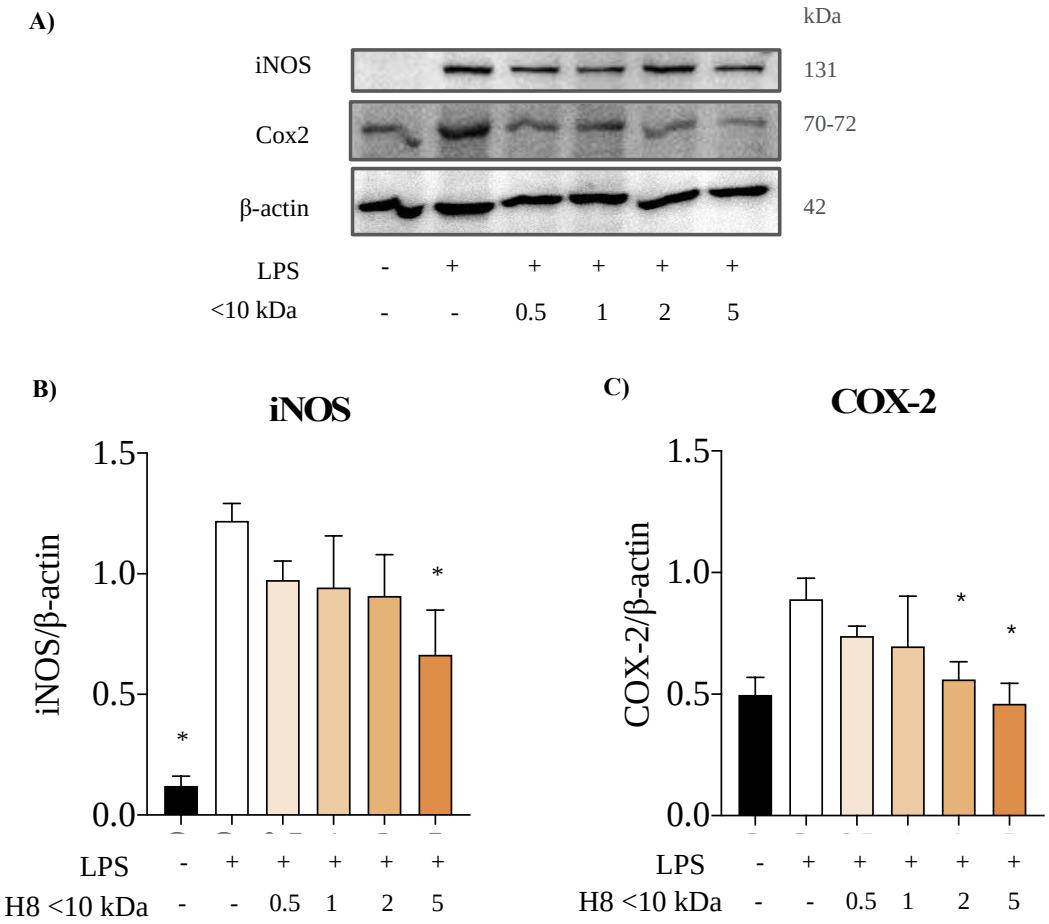

Figure 5. Effect of different doses of the H8p <10 kDa fraction on the inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) protein expression of LPS-stimulated RAW 264.7 macrophages. **A)** Representatives immunoblot for ß-actin, iNOS, and COX-2 proteins. **B)** Relative protein quantification of iNOS enzyme after normalization with ß-actin values. **C)** Relative protein quantification of COX-2 enzyme after normalization with ß-actin values. Data are expressed as the mean (n = 3) ± standard deviation. * indicates significant differences with LPS + ($p < 0.05$, T-test).

This effect was partially in concordance with the results obtained in the NO measurement, where we observed a slight reduction in NO production at 2 mg/mL. This reduction in NO may also be mediated by the activity of molecules with additional mechanisms of action, such as NO-scavengers and antioxidant enzymes (catalase, superoxide dismutase, horseradish peroxidase, or myeloperoxidase) [61,62]. For instance, NO-scavenging activity was shown by different hydrolysates obtained from peanut worms, with an IC_{50} ranging between 19.9 and 1.2 µg/mL [63]. Moreover, many

food-derived peptides and protein hydrolysates have shown other scavenging activities *in vitro* and antioxidant effects *in vivo* [14,64]. Several studies in animal models reported an increase in liver antioxidant molecules, such as reduced glutathione, and antioxidant enzymes, such as catalase and superoxide dismutase, after food-derived hydrolysate treatments [65]. In addition, the oral consumption of a chicken-bone-collagen hydrolysate improved the skin's UV-induced inflammation and oxidative stress by reducing levels of IL-1α and increasing the activity of the aforementioned antioxidant enzymes in the skin of BALB/C hairless mice [66]. The reduction in reactive oxygen species (through different pathways) has been also reported [20]. However, this bioactivity has not been evaluated in the present study.

Regarding COX-2 analysis, treatment with the <10 kDa fraction significantly reduced the protein expression of this enzyme in LPS-stimulated cells at the two highest doses (40 and 50 % reduction for 2 and 5 mg/mL, respectively) compared to the non-treated LPS-stimulated cells ($p < 0.05$) (Figure 5A and C). Moreover, there were no significant differences in COX-2 protein expression between the two highest doses and control cells (cells without LPS stimulation) ($p = 0.99$). Therefore, collagen-derived peptides not only inhibit COX-2 activity but also its protein expression.

Several studies have shown that some food-derived peptides or hydrolysates that affect NO production in LPS-stimulated cells can also downregulate iNOS and COX-2 protein and/or gene expression [40,56]. For instance, egg yolk livetins hydrolysated with pepsin downregulated iNOS protein expression in a dose-dependent manner (0.1 - 0.4 mg/mL) in LPS-stimulated RAW 264.7 cells, whereas that obtained using Alcalase downregulated COX-2 protein at 0.4 mg/mL [59]. In addition, pretreatment of LPS-stimulated RAW 264.7 cells, with the > 5 kDa fraction of a pepsin-digested hydrolysate obtained from *Mytilus edulis*, downregulated iNOS and COX-2 protein and gene expressions [67]. In the same cellular model, both enzymes were also downregulated at gene expression level by an isolated peptide fraction obtained from salmon bones hydrolyzed with papain (1.25 - 5 µg/mL) [68]. Interestingly, a crosstalk in the regulation of these two inflammatory pathways has been described. Thus, high levels of NO can upregulate the *iNos* mRNA expression and increase COX-2 activity and protein levels. Moreover, iNOS can enhance COX-2 activity by its binding [69].

3.5. *Anti-nociceptive effects of the fraction <10 kDa on the tail-withdrawn test*

Finally, considering that some natural anti-inflammatory compounds [70–72] and selective COX-2 drugs [45] have anti-nociceptive effects, this activity was evaluated in the H8p <10 kDa fraction. For this purpose, the acute thermal anti-nociceptive effect was evaluated by the tail-withdrawal test using water at two temperatures (48 and 55 °C). This is a well-known test used to determine the nociceptive response to painful stimuli, where the rat tail is submerged in 40 - 55 °C warm water, and the latency to purposely withdraw the tail from the water is measured [73]. In the present study, baseline tail-withdrawal latencies were 9.4 and 2.5 seconds at 48 and 55 °C, respectively. Similar latency responses have been observed in other studies using several rat strains [74,75]. Moreover, an increase in water temperature has been linked to a quicker tail-withdrawal response [35,76]. No differences were found between the treatment groups and control group in the 48 ºC tail-withdrawal latency (Figure 6A, p = 0.30). Interestingly, the fraction <10 kDa was retarded by 1.4-fold the time required to flick the tail compared to non-treated rats in the 55 ºC tail-withdrawal test (Figure 6A, p < 0.001). The anti-nociceptive effect of <10 kDa fraction was similar to that of sodium diclofenac (Figure 6B). Several studies have demonstrated the anti-nociceptive effect of diclofenac in rats after oral, topical, intraperitoneal, and intrathecal administration using 55 °C tail-flick tests [77–79].

There is scarce evidence regarding the anti-nociceptive effects of protein hydrolysates and dietary-derived peptides. In this regard, the consumption of proteins isolated from *Chelidonium majus* L. (200 mg/kg bw) exerted an analgesic effect (48 °C hot-plate test) increasing mice hind paw licking from 31.9 to 50.1 seconds 3 h post-administration [80]. Moreover, the tail-flick latency, measured using radiant heat, increased from 3.5 to 6.3 seconds, 60 min after the intraperitoneal injection of Lys-Arg-Asp-Ser (80 mg/kg) in Wistar rats in a dose-dependent manner [81]. Finally, Tavares *et al.* (2013) demonstrated the anti-nociceptive and anti-inflammatory effects of a whey protein hydrolysate after oral ingestion [51]. This hydrolysate showed non-dose-dependent analgesic effects reducing by 39 - 42 % the acetic acid-induced writhings (100 - 1,000 mg/kg); however, no effects were observed in the hot-plate test (1 g/kg bw). The differing effects shown by this reported hydrolysate might be attributed to the distinct pain phases evaluated by each test. The hot-plate test and tail-flick/withdrawal test evaluate phasic pain (a short-lasting form of pain), whereas the writhing test evaluates tonic and visceral pain (inflammatory pain) [82]. Considering that phasic pain

is not an inflammation-induced pain, COX-2 might not be involved in the anti-nociceptive effect shown by the H8p <10 kDa fraction, as other NSAIDs with effects on the tail-withdrawal test (Miranda et al. 2001). Several compounds with anti-nociceptive effects tested in these non-inflammatory-induced pain assays have been proven to be mediated via opioidergic, adrenergic, serotonergic, purinergic, histaminergic, and cannabinoidergic mechanisms, among others [82]. There is no evidence of how dietary-derived peptides could retard the heat and acute nociceptive response. However, it has been shown that some dietary peptides can exert biological effects by acting on opioid, histamine, and serotonin receptors [83–86]. The anti-nociceptive effects exhibited by the H8p <10 kDa fraction are in concordance with its anti-inflammatory effects, which suggested that additional mechanisms to COX-2 inhibition might be involved in its *in vivo* effect.

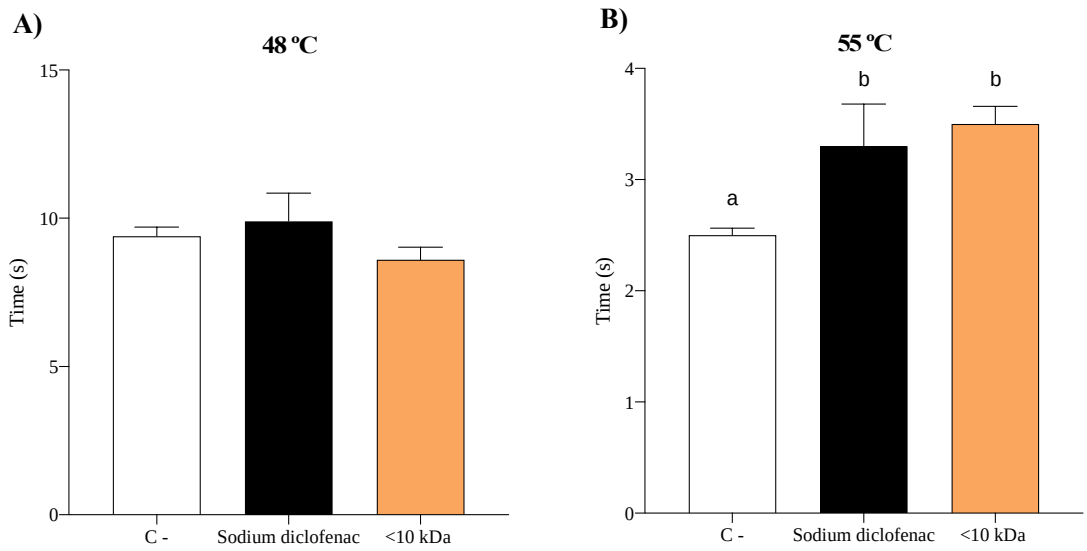

Figure 6. Tail withdrawal latency for rats topically treated with 300 mg of a base cream (control group), a commercial cream containing 11.6 mg/g of sodium diclofenac (sodium diclofenac group), or a cream containing 5 % of the H8p <10 kDa fraction (<10 kDa group), in response to warm water at 48 ºC **(A)** and 55 ºC **(B)**. Data are represented as mean (n = 6 per group) ± standard error of the mean. Different letters represent significant differences between groups ($p < 0.05$, one-way ANOVA, Bonferroni post-hoc test).

4. Conclusions

Pigskin collagen can be a good source of COX-2 inhibitory peptides although the selection of hydrolysis conditions mainly enzyme type and hydrolysis time is crucial.

The <10 kDa fraction of the collagen hydrolysate H8 elaborated on a pilot scale (H8p) exhibited selective *in vitro* inhibition of COX-2. *In vivo* experiments revealed the anti-inflammatory effects of this peptide fraction after topical application in a carrageenan-induced paw edema model. In LPS-stimulated RAW 264.7 cells, the peptide fraction reduced NO production, iNOS and COX-2 protein expression, and COX-2 activity, which could be some of the mechanisms involved in the anti-inflammatory effects of this fraction. Moreover, the H8p fraction also exerted anti-nociceptive effects in 55 °C warm-water tail-withdrawal tests. The obtained results encourage further studies to determine the peptides responsible for the observed effects and potentiate their use as novel anti-inflammatory and analgesic therapies.

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7. Conflicts of Interest

The authors declare no conflict of interest.

8. Author contributions

Conceptualization, J.M. dB., X.E, M.M, B.M, and F.I.B.; formal analysis, N.I-B; funding acquisition, M.M, B.M, and F.I.B.; methodology, N.I-B, X.E, E.C, A.J.C-E; supervision, F.I.B.; writing-original draft, N.I-B, and F.I.B.; writing-review and editing, N.I-B, X.E, F.I.B. All authors have read and agreed to the published version of the manuscript.

REFERENCES

1. Medzhitov, R. Origin and Physiological Roles of Inflammation. *Nature* **2008**, *454*, 428–435.
2. Medrano-Bosch, M.; Simón-Codina, B.; Jiménez, W.; Edelman, E.R.; Melgar-Lesmes, P. Monocyte-Endothelial Cell Interactions in Vascular and Tissue Remodeling. *Front Immunol* **2023**, *14*, 1196033.
3. Lee, Y.; Rodriguez, C.; Dionne, R. The Role of COX-2 in Acute Pain and the Use of Selective COX-2 Inhibitors for Acute Pain Relief. *Curr Pharm Des* **2005**, *11*, 1737–1755.
4. Williams, J.A.; Shacter, E. Regulation of Macrophage Cytokine Production by Prostaglandin E2. *Journal of Biological Chemistry* **1997**, *272*, 25693–25699.
5. Kidd, B.L.; Urban, L.A. Mechanisms of Inflammatory Pain. *Br J Anaesth* **2001**, *87*, 3–11.
6. Leite, C.B.G.; Merkely, G.; Charles, J.F.; Lattermann, C. From Inflammation to Resolution: Specialized Pro-Resolving Mediators in Posttraumatic Osteoarthritis. *Curr Osteoporos Rep* **2023**, 1–13.
7. Wang, R.; Zhou, M.; Ma, H.; Qiao, Y.; Li, Q. The Role of Chronic Inflammation in Various Diseases and Anti-inflammatory Therapies Containing Natural Products. *ChemMedChem* **2021**, *16*, 1576–1592.
8. Tsatsanis, C.; Androulidaki, A.; Venihaki, M.; Margioris, A.N. Signalling Networks in Focus Signalling Networks Regulating Cyclooxygenase-2. *Int J Biochem Cell Biol* **2006**, *38*, 1654–1661.
9. Ju, Z.; Li, M.; Xu, J.; Howell, D.C.; Li, Z.; Chen, F.E. Recent Development on COX-2 Inhibitors as Promising Anti-Inflammatory Agents: The Past 10 Years. *Acta Pharm Sin B* **2022**, *12*, 2790–2807.
10. Rocha-González, H.I.; Sánchez-Mendoza, M.E.; Cruz-Antonio, L.; Flores-Murrieta, F.J.; Cornelio-Huerta, X.I.; Arrieta, J. Antinociceptive Interaction and Pharmacokinetics of the Combination Treatments of Methyleugenol Plus Diclofenac or Ketorolac. *Molecules* **2020**, *25*.
11. Stiller, C.; Hjemdahl, P. Lessons from 20 Years with COX-2 Inhibitors: Importance of Dose–Response Considerations and Fair Play in Comparative Trials. *J Intern Med* **2022**, *292*, 557–574.
12. Santiago-Lopez, L.; F. Gonzalez-Cordova, A.; Hernandez-Mendoza, A.; Vallejo-Cordoba, B. Potential Use of Food Protein-Derived Peptides in the Treatment of Inflammatory Diseases. *Protein Pept Lett* **2017**, *24*, 137–145.
13. Mora, L.; Toldrá, F. Advanced Enzymatic Hydrolysis of Food Proteins for the Production of Bioactive Peptides. *Curr Opin Food Sci* **2023**, *49*, 100973.
14. Ibarz-Blanch, N.; Alcaide-Hidalgo, J.M.; Cortés-Espinar, A.J.; Albi-Puig, J.; Suárez, M.; Mulero, M.; Morales, D.; Bravo, F.I. Chicken Slaughterhouse By-Products: A Source of Protein Hydrolysates to Manage Non-Communicable Diseases. *Trends Food Sci Technol* **2023**, *139*, 104125.
15. Chew, L.Y.; Toh, G.T.; Ismail, A. *Application of Proteases for the Production of Bioactive Peptides*; 2019; pp. 247–261.
16. Liu, Y.-F.; Oey, I.; Bremer, P.; Carne, A.; Silcock, P. Bioactive Peptides Derived from Egg Proteins: A Review. *Crit Rev Food Sci Nutr* **2018**, *58*, 2508–2530.
17. Liu, W.; Chen, X.; Li, H.; Zhang, J.; An, J.; Liu, X. Anti-Inflammatory Function of Plant-Derived Bioactive Peptides: A Review. *Foods* **2022**, *11*, 2361.

18. Gómez-Guillén, M.C.; Giménez, B.; López-Caballero, M.E.; Montero, M.P. Functional and Bioactive Properties of Collagen and Gelatin from Alternative Sources: A Review. *Food Hydrocoll* **2011**, *25*, 1813–1827.
19. Brandao-Rangel, M.A.R.; Oliveira, C.R.; da Silva Olímpio, F.R.; Aimbire, F.; Mateus-Silva, J.R.; Chaluppe, F.A.; Vieira, R.P. Hydrolyzed Collagen Induces an Anti-Inflammatory Response That Induces Proliferation of Skin Fibroblast and Keratinocytes. *Nutrients* **2022**, *14*, 4975.
20. Galland, F.; de Espindola, J.S.; Lopes, D.S.; Taccola, M.F.; Pacheco, M.T.B. Food-Derived Bioactive Peptides: Mechanisms of Action Underlying Inflammation and Oxidative Stress in the Central Nervous System. *Food Chemistry Advances* **2022**, *1*, 100087.
21. Millán-Linares, M.D.C.; Yust, M.D.M.; Alcaide-Hidalgo, J.M.; Millán, F.; Pedroche, J. Lupine Protein Hydrolysates Inhibit Enzymes Involved in the Inflammatory Pathway. *Food Chem* **2014**, *151*, 141–147.
22. Hayes, M.; Aluko, R.E.; Aurino, E.; Mora, L. Generation of Bioactive Peptides from Porphyridium Sp. and Assessment of Their Potential for Use in the Prevention of Hypertension, Inflammation and Pain. *Mar Drugs* **2023**, *21*, 422.
23. Larder, C.E.; Iskandar, M.M.; Kubow, S. Collagen Hydrolysates: A Source of Bioactive Peptides Derived from Food Sources for the Treatment of Osteoarthritis. *Medicines (Basel)* **2023**, *10*.
24. Fu, Y.; Therkildsen, M.; Aluko, R.E.; Lametsch, R. Exploration of Collagen Recovered from Animal By-Products as a Precursor of Bioactive Peptides: Successes and Challenges. *Crit Rev Food Sci Nutr* **2019**, *59*, 2011–2027.
25. Ramakrishnan, S.R.; Jeong, C.-R.; Park, J.-W.; Cho, S.-S.; Kim, S.-J. A Review on the Processing of Functional Proteins or Peptides Derived from Fish By-Products and Their Industrial Applications. *Heliyon* **2023**, *9*, e14188.
26. Lupu, M.-A.; Gradisteanu Pircalabioru, G.; Chifiriuc, M.-C.; Albulescu, R.; Tanase, C. Beneficial Effects of Food Supplements Based on Hydrolyzed Collagen for Skin Care (Review). *Exp Ther Med* **2020**, *20*, 12–17.
27. Bhat, C.; Rosenberg, H.; James, D. Topical Nonsteroidal Anti-Inflammatory Drugs. *Can Med Assoc J* **2023**, *195*, E1231–E1231.
28. Kumar, S.; Zakrewsky, M.; Chen, M.; Menegatti, S.; Muraski, J.A.; Mitragotri, S. Peptides as Skin Penetration Enhancers: Mechanisms of Action. *Journal of Controlled Release* **2015**, *199*, 168–178.
29. Chaulagain, B.; Jain, A.; Tiwari, A.; Verma, A.; Jain, S.K. Passive Delivery of Protein Drugs through Transdermal Route. *Artif Cells Nanomed Biotechnol* **2018**, *46*, 472–487.
30. Chromý, V.; Vinklárková, B.; Šprongl, L.; Bittová, M. The Kjeldahl Method as a Primary Reference Procedure for Total Protein in Certified Reference Materials Used in Clinical Chemistry. I. A Review of Kjeldahl Methods Adopted by Laboratory Medicine. *Crit Rev Anal Chem* **2015**, *45*, 106–111.
31. Cunniff, P. *Official Methods of Analysis of AOAC International*; Journal of AOAC international, 1995; ISBN 0935584544.
32. Adler-Nissen, J. Determination of the Degree of Hydrolysis of AOAC International.; Food Protein Hydrolysates by Trinitrobenzenesulfonic Acid. *J Agric Food Chem* **1979**, *27*.
33. Government, M. of R. with the C. and the S. of the BOE-A-1991-18408. *BOE (Spanish)* 1991, 23725–23730.

34. Safety, F.O. of C.P. and F. BVL - Official Collection of Methods of Analysis Available online: https://www.bvl.bund.de/EN/Tasks/09_Laboratories/01_Tasks/04_official_collection_methods_analysis/official_collection_methods_analysis_node.html (accessed on 20 March 2024).
35. Withey, S.L.; Paronis, C.A.; Bergman, J. Concurrent Assessment of the Antinociceptive and Behaviorally Disruptive Effects of Opioids in Squirrel Monkeys. *J Pain* **2018**, *19*, 728–740.
36. Inkanuwat, A.; Sukaboon, R.; Reamtong, O.; Asawanonda, P.; Pattaratanakun, A.; Saisavoey, T.; Sangtanoo, P.; Karnchanatat, A. Nitric Oxide Synthesis Inhibition and Anti-Inflammatory Effect of Polypeptide Isolated from Chicken Feather Meal in Lipopolysaccharide-Stimulated RAW 264.7 Macrophages. *Food Technol Biotechnol* **2019**, *57*, 200–212.
37. Miranda, K.M.; Espey, M.G.; Wink, D.A. A Rapid, Simple Spectrophotometric Method for Simultaneous Detection of Nitrate and Nitrite. **2001**.
38. Everts, B.; Währborg, P.; Hedner, T. COX-2-Specific Inhibitors--the Emergence of a New Class of Analgesic and Anti-Inflammatory Drugs. *Clin Rheumatol* **2000**, *19*, 331–343.
39. Zielińska, E.; Baraniak, B.; Karaś, M. Antioxidant and Anti-Inflammatory Activities of Hydrolysates and Peptide Fractions Obtained by Enzymatic Hydrolysis of Selected Heat-Treated Edible Insects. *Nutrients* **2017**, *9*, 970.
40. Rivera-Jiménez, J.; Berraquero-García, C.; Pérez-Gálvez, R.; García-Moreno, P.J.; Espejo-Carpio, F.J.; Guadix, A.; Guadix, E.M. Peptides and Protein Hydrolysates Exhibiting Anti-Inflammatory Activity: Sources, Structural Features and Modulation Mechanisms. *Food Funct* **2022**, *13*, 12510–12540.
41. Hong, H.; Fan, H.; Chalamaiah, M.; Wu, J. Preparation of Low-Molecular-Weight, Collagen Hydrolysates (Peptides): Current Progress, Challenges, and Future Perspectives. *Food Chem* **2019**, *301*, 125222.
42. Shoulders, M.D.; Raines, R.T. Collagen Structure and Stability. *Annu Rev Biochem* **2009**, *78*, 929–958.
43. Maswadeh, H.M.; Semreen, M.H.; Naddaf, A.R. Anti-Inflammatory Activity of Achillea and Ruscus Topical Gel on Carrageenan-Induced Paw Edema in Rats. *Acta Pol Pharm* **2006**, *63*, 277–280.
44. Gupta, A.K.; Parasar, D.; Sagar, A.; Choudhary, V.; Chopra, B.S.; Garg, R.; Ashish; Khatri, N. Analgesic and Anti-Inflammatory Properties of Gelsolin in Acetic Acid Induced Writhing, Tail Immersion and Carrageenan Induced Paw Edema in Mice. *PLoS One* **2015**, *10*, e0135558.
45. European Medicines Agency *New Safety Advice for Diclofenac-CMDh Endorses PRAC Recommendation*; Lancet Publishing Group, **2013**; Vol. 382.
46. Smith, H.S. Nonsteroidal Anti-Inflammatory Drugs; Acetaminophen. In *Encyclopedia of the Neurological Sciences*; Aminoff, M.J., Daroff, R.B., Eds.; Elsevier, **2014**; pp. 610–613.
47. Pop, R.M.; Sabin, O.; Suciu, Șoimița; Vesa, S.C.; Socaci, S.A.; Chedea, V.S.; Bocsan, I.C.; Buzoianu, A.D. Nigella Sativa's Anti-Inflammatory and Antioxidative Effects in Experimental Inflammation. *Antioxidants* **2020**, *9*, 921.
48. Crunkhorn, P.; Meacock, S.C.R. Mediators of the Inflammation Induced in the Rat Paw by Carrageenin. *Br J Pharmacol* **1971**, *42*, 392–402.

49. Guay, J.; Bateman, K.; Gordon, R.; Mancini, J.; Riendeau, D. Carrageenan-Induced Paw Edema in Rat Elicits a Predominant Prostaglandin E2 (PGE2) Response in the Central Nervous System Associated with the Induction of Microsomal PGE2 Synthase-1. *Journal of Biological Chemistry* **2004**, *279*, 24866–24872.
50. Chan-Zapata, I.; Arana-Argáez, V.E.; Torres-Romero, J.C.; Segura-Campos, M.R. Anti-Inflammatory Effects of the Protein Hydrolysate and Peptide Fractions Isolated from *Salvia Hispanica* L. Seeds. *Food Agric Immunol* **2019**, *30*, 786–803.
51. Tavares, T.G.; Spindola, H.; Longato, G.; Pintado, M.E.; Carvalho, J.E.; Malcata, F.X. Antinociceptive and Anti-Inflammatory Effects of Novel Dietary Protein Hydrolysate Produced from Whey by Proteases of Cynara Cardunculus. *Int Dairy J* **2013**, *32*, 156–162.
52. de Carvalho-Silva, L.B.; Bertoldo-Pacheco, M.T.; Bertoldo, R.; de Carvalho-Veloso, C.; Costa-Teodoro, L.; Giusti-Paiva, A.; Barboza-Lollo, P.C.; Soncini, R. Anti-Inflammatory Activities of Enzymatic (Alcalase) Hydrolysate of a Whey Protein Concentrate. *Afr J Biotechnol* **2012**, *11*.
53. Bird, D.; Ravindra, N.M. Transdermal Drug Delivery and Patches—An Overview. *Med Devices Sens* **2020**, *3*.
54. Tak, P.P.; Firestein, G.S. NF-KB: A Key Role in Inflammatory Diseases. *Journal of Clinical Investigation* **2001**, *107*, 7–11.
55. Facchin, B.M.; dos Reis, G.O.; Vieira, G.N.; Mohr, E.T.B.; da Rosa, J.S.; Kretzer, I.F.; Demarchi, I.G.; Dalmarco, E.M. Inflammatory Biomarkers on an LPS-Induced RAW 264.7 Cell Model: A Systematic Review and Meta-Analysis. *Inflammation Research* **2022**, *71*, 741–758.
56. Zhu, W.; Ren, L.; Zhang, L.; Qiao, Q.; Farooq, M.Z.; Xu, Q. The Potential of Food Protein-Derived Bioactive Peptides against Chronic Intestinal Inflammation. *Mediators Inflamm* **2020**, *2020*, 1–15.
57. Spiller, F.; Oliveira Formiga, R.; Fernandes da Silva Coimbra, J.; Alves-Filho, J.C.; Cunha, T.M.; Cunha, F.Q. Targeting Nitric Oxide as a Key Modulator of Sepsis, Arthritis and Pain. *Nitric Oxide* **2019**, *89*, 32–40.
58. Kim, H.S.; Lee, J.H.; Moon, S.H.; Ahn, D.U.; Paik, H.-D. Ovalbumin Hydrolysates Inhibit Nitric Oxide Production in LPS-Induced RAW 264.7 Macrophages. *Food Sci Anim Resour* **2020**, *40*, 274–285.
59. Meram, C.; Wu, J. Anti-Inflammatory Effects of Egg Yolk Livetins (α, β, and γ-Livetin) Fraction and Its Enzymatic Hydrolysates in Lipopolysaccharide-Induced RAW 264.7 Macrophages. *Food Research International* **2017**, *100*, 449–459.
60. Fang, Y.; Yang, L.; He, J. Plantanone C Attenuates LPS-Stimulated Inflammation by Inhibiting NF-KB/INOS/COX-2/MAPKs/Akt Pathways in RAW 264.7 Macrophages. *Biomedicine & Pharmacotherapy* **2021**, *143*, 112104.
61. Han, Y.-J.; Kwon, Y.-G.; Chung, H.-T.; Lee, S.-K.; Simmons, R.L.; Billiar, T.R.; Kim, Y.-M. Antioxidant Enzymes Suppress Nitric Oxide Production through the Inhibition of NF-KB Activation: Role of H2O2 and Nitric Oxide in Inducible Nitric Oxide Synthase Expression in Macrophages. *Nitric Oxide* **2001**, *5*, 504–513.
62. Kang, S.G.; Lee, G.B.; Vinayagam, R.; Do, G.S.; Oh, S.Y.; Yang, S.J.; Kwon, J.B.; Singh, M. Anti-Inflammatory, Antioxidative, and Nitric Oxide-Scavenging Activities of a Quercetin Nanosuspension with Polyethylene Glycol in LPS-Induced RAW 264.7 Macrophages. *Molecules* **2022**, *27*, 7432.
63. Sangtanoo, P.; Srimongkol, P.; Saisavoey, T.; Reamtong, O.; Karnchanatat, A. Anti-Inflammatory Action of Two Novel Peptides Derived from Peanut Worms (*Sipunculus*

Nudus) in Lipopolysaccharide-Induced RAW264.7 Macrophages. *Food Funct* **2020**, *11*, 552–560.

64. Deng, G.; Huang, K.; Jiang, X.; Wang, K.; Song, Z.; Su, Y.; Li, C.; Zhang, S.; Wang, S.; Huang, Y. Developments for Collagen Hydrolysates as a Multifunctional Antioxidant in Biomedical Domains. *Collagen and leather* **2023**, *5*, 1–21.
65. Esfandi, R.; Walters, M.E.; Tsopmo, A. Antioxidant Properties and Potential Mechanisms of Hydrolyzed Proteins and Peptides from Cereals. *Heliyon* **2019**, *5*, e01538.
66. Cao, C.; Xiao, Z.; Tong, H.; Liu, Y.; Wu, Y.; Ge, C. Oral Intake of Chicken Bone Collagen Peptides Anti-Skin Aging in Mice by Regulating Collagen Degradation and Synthesis, Inhibiting Inflammation and Activating Lysosomes. *Nutrients* **2022**, *14*, 1622.
67. Kim, Y.-S.; Ahn, C.-B.; Je, J.-Y. Anti-Inflammatory Action of High Molecular Weight Mytilus Edulis Hydrolysates Fraction in LPS-Induced RAW264.7 Macrophage via NF-KB and MAPK Pathways. *Food Chem* **2016**, *202*, 9–14.
68. Saisavoey, T.; Sangtanoo, P.; Reamtong, O.; Karnchanatat, A. Free Radical Scavenging and Anti-inflammatory Potential of a Protein Hydrolysate Derived from Salmon Bones on RAW 264.7 Macrophage Cells. *J Sci Food Agric* **2019**, *99*, 5112–5121.
69. Salvemini, D.; Kim, S.F.; Mollace, V. Reciprocal Regulation of the Nitric Oxide and Cyclooxygenase Pathway in Pathophysiology: Relevance and Clinical Implications. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **2013**, *304*, 473–487.
70. Sofidiya, M.O.; Imeh, E.; Ezeani, C.; Aigbe, F.R.; Akindele, A.J. Antinociceptive and Anti-Inflammatory Activities of Ethanolic Extract of Alafia Barteri. *Revista Brasileira de Farmacognosia* **2014**, *24*, 348–354.
71. Islam, S.; Shajib, Md.S.; Rashid, R. Bin; Khan, M.F.; Al-Mansur, Md.A.; Datta, B.K.; Rashid, M.A. Antinociceptive Activities of Artocarpus Lacucha Buch-Ham (Moraceae) and Its Isolated Phenolic Compound, Catechin, in Mice. *BMC Complement Altern Med* **2019**, *19*, 214.
72. Galehdar, N.; Rezaeifar, M.; Rezaeifar, M.; Rezaeifar, M. Antinociceptive and Anti-Inflammatory Effects of Amygdalus Eburnea Shell Root Extract in Mice. *Biomedical Research and Therapy* **2018**, *5*, 2746–2751.
73. Langford, D.J.; Mogil, J.S. Pain Testing in the Laboratory Mouse. In *Anesthesia and Analgesia in Laboratory Animals*; Fish, Richard.E., Brown, M.J., Danneman, P.J., Karas, A.Z., Eds.; Elsevier, **2008**; pp. 549–560.
74. Avsaroglu, H.; Van Der Sar, A.S.; Van Lith, H.A.; Van Zutphen, M.; Hellebrekers, L.J. Differences in Response to Anaesthetics and Analgesics between Inbred Rat Strains. *Lab Anim* **2007**, *41*, 337–344.
75. Morgan, M.M.; Fossum, E.N.; Stalding, B.M.; King, M.M. Morphine Antinociceptive Potency on Chemical, Mechanical, and Thermal Nociceptive Tests in the Rat. *Journal of Pain* **2006**, *7*, 358–366.
76. Udell, M.E.; Garcia Martinez, A.; Wang, T.; Ni, J.; Hurt, C.; Redei, E.E.; Chen, H. TailTimer: An Open-Source Device for Automating the Rodent 1 Tail Immersion Assay. *PlosOne* **2021**, *16*, e0256264.
77. Kaushik, D.; Kumar, A.; Kaushik, P.; Rana, A.C. Analgesic and Anti-Inflammatory Activity of Pinus Roxburghii Sarg. *Adv Pharmacol Sci* **2012**, *2012*.
78. Karrat, L.; Abajy, M.Y.; Nayal, R. Investigating the Anti-Inflammatory and Analgesic Properties of Leaves Ethanolic Extracts of Cedrus Libani and Pinus Brutia. *Heliyon* **2022**, *8*, e09254.

79. Miranda, H.F.; Lopez, J.; Dds, S.; Correa, A.; Pinardi, G. NSAID Antinociception Measured in a Chemical and a Thermal Assay in Mice. *Pain Res Manage* **2001**, *6*.
80. Mikolajczak, P.L.; Kêdzia, B.; Marcin, O.; Kujawski, R.; Bogacz, A.; Bartkowiak-Wieczorek, J.; Bialas, W.; Gryszczyñska, A.; Buchwald, W.; Szulc, M.; et al. Evaluation of Anti-Inflammatory and Analgesic Activities of Extracts from Herb of Chelidonium Majus L. *Central European Journal of Immunology* **2015**, *40*, 400–410.
81. Narayana Raju, K.V.S.; Ashok Kumar, D.; Arutselvan, N.; Thejomoorthy, P.; Puvanakrishnan, R. Antinociceptive and Antipyretic Effects of a Derivatized Tetrapeptide from Lactoferrin in Rats. *Peptides (N.Y.)* **2005**, *26*, 615–619.
82. Yam, M.F.; Loh, Y.C.; Oo, C.W.; Basir, R. Overview of Neurological Mechanism of Pain Profile Used for Animal "Pain-like" Behavioral Study with Proposed Analgesic Pathways. *Int J Mol Sci* **2020**, *21*, 4355.
83. Buey, B.; Layunta, E.; Latorre, E.; Mesonero, J.E. Potential Role of Milk Bioactive Peptides on the Serotonergic System and the Gut-Brain Axis. *Int Dairy J* **2023**, *137*, 105534.
84. Tyagi, A.; Daliri, E.B.-M.; Kwami Ofosu, F.; Yeon, S.-J.; Oh, D.-H. Food-Derived Opioid Peptides in Human Health: A Review. *Int J Mol Sci* **2020**, *21*, 8825.
85. Mas-Capdevila, A.; Iglesias-Carres, L.; Arola-Arnal, A.; Aragonès, G.; Muguerza, B.; Bravo, F.I. Implication of Opioid Receptors in the Antihypertensive Effect of a Novel Chicken Foot-Derived Peptide. *Biomolecules* **2020**, *10*, 992.
86. Vo, T.-S.; Kim, S.-K. Down-Regulation of Histamine-Induced Endothelial Cell Activation as Potential Anti-Atherosclerotic Activity of Peptides from Spirulina Maxima. *European Journal of Pharmaceutical Sciences* **2013**, *50*, 198–207.

Manuscript 2

Objective: To identify the peptides responsible for the anti-inflammatory effects of the H8 pigskin-collagen hydrolysate, evaluating their bioactivity in LPS-stimulated RAW 264.7 cells.

Pigskin collagen peptides alleviate LPS-induced inflammatory response in RAW 264.7 macrophages

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Abstract

Bioactive peptides are emerging as novel anti-inflammatory preventive treatments. In a previous study, <10 kDa fraction of a collagen-derived hydrolysate (H8) showed anti-inflammatory effects in carrageenan-induced rat paw edema. This study aimed to identify the peptides responsible for its effect. The <3 kDa fraction of H8 was obtained and further fractionated by RP- HPLC. Of the 22 collected fractions, F3 and F20 showed the highest *in vitro* cyclooxigenase-2 (COX-2) inhibitory activities. Nine peptides were identified by a UHPLC-Orbitrap MS/MS in F3 and F20. AGERGEQ, GSKGRPG, AGPAGKP, and PGPAGPV showed selective *in vitro* COX-2 inhibition and reduced nitrite production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. AGERGEQ and GSKGRPG also decreased inducible nitric oxide synthase (iNOS) and COX-2 protein expression in these macrophages. These peptides may be responsible for the anti-inflammatory effects of the hydrolysate. This study provides evidence for the potential use of collagen-derived peptides as functional ingredients to prevent inflammation.

Keywords: anti-inflammatory activity, cyclooxygenase-2 inhibitory activity, inducible nitric oxide synthase, nitrite production, UHPLC-Orbitrap MS/MS

1. Introduction

Inflammation, a natural defense process in living organisms, can lead to severe consequences if persistent [1]. It is implicated in the development of numerous diseases, including cardiovascular, intestinal, skin, or neurodegenerative diseases [2–5]. There are a broad variety of anti-inflammatory drugs that use different molecular approaches. One of them is the group of non-steroidal anti-inflammatory drugs (NSAIDs), with cyclooxygenase enzyme (COX) inhibition being the main characteristic target [6]. This enzyme, specifically isoenzyme 2 (COX-2), is responsible for the production of prostaglandins, a group of pro-inflammatory molecules derived from arachidonic acid [7]. ibuprofen, naproxen, and aspirin are some of the best-known NSAIDs. However, many undesired side effects have been reported with the use of NSAIDs and other anti-inflammatory drugs, especially in older populations and patients with cardiovascular, renal, or hepatic complications [8,9]. In this regard, the search for new anti-inflammatory compounds, mainly obtained from natural sources as bioactive peptides, that can be used as functional ingredients, is a promising research field [10–12].

Bioactive peptides are small amino acid sequences (3-12 amino acids), that once released from the native protein by enzymatic hydrolysis, microbial fermentation, or gastrointestinal digestion, can exert beneficial effects on the host organism [8,13]. A wide range of bioactivities has been described for bioactive peptides, such as antioxidant, antihypertensive, neuroprotective, antidiabetic, or anti-inflammatory [14], with the latter being among the most widely investigated. Anti-inflammatory peptides have traditionally been obtained from many protein sources, such as eggs, dairy products, or legumes [15–19]. Nonetheless, the search for anti-inflammatory peptides from other sources is emerging [8,13]. The most commonly used techniques for evaluating the anti-inflammatory potential of peptides and protein hydrolysates are the quantification in nitrite production and the evaluation of changes in the protein and mRNA expression of cytokines and pro-inflammatory factors in lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophages [8]. In this regard, in a previous study by our group, a collagen-derived hydrolysate (H8) with *in vitro* COX-2 inhibitory activity was obtained. Moreover, its <10 kDa fraction inhibited the COX-2 enzyme *in vitro* and also reduced the production of nitrites in LPS-stimulated RAW 264.7 macrophages. In *in vivo* models, topical administration of the <10 kDa fraction increased pain tolerance in a 55 °C tail-withdrawal test (anti-nociceptive effect) and reduced paw edema formation after carrageenan induction [manuscript 1]. However, the

peptides responsible for these effects remain unclear. Therefore, the objective of this study was to identify the peptides responsible for the anti-inflammatory effects of this collagen-derived hydrolysate.

2. Materials and methods

2.1. Materials and reagents

Porcine skin collagen was supplied by Juncà Gelatines S.L. (Girona, Spain). The <10 kDa fraction of the pigskin collagen hydrolysate H8 was obtained as previously described in a pilot scale [manuscript 1]. This fraction was characterized as follows: total protein content was 88.96 % ± 0.66 (w/w) (determined by the Kjeldahl method, with 5.55 as protein factor [20]), moisture was 3.18 % ± 0.11 (w/w) (determined by AOAC official method [21]), the degree of hydrolysis was 63.20 % ± 1.70 (analyzed by TNBS method [22]), and the *in vitro* COX-2 inhibition was 74.04 % ± 8.89 (determined as described in section 2.3.2). The COX colorimetric inhibitor screening assay kit (ref. 701050) was purchased from Cayman Chemical (Michigan, USA). HPLC-grade acetonitrile was provided by Carlo Erba Reagents SRL (Milan, Italy). Peptides (AGERGEQ, GPAGSVG, GSKGRPG, AGPAGKP, AGPPGAK, GPAGPAG, GPAGPAGPRG, PGPAGPV, TGPIGPPGPA) were purchased from Caslo ApS (Lyngby, Denmark). Murine macrophage cell line RAW 264.7 was purchased from the American Type of Culture Collection (ATCC, Manassas, VA). Penicillin-streptomycin, L-glutamine, *E. coli* LPS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), glycine, sulfanilamide, N-(1-Naphthyl)ethylenediamine (NED), Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Merck Millipore (Madrid, Spain). BCA™ Protein Assay Kit and protease and phosphatase inhibitors cocktails were provided by Thermo Fisher Scientific (Madrid, Spain).

2.2. Identification of peptides from the collagen-derived hydrolysate H8

2.2.1. Peptide isolation

The <10 kDa fraction was reconstituted in Milli-Q water, and the peptide fraction lower than 3 kDa was obtained using centrifugal filter units with a 3 kDa cut-off membrane (Amicon® Ultra 15, Merck Millipore, Madrid, Spain). The obtained fraction was freeze-dried and stored at -20 ºC. Subsequently, peptides contained in the <3 kDa fraction were fractionated by semi-preparative High-Performance Liquid

Chromatography in reversed-phase (RP-HPLC) (Agilent Technologies 1260 series, Santa Clara, CA USA) using a Europa peptide column (120 Å pore size, 25 x 1 cm i.d. 5 µm silica particle size) (Teknokroma, Barcelona, Spain) according to *Bravo et al. 2022* [23]. The mobile phase was water: trifluoroacetic acid (1000:1, v:v) as solvent A and acetonitrile: trifluoroacetic acid (1000:0.8, v:v) was the solvent B. Elution flow was set at 4 mL/min using the following gradient: 0 to 23.5 % B in 59.2 min, 23.5 - 90 % B in 5.8 min, and 90 - 0 % B in 2 min. The column was allowed to equilibrate with 100 % solvent A for 13 min. The sample injection volume was 400 µL at a concentration of 300 mg of sample/mL dissolved in Milli-Q water, and the absorbance was measured at 214 nm. Different peptide fractions were collected during the chromatographic separation, lyophilized, and kept at -20 ºC. Finally, the *in vitro* COX-2 inhibitory activity of the fractions, reconstituted in Milli-Q, was determined at a protein concentration of 45 µg/mL in the well (method described in section 2.3.1). The protein content was quantified using the bicinchoninic acid method with a BCA™ protein assay kit according to the manufacturer's instructions. The two fractions with the highest activity were further analyzed for peptide identification.

2.2.2. Peptide identification using a UHPLC-Orbitrap MS/MS)

A rapid-resolution liquid chromatograph (UHPLC, Agilent Technologies, California, USA) coupled to an Orbitrap MS/MS (LTQ-Orbitrap Velos Pro mass spectrometer; Thermo Fisher Scientific, CA, USA) was used to determine the peptide composition of the selected RP-HPLC-derived fractions (F3 and F20). The peptides were separated on a Peptide BEH C18 reverse phase column (Waters, Massachusetts, USA) coupled to the UHPLC for 70 min. Phase A consisted of Milli-Q water containing 0.1 % formic acid, and phase B consisted of 100 % acetonitrile. The elution flow rate was set to 0.4 mL/min. For real-time peptide ionization and fragmentation, an enhanced high-resolution FT spectrum (resolution = 30,000 FHMW), followed by a data-dependent FT-MS/MS scan, was employed. This scan focused specifically on the ten most intense parent ions with a charge state of one or higher. Fragmentation was achieved by high-energy collision-induced dissociation with a standardized collision energy set at 40 %. A dynamic exclusion strategy with a period of 0.5 min was applied to avoid redundancies. Subsequently, the tandem mass spectra were processed with Proteome Discoverer version 1.4.0.288 (ThermoFisher Scientific) to deconvolute the charge state. MS and MS/MS samples were analyzed with the Mascot (Version 2.5)

search engine using one node with the proteome of *Sus Scrofa* (26103 entries) from UniProt. This search assumed no enzyme digestion and an error of 40 mmu for fragment ion mass, and 20 ppm for precursor ions. Oxidation of methionine and acetylation of the N-termini were specified as variable modifications. For the peptide identification, visual verification of fragmentation spectra was performed and only the peptides found in both replicates were considered.

To evaluate the bioactivity of the identified peptides, they were chemically synthesized by Caslo ApS using the phase Fmoc method and a synthesizer (Model 431A; Applied Biosystems Inc., Überlingen, Germany). The purity of all the synthesized peptides was greater than 96 %.

2.3. Evaluation of the in vitro anti-inflammatory effect of the RP-HPLC fractions and identified peptides

2.3.1. Prediction of bioactivity

The potential bioactivity of peptides was evaluated using the BioPep database (https://biochemia.uwm.edu.pl/biopep/start_biopep.php, accessed on 14 February 2024) [24], and the software PeptideRanker (http://distilldeep.ucd.ie/PeptideRanker/, accessed on 14 February 2024) [25].

2.3.2. In vitro COX-2 and COX-1 inhibitory activity

COX-2 inhibitory activity of the <10 kDa fraction of the collagen-derived hydrolysate, RP-HPLC fractions, and synthetic peptides was tested using a colorimetric inhibitor screening assay kit, according to the procedure given by the manufacturer (Cayman Chemical). Briefly, the enzyme, samples, and hemin, as a co-factor, were incubated for 5 min at room temperature. The reaction was initiated by the addition of the colorimetric substrate TMPD (N, N, N', N'-tetramethyl-p-phenylenediamine) and arachidonic acid. After 2 min of reaction, the absorbance was measured at 590 nm using a spectrophotometer (Eon BioTek Izasa, Barcelona, Spain). <10 kDa fraction, lyophilized RP-HPLC fractions, and synthetic peptides, dissolved in Milli-Q water, were tested in triplicate at a protein/peptide concentration of 1 mg/mL for the fractions and 0.2 mg/mL for the peptides (well concentration of 45 µg/mL and 9 µg/mL, respectively). The percentage of inhibition was calculated as follows:

$$\%\,Inhibition = \left(1 - \frac{Abs_{590nm}\,S\; - Abs_{590nm}\,BS}{Abs_{590nm}\,C^{+}\; - Abs_{590nm}\,B}\right) * 100$$

where C^+ is the enzyme without inhibitors, B is the background of the reagents without enzyme, S is the enzyme incubated with the sample, and BS is the background of the reagents and sample without the enzyme.

COX-1 inhibitory activity was also evaluated for synthetic peptides (9 µg/mL) using the same enzymatic kit as COX-2, following the manufacturer's instructions (Cayman Chemical).

2.3.3. Cell culture

Murine macrophages RAW 264.7 were cultured at 37 °C in DMEM supplemented with 10 % FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and L-glutamine in a humidified 5 % CO_2 incubator. The cells were then dislodged using the flask with a cell scraper. All samples and compounds were filtered using a 0.22 µm filter before added to the culture.

2.3.4. Determination of cell viability

Cell viability was determined using the MTT assay adapted from Inkanuwat et al. 2019 [26]. RAW 264.7 macrophages were seeded at a concentration of 1 x 10^5 cells/mL in a 96-well plate and cultured overnight with supplemented DMEM. Cells were incubated for 48 h with the peptides (final concentration of 0.1 mg/mL) or DMEM. Then, the medium was replaced with fresh medium containing 0.5 mg/mL MTT (previously dissolved in PBS at 5 mg/mL). After 2 h of incubation in the dark at 37 ºC, the 96-well plates were centrifuged at 700 *x g* for 3 min, and the supernatants were discarded. Dimethyl sulfoxide and 0.1 M glycine buffer were added to the wells to dissolve the formed crystals. Then, absorbance was read at 570 nm. Results were expressed as a percentage of viability, considering untreated cells (DMEM cells) as 100 % viability. The treatments were assayed in triplicate.

2.3.5. Determination of nitrite production in LPS-stimulated RAW 264.7 cells

On the first day of the experiment, 1 x 10^5 cells/mL were seeded in 24-well plates and cultured overnight in DMEM supplemented at 37 ºC. Then, samples were filtered by a 0.22 µm filter, and macrophages were pre-incubated for 24 h with the peptides at a concentration of 0.1 mg/mL in the well or sterile-filtered PBS for the negative control, in triplicate. The cells were then stimulated with LPS (1 µg/mL) for 24 h, except in the negative control wells. The supernatant was collected, and nitrite concentration was

determined using the Griess method, with modifications [16]. Briefly, 50 µL of the supernatants, in triplicate, were incubated for 30 min at 4 ºC with 100 µL of 1.5 % sulfanilamide in HCl 3.25 M and 50 µL of NED (14 mM). Absorbance was measured at 540 nm. Sodium nitrate at different concentrations (0-200 mM) was used as the standard. Cells were collected in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl; pH 7.4, 1% Tween 20, 0.25% Na-deoxycholate) containing protease and phosphatase inhibitors cocktails and phenylmethylsulfonyl fluoride (PMSF) for protein quantification. The nitrite concentration in the supernatants was standardized using the amount of protein in each well, which was determined using the BCA™ Protein Assay Kit. Results were expressed as a percentage of reduction of nitrites in the supernatants, considering the control with LPS wells as 100 % of nitrite secretion. Maximum production of nitrites was considered for the LPS-stimulated RAW 264.7 macrophages treated with PBS. Cells were treated in triplicate and supernatants were analyzed in triplicate.

2.3.6. Immunoblot analysis

As mentioned, RAW 264.7 cells incubated with the different peptides and stimulated with LPS were collected for the analysis of inducible nitric oxide synthase (iNOS) and COX-2 protein by Western Blot. RIPA buffer containing protease and phosphatase inhibitor cocktails and PMSF were used to lyse the cells and protein was extracted.

Then, 20 µg of the denatured proteins were loaded in a 10 % SDS-polyacrylamide gel and transferred onto a 0.45 µm methanol-activated PVDF membrane. Milk powder (5 % in 0.2 % TBS-Tween buffer) was used to block the membranes for 1 h. Immunoblot was carried out by using polyclonal antibodies against iNOS, COX-2, and β-actin which was used as loading control (Santa Cruz Biotechnology, Heidelberg, Germany). Proteins were detected with the chemiluminescent reagent ECL Select Western Blotting Detection Reagent (GE Healthcare, Barcelona, Spain). Images were captured using a G:BOX Chemi XL1.4 (Syngene, Cambridge, UK), and densitometry quantification was performed using ImageJ Software 1.54 (NIH, Bethesda, MD, USA). The assay was performed in duplicate.

2.4. Statistical analysis

Values were expressed as the mean ± standard deviation (SD). Outliers were identified using the Grubbs' test and removed before statistical analysis. Normality and homogeneity were evaluated by using Shapiro–Wilk's and Levene's tests, respectively. Both statistical analysis and graphics were performed with Prism v.9 software (GraphPad Software, San Diego, CA, USA). One-way analyses of variance (ANOVA) were used to compare groups (Bonferroni post-hoc test). An obtained p-value < 0.05 was considered statistically significant.

3. Results and discussion

Inflammation can become a serious issue if not properly resolved [1]. Moreover, it is implicated in the development of multiple pathologies such as cardiovascular, respiratory, or neurodegenerative diseases [2]. The numerous side effects of the current anti-inflammatory drugs lead to the search for new therapies [27–29]. Bioactive compounds, such as bioactive peptides, have emerged as promising candidates for the prevention of inflammation [16,17,30,31]. In a previous study, we obtained a <10 kDa fraction from a collagen-derived hydrolysate with *in vitro* COX-2 inhibitory activity and *in vivo* anti-inflammatory effects [manuscript 1]. However, the peptides responsible for these effects were not identified. Therefore, their identification was the objective of the current study.

3.1. Identification of the peptides present in the anti-inflammatory collagen-derived hydrolysate

Several studies have shown that most bioactive peptides, including anti-inflammatory peptides, tend to be smaller in size [8,32,33]. This may be because these peptides are less susceptible to hydrolysis by gastrointestinal digestion, are more easily absorbed by cells, and are more accessible to enzymes to produce their inhibition [8,34,35]. Thus, to identify the peptides contained in the anti-inflammatory collagen hydrolysate, the peptide <3 kDa fraction was obtained by ultrafiltration to eliminate larger peptides and non-digested proteins. This peptide size fraction was also used to identify anti-inflammatory peptides from digested velvet antler proteins [36]. Moreover, *Gao et al., (2020)* observed that the <3 kDa fraction obtained from alcalase-digested sturgeon muscle showed the highest anti-inflammatory effects on LPS-stimulated RAW 264.7 macrophages compared to the 3 -10 kDa and >10 kDa fractions [37]. RP-HPLC

was used in the present study to further isolate the peptides in the <3 kDa fraction based on their polarity. The separation was carried out by decreasing the polarity of the mobile phase (increasing the percentage of acetonitrile), and 22 fractions (F1 - F22) were collected (Figure 1A). The most hydrophilic peptides were collected in F1 (approximately 2.4 % of acetonitrile), and the least hydrophilic peptides were collected in F22, where the percentage of acetonitrile reached 90 %. The chromatogram shown in Figure 1A reflects the complexity and variety of peptides present in the collagen-derived hydrolysate H8.

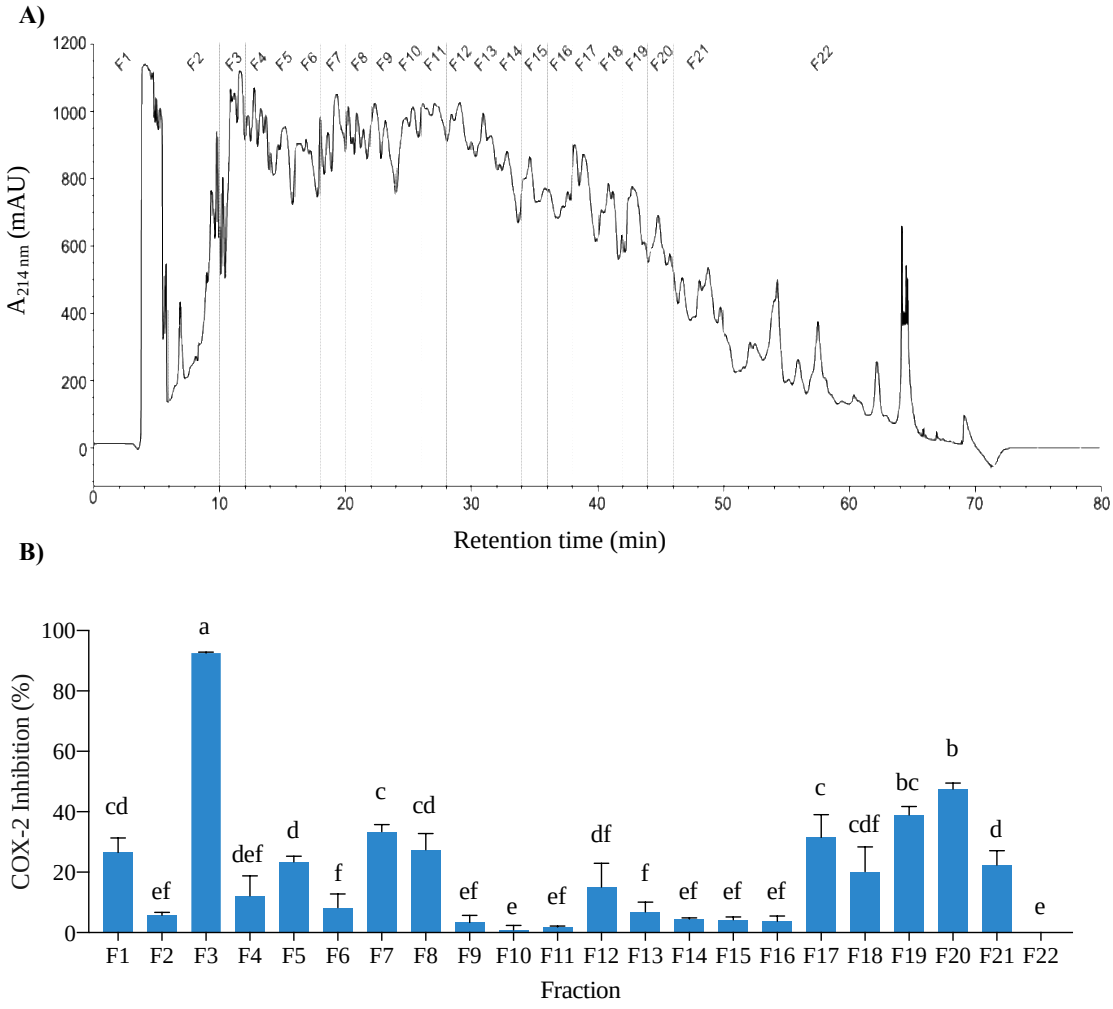

Figure 1. A) RP-HPLC chromatogram of the <3 kDa fraction of the anti-inflammatory collagen-derived hydrolysate and collected fractions. **B)** *In vitro* cyclooxygenase-2 (COX-2) inhibitory activity of RP-HPLC fractions at 45 µg/mL. The percentage of inhibition is expressed as the mean ± standard deviation (n=3). Significant differences between fractions are represented by different letters (p <0.05; one-way ANOVA; post-hoc: Bonferroni test).

The inhibitory capacity against COX-2 enzyme was tested for all 22 fractions *in vitro* at a final protein concentration of 45 µg protein/mL in the well (Figure 1B). This anti-inflammatory bioactivity was selected based on the *in vitro* COX-2 inhibition activity shown by the collagen-derived hydrolysate [manuscript 1]. This enzyme, expressed in response to inflammatory factors, catalyzes the synthesis of prostaglandin H from arachidonic acid, which is further transformed into the pro-inflammatory compound prostaglandin E_2 [7]. Its inhibition is considered an anti-inflammatory target for different drugs known as NSAIDs [38]. Moreover, COX-2 inhibition has also been used to evaluate the anti-inflammatory effects of protein hydrolysates, such as a hydrolysate obtained from millet grains under *in vitro* gastrointestinal conditions and different lupine protein hydrolysates [17,39]. In the present study, the RP-HPLC fractions exerted a diverse range of COX-2 inhibition (between 92.6 % and 0 %). F3 was clearly the most bioactive fraction, with 92.6 % ± 0.3 of COX-2 inhibition, followed by fraction F20 (47.5 % ± 2.0) (Figure 1B). Curiously, the most active fractions were collected at the opposite ends of the chromatogram, indicating significant differences in polarity owing to their acetonitrile percentages (approximately 4.8 % for F3 and 18.3 % for F20). By contrast, fractions such as F10 and F22 did not show any COX-2 inhibitory activity at the tested protein concentration.

Fractions F3 and F20 were selected for peptide identification by UHPLC-Orbitrap MS/MS and searching for sequences in the *Sus scrofa* proteome. The amino acid sequences and the fraction of origin, native protein, mass, charge state, and m/z ratio, as well as the percentage of hydrophobic amino acids, are listed in Table 1.

Table 1. Amino acid sequences identified in the RP-fractions F3 and F20 and their characteristics.

Peptide label	Sequence*	Fraction	MH+ (Da)	Charge	m/z (Da)	Protein	Hydrophobic aa (%)	PR score
P1	AGPAGKP	F20	597.33	2	299.17	Collagen type XI	57.14	0.672
P2	GSKGRPG	F3	658.36	2	329.68	Collagen type VI	14.29	0.506
P3	AGPPGAK	F20	597.33	2	299.17	Collagen type XI	57.14	0.660
P4	GPAGPAG	F20	526.26	1	526.26	Collagen type XI	57.14	0.734
P5	PGPAGPV	F20	594.32	1	594.32	-	71.43	0.665
P6	AGERGEQ	F3	746.33	2	373.67	Collagen type II	14.29	0.084
P7	GPAGSVG	F3	544.27	2	272.30	Collagen type VII	42.86	0.316
P8	GPAGPAGPRG	F20	836.43	2	418.72	-	50.00	0.908
P9	TGPIGPPGPA	F20	863.47	1	863.47	Collagen type II	60.00	0.528

[a]Amino acids are designated using their one-letter codes. Abbreviations: aa: amino acid residues; m/z: mass/charge; PR: PeptideRanker.

Three amino acid sequences (AGERGEQ, GPAGSVG, and GSKGRPG) were identified in fraction F3, whereas six peptides (AGPAGKP, AGPPGAK, GPAGPAG, GPAGPAGPRG, PGPAGPV, and TGPIGPPGPA) were found in the fraction F20. The MS/MS spectra of three of them are shown as an example in Figure 2.

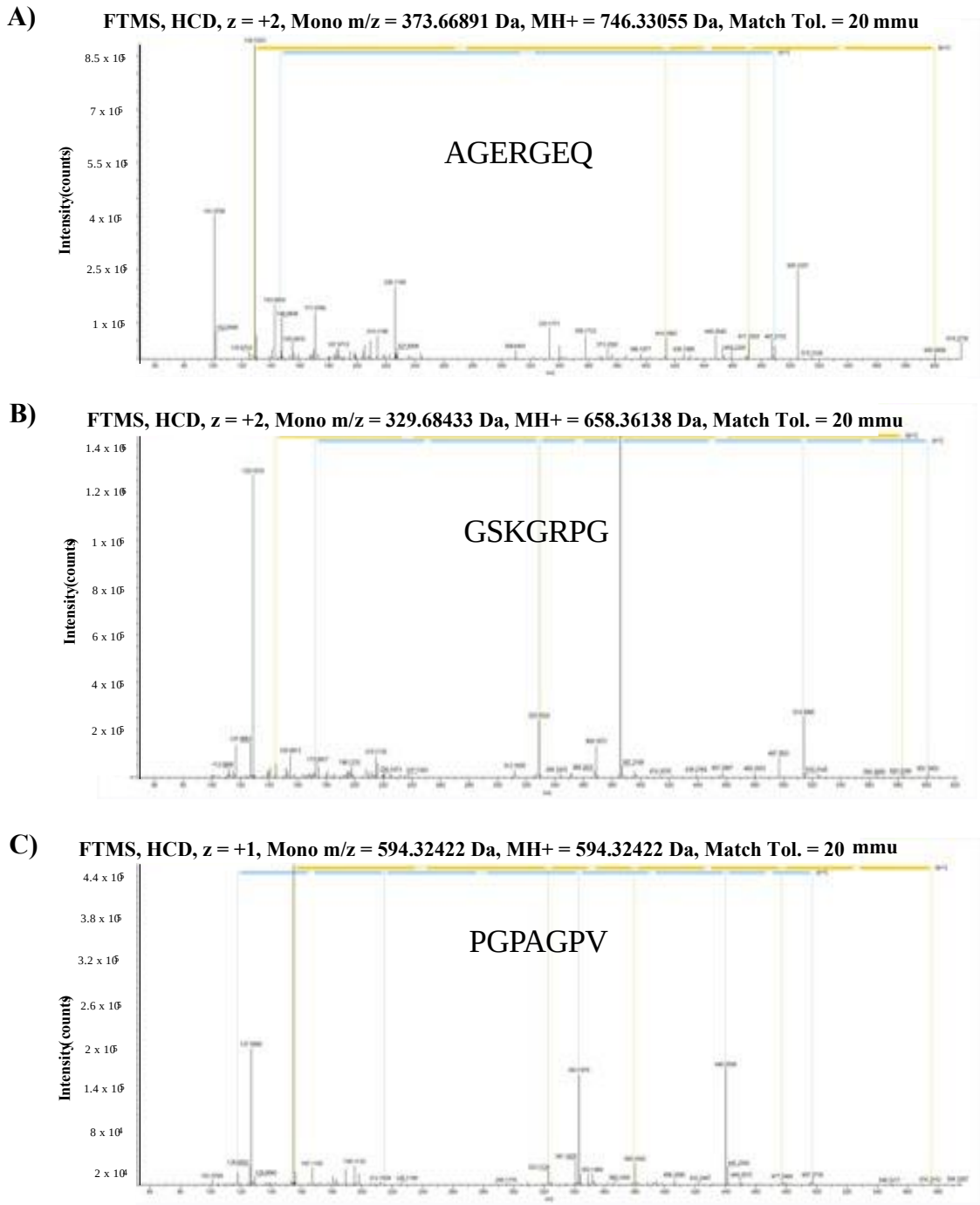

Figure 2. MS/MS spectrum of the doubly charged ion m/z 373.67 **(A)** and 329.68 **(B)**, and mono-charged ion m/z 594.32 **(C)**. After sequence interpretation and database search, the peptides were identified as AGERGEQ, GSKGRPG, and PGPAGPV, respectively. The MS/MS spectra were acquired using a linear trap quadrupole-orbitrap mass spectrometer. The sequences are shown with the observed fragmented ions in the spectra.

As expected, diverse collagen types from *Sus scrofa* were identified as native proteins of the identified peptides, except for GPAGPAGPRG and PGPAGPV, whose protein of origin was not identified (Table 1). In concordance with the polarity found in the RP-HPLC fractions, polar amino acids were predominant in the peptide sequences found in F3 (57 – 86 % of polar amino acids), such as Gly, Ser, or Gln, whereas non-polar amino acids such as Pro, Ala, or Ile were more abundant in the peptides identified in F20 (50 – 71 % of non-polar amino acids). According to recent studies, known peptides with anti-inflammatory activities do not share clear common characteristics. However, length (2-20 amino acids), low molecular weight (<1 kDa), the presence of hydrophobic amino acids (Val, Ile, Pro), or positively charged amino acids (His, Arg, Lys) could enhance the anti-inflammatory effect [8,18]. Analyzing the obtained peptides in the present study, all identified sequences were composed of 7 - 10 amino acids, and their molecular weight was lower than 900 Da. The sequences and physical characterization of the identified peptides suggest great potential bioactivity for some of them. For example, the PGPAGPV peptide (P5) was rich in hydrophobic amino acids (71.43 %), particularly Pro, and only two of the 7 amino acid residues were polar (Gly). This result was expected given that this peptide was obtained from fraction F20. On the other hand, the GSKGRPG (P2) peptide from F3, as expected, had a lower content of hydrophobic amino acids (14.29 %) but contained positively charged amino acids (Arg and Lys), which could confer anti-inflammatory bioactivity.

3.2. Evaluation of the in vitro anti-inflammatory effect of the peptides

We did not find in the BioPep database any previously described bioactivity for the nine identified sequences [24]. The peptide GPAGPAG (P4) was found within the peptide GETGPAGPAGAAGPAGPR, which showed angiotensin-converting enzyme inhibitory activity. In addition, the sequences of the peptides were also introduced to the software PeptideRanker to predict any potential bioactivity [25]. The PeptideRanker scores for most peptides ranged from 0.5 - 0.9, except for the amino acid sequences AGERGEQ (P6, 0.08) and GPAGSVG (P7, 0.316). The peptide with the highest score was GPAGPAGPRG (P8, 0.908). The higher the predicted probability approaches 1, the greater PeptideRanker's confidence in the peptide being bioactive [25]. The probability values considered acceptable for bioactivity fall within the range of 0.5 to 1.0. For instance, the peptides AIPAAPAAPAGPKLY, and LIHADPPGVGL with PeptideRanker scores of 0.768, and 0.743, respectively, showed COX-1 inhibitory activity [40]. Thus,

the next step of the study was to test the anti-inflammatory activity of the identified peptides. This bioactivity was evaluated through the COX-2 inhibition activity *in vitro*, the reduction of the nitrite production of LPS-stimulated macrophages, and the detection of COX-2 and iNOS by immunoblot analysis. For this purpose, the nine peptides identified in the F3 and F20 fractions were chemically synthesized.

3.2.1. Identification of selective COX-2 but no COX-1 inhibitors

Considering the COX-2 inhibitory effect of the anti-inflammatory collagen hydrolysate, this bioactivity was first tested in the synthetic peptides (Figure 3A). All the sequences inhibited the COX-2 enzyme when tested at 9 µg/mL. The most active peptides were PGPAGPV (P5, 41.3 %), GPAGSVG (P7, 40.1 %), AGERGEQ (P6, 37.6 %), GSKGRPG (P2, 37.5 %), and AGPAGKP (P1, 32.2 %). TGPIGPPGPA (P9) was the peptide with the lowest COX-2 inhibitory activity (14.5 %). There is limited evidence regarding peptides inhibiting COX-2. In this regard, *Zielinska et al. (2018)* identified 12 amino acid sequences with this bioactivity, which were generated after subjecting different edible insect species (*Gryllodes sigillatus, Schistocerca gragaria*, and *Tenebrio molitor)* to *in vitro* gastrointestinal digestion [41]. Their COX-2 inhibitory activity ranged between 7.4 and 9.75 mg/mL (IC_{50}), with the amino acid sequence FDPFPK exhibiting the highest activity. The most active peptides found in our study were markedly more active than the insect-derived peptides, given that five peptides (PGPAGPV, GPAGSVG, AGERGEQ, GSKGRPG and AGPAGKP) showed approximately 40 % of COX-2 inhibition at 9 µg/mL, whereas the most active insect-derived peptide needed 7.40 mg/mL to inhibit 50 % of the enzyme activity.

Similarly, these peptides also showed more COX-2 inhibitory activity than the peptides GVDYVRFF, AIPAAPAAPAGPKLY, and LIHADPPGVGL, identified in a hydrolysate obtained from a red microalga (*Porphyridium* sp) using Viscozyme® and Alcalase®. In this case, the maximum inhibition achieved was 30 % at 1 mg/mL [40].

Given the potential of these five identified peptides (PGPAGPV, GPAGSVG, AGERGEQ, GSKGRPG, and AGPAGKP) to inhibit COX-2 activity, COX-1 inhibition was also evaluated for these peptides (Figure 3B). It is important to note that non-selective COX inhibition has been associated with undesirable side effects of NSAIDs, especially in the gastrointestinal tract [29,38]. In our results, only GPAGSVG inhibited COX-1 (29.5 %) at a concentration of 9 µg/mL. Previous studies found that peptides such as AIPAAPAAPAGPKLY and LIHADPPGVGL, obtained from a red microalga

Porphyridium sp hydrolysate, also inhibited COX-1 (IC_{50} values of 0.23 and 0.22 mg/mL) [40]. These results evidenced that the peptides AGERGEQ, GSKGRPG, AGPAGKP, and PGPAGPV selectively inhibited COX-2.

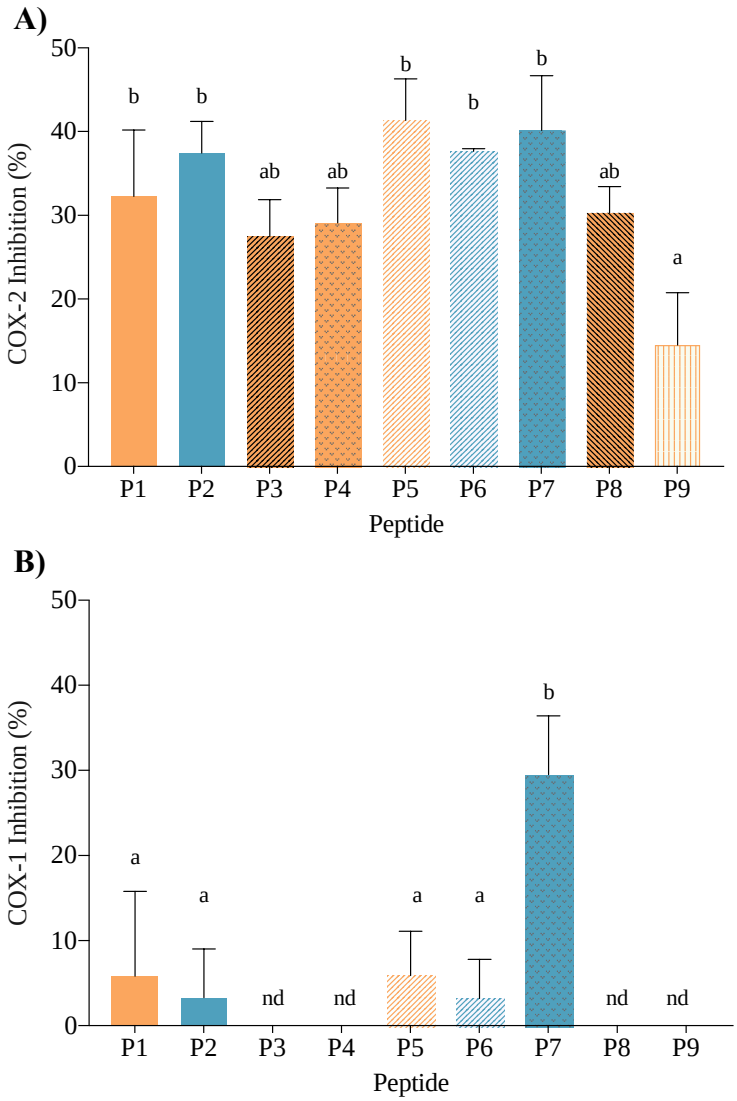

Figure 3. *In vitro* cyclooxygenase (COX) isoforms 2 (COX-2, **A**) and 1 (COX-1, **B**) inhibitory activity for the peptides identified in fractions F3 and F20 at 9 µg/mL. Data are expressed as the mean ± standard deviation (n=3). Significant differences between groups are represented by different letters (p <0.05; one-way ANOVA; post-hoc: Bonferroni test). P1: AGPAGKP; P2: GSKGRPG; P3: AGPPGAK; P4: GPAGPAG; P5 PGPAGPV; P6: AGERGEQ; P7: GPAGSVG; P8: GPAGPAGPRG; P9: TGPIGPPGPA.

3.2.2. Anti-inflammatory effect of peptides in LPS-stimulated RAW 264.7 macrophages.

The capacity of the peptides to reduce the secretion of nitrites of LPS-stimulated RAW 264.7 murine macrophages was tested. Stimulation with LPS promotes the overexpression of iNOS and other pro-inflammatory markers. This enzyme catalyzes the reaction of L-arginine into L-citrulline, releasing nitric oxide (NO) during the process, which acts as a pro-inflammatory mediator [42]. The production and subsequent secretion of NO are directly linked to the presence and activity of iNOS, and the secreted NO (or its derivatives) can be quantified using the Griess method. This methodology has been widely used to screen and evaluate the anti-inflammatory activity of peptides and other bioactive compounds [8,43].

Firstly, the viability of RAW 264.7 cells after being treated with the peptides at 0.1 mg/mL was determined by the MTT assay (Figure 4A). None of the treatments exerted a statistically negative effect on the viability of the macrophages related to the untreated control group survivance. Figure 4B shows the percentage of nitrite reduction observed in the supernatant of LPS-stimulated RAW 264.7 cells incubated with the identified peptides (0.1 mg/mL) compared to the untreated LPS-stimulated RAW 264.7 macrophages (positive control). The peptides GSKGRPG (P2, 23.4 %) and AGERGEQ (P6, 17.9 %), identified in the fraction F3, and peptides PGPAGPV (P5, 20.8 %) and AGPAGKP (P1, 18.2 %), identified in the fraction F20, were able to reduce of LPS-induced nitrite production in macrophages. Peptides from many different sources, including collagen-rich sources, have been previously reported to reduce nitrite production [8,13]. For instance, the peptide SNPSVAGVR identified in a chicken feather meal hydrolysate reduced nitrite production (estimated value of 10-82 %) by LPS-stimulated RAW 264.7 cells in a dose-dependent manner (15-120 mM) [26]. The tripeptide PAY (0.75 mM) obtained from salmon (*Salmo salar*) also reduced nitrite production by 63.8 % in the same cell model [44]. As our peptides AGERGEQ (P6) and GSKGRPG (P2) were tested at 0.013 mM and 0.015 mM respectively, the results indicate that these peptides might exert higher anti-inflammatory effects than the mentioned reported peptides.

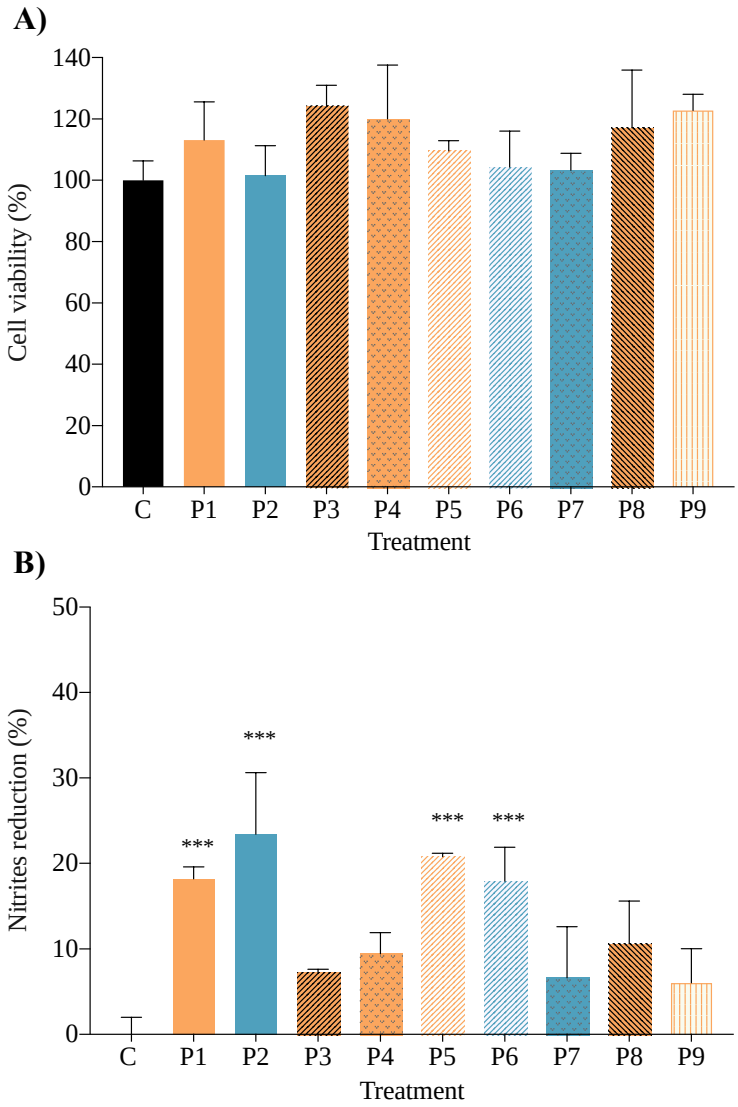

Figure 4. **(A)** MTT cell viability assay of RAW 264.7 cells treated with peptides. 1 x 10^5 cells/mL were seeded in a 96-well plate, cultured overnight, and treated with peptides (100 µg/mL) or medium (negative control) for 48 h. **(B)** Inhibition of the nitrite production of lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophages produced by peptide treatment. RAW 264.7 cells were pretreated with 100 µg/mL of peptides for 24 h, and then exposed to LPS (1 µg/mL) for 24 h. Nitrite levels were analyzed in the culture media. Nitrite levels determined in untreated LPS-stimulated cells were considered 100 %. Results are expressed as the mean ± standard deviation (n=3). *** indicates significant differences between each peptide treatment and control (C: PBS-treated LPS-stimulated cells) (p <0.001, one-way ANOVA; post-hoc: Bonferroni test). P1: AGPAGKP; P2: GSKGRPG; P3: AGPPGAK; P4: GPAGPAG; P5 PGPAGPV; P6: AGERGEQ; P7: GPAGSVG; P8: GPAGPAGPRG; P9: TGPIGPPGPA.

Protein expression of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells treated with the peptides (P1, P2, P5, and P6) was also analyzed by western blotting (Figure 5).

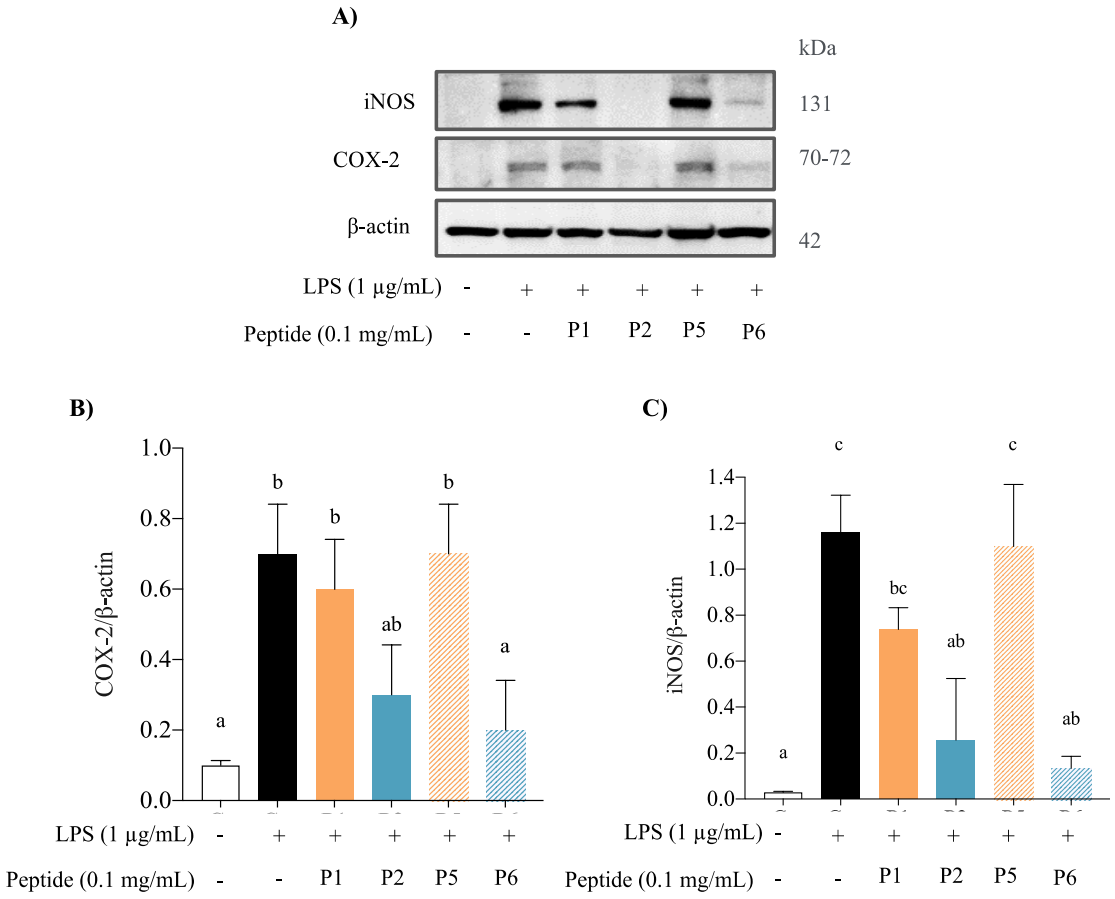

Figure 5. Effects of peptides in inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein expression in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. RAW 264.7 cells were pretreated with 100 µg/mL of peptides for 24 h, and then exposed to LPS (1 µg/mL) for 24 h. Cells were lysed for protein extraction and prepared for western blot analysis. Representative Western blot analysis of iNOS, COX-2, and β-actin **(A)**. Representative densitometric analysis of iNOS **(B)** and COX-2 **(C)**. β-actin was used to normalize between groups. Data are expressed as the mean ± standard error of the mean of two separate experiments. Significant differences between groups are represented by different letters (p <0.05; One-way ANOVA; post-hoc: Bonferroni test). P1: AGPAGKP; P2: GSKGRPG; P5 PGPAGPV; P6: AGERGEQ.

Treatment with AGERGEQ (P6) and GSKGRPG (P2) strongly reduced the relative protein expression of iNOS (by 90 % and 76 %, respectively) and COX-2 (76 % and 56

%, respectively), with no significant differences compared to the values observed in the non-LPS control group. No changes in iNOS and COX-2 protein expression were observed in macrophages treated with AGPAGKP (P1) or PGPAGPV (P5) compared with those observed in the positive control (LPS-control group) (Figure 5). These results are in line with another study in which iNOS and COX-2 protein expressions were also attenuated by 0.75 and 0.48-fold, respectively, after treatment with PAY (0.75 mM) [44]. Similarly, the expression of both proteins was also reduced in the same cell model after treatment with the peptide NCWPFQGVPLGFQAPP, present in clam worms (*Marphysa sanguinea)* [45]. Other anti-inflammatory peptides have also been shown to downregulate *iNos* and *Cox-2* mRNA expression in LPS-stimulated RAW 264.7 cells [46].

The results of the present study indicated that the peptides AGERGEQ (P6) and GSKGRPG (P2) exerted anti-inflammatory effects in LPS-stimulated macrophages, given their ability to reduce LPS-induced nitrite production. This effect could be explained by the reduction in iNOS protein expression observed in the peptide-treated macrophages. However, other mechanisms could also be involved in NO reduction, such as the inhibition of iNOS activity or NO scavenging [26]. In this regard, *in silico* studies have shown that different peptides produced by broccoli fermentation can interact with iNOS [47]. Moreover, a hydrolysate of chicken feather meal and several of their peptide fractions showed NO radical-scavenging activity (IC_{50}= 5.5 μg/mL) and also reduced nitrite production in LPS-stimulated RAW 264.7 cells [26]. In addition to this effect, these two peptides reduced LPS-induced COX-2 protein expression in macrophages. As mentioned, this enzyme plays a key role in the inflammatory process and is responsible for the synthesis of pro-inflammatory prostaglandins [7]. Thus, this diminished expression of COX-2 would tend to lower prostaglandin E levels. Moreover, it is known that COX-2 enzyme activity is enhanced by NO levels, and by the direct binding of iNOS [7]. In line with this observation, the P2 and P6 peptides reduced NO levels and iNOS protein expression in LPS-stimulated macrophages, and were two of the most active in reducing COX-2 activity in these cells (Figure 3A). Interestingly, peptides AGPAGKP (P1) and PGPAGPV (P5), also showed anti-inflammatory effects by reducing LPS-induced nitrite production in RAW 264.7 cells. However, the treatment with these peptides neither reduced iNOS protein expression nor COX-2 protein expression, inciting to further studies to decipher the mechanisms underlying their anti-inflammatory effects. It should be mentioned that the PeptideRanker score

was not a good predictor for the anti-inflammatory effects of the peptides, given that AGERGEQ (P6), which showed anti-inflammatory activity, was the one having the lowest score (0.08), whereas peptide GPAGPAGPRG, which did not show anti-inflammatory activity, was the one with the highest score (0.908). However, it is important to note that this score is not specific for predicting anti-inflammatory activity.

The peptides AGPAGKP (P1), GSKGRPG (P2), PGPAGPV (P5), and AGERGEQ (P6), could be responsible for the anti-inflammatory activity of the collagen-derived hydrolysate shown in the model of carrageenan-induced rat paw edema.

4. Conclusions

Nine peptides were identified in the anti-inflammatory collagen-derived hydrolysate. Its anti-inflammatory effect may be associated with the activity of five out of nine identified peptides, specifically AGERGEQ, GSKGRPG, PGPAGPV, and AGPAGKP. Our findings support the idea that peptides AGERGEQ (P6) and GSKGRPG (P2) might target multiple components of the inflammatory response: i) reducing the production of nitric oxide-derived compounds by negative regulating iNOS protein expression and ii) inhibiting the synthesis of prostaglandins through the inhibition of COX-2 enzyme and reducing its protein expression. Results suggest the potential of the use of these peptides as ingredients for inflammation management. Further studies for a better comprehension of the anti-inflammatory activity of these peptides would enhance their application in dermo-cosmetics or as new anti-inflammatory therapies.

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7. Conflict of interest

The authors declare no conflict of interest.

8. Author contributions

Conceptualization, M.M, B.M, and F.I.B.; formal analysis, N.I-B; funding acquisition, M.M, B.M, and F.I.B.; methodology, N.I-B, J.M.A-H., E.C, A.J.C-E; supervision, F.I.B.; writing—original draft, N.I-B.; writing—review and editing, N.I-B, F.I.B. All authors have read and agreed to the published version of the manuscript.

REFERENCES

1. Medzhitov, R. Origin and Physiological Roles of Inflammation. *Nature* **2008**, *454*, 428–435.
2. Wang, R.; Zhou, M.; Ma, H.; Qiao, Y.; Li, Q. The Role of Chronic Inflammation in Various Diseases and Anti-inflammatory Therapies Containing Natural Products. *ChemMedChem* **2021**, *16*, 1576–1592.
3. Nosalski, R.; Guzik, T.J. Perivascular Adipose Tissue Inflammation in Vascular Disease. *Br J Pharmacol* **2017**, *174*, 3496–3513.
4. Brennan, C.A.; Garrett, W.S. Gut Microbiota, Inflammation, and Colorectal Cancer. *Annu Rev Microbiol* **2016**, *70*, 395–411.
5. Ano, Y.; Yoshino, Y.; Uchida, K.; Nakayama, H. Preventive Effects of Tryptophan-Methionine Dipeptide on Neural Inflammation and Alzheimer's Pathology. *Int J Mol Sci* **2019**, *20*.
6. Leite, C.B.G.; Merkely, G.; Charles, J.F.; Lattermann, C. From Inflammation to Resolution: Specialized Pro-Resolving Mediators in Posttraumatic Osteoarthritis. *Curr Osteoporos Rep* **2023**, 1–13.
7. Tsatsanis, C.; Androulidaki, A.; Venihaki, M.; Margioris, A.N. Signalling Networks in Focus Signalling Networks Regulating Cyclooxygenase-2. *Int J Biochem Cell Biol* **2006**, *38*, 1654–1661.
8. Rivera-Jiménez, J.; Berraquero-García, C.; Pérez-Gálvez, R.; García-Moreno, P.J.; Espejo-Carpio, F.J.; Guadix, A.; Guadix, E.M. Peptides and Protein Hydrolysates Exhibiting Anti-Inflammatory Activity: Sources, Structural Features and Modulation Mechanisms. *Food Funct* **2022**, *13*, 12510–12540.
9. Everts, B.; Währborg, P.; Hedner, T. COX-2-Specific Inhibitors--the Emergence of a New Class of Analgesic and Anti-Inflammatory Drugs. *Clin Rheumatol* **2000**, *19*, 331–343.
10. Khursheed, M.; Ghelani, H.; Jan, R.K.; Adrian, T.E. Anti-Inflammatory Effects of Bioactive Compounds from Seaweeds, Bryozoans, Jellyfish, Shellfish and Peanut Worms. *Mar Drugs* **2023**, *21*.
11. Zhang, H.; Tsao, R. Dietary Polyphenols, Oxidative Stress and Antioxidant and Anti-Inflammatory Effects. *Curr. Opin. Food Sci*. **2016**, *8*, 33-42.
12. Chakrabarti, S.; Jahandideh, F.; Wu, J. Food-Derived Bioactive Peptides on Inflammation and Oxidative Stress. *Biomed Res Int* **2014**, 2014, 608979.
13. Abril, A.G.; Pazos, M.; Villa, T.G.; Calo-Mata, P.; Barros-Velázquez, J.; Carrera, M. Proteomics Characterization of Food-Derived Bioactive Peptides with Anti-Allergic and Anti-Inflammatory Properties. *Nutrients* **2022**, *14*.
14. Ibarz-Blanch, N.; Alcaide-Hidalgo, J.M.; Cortés-Espinar, A.J.; Albi-Puig, J.; Suárez, M.; Mulero, M.; Morales, D.; Bravo, F.I. Chicken Slaughterhouse By-Products: A Source of Protein Hydrolysates to Manage Non-Communicable Diseases. *Trends Food Sci Technol* **2023**, *139*, 104125.
15. Öztürk, H.İ.; Oraç, A.; Akın, N. Characterization of Bioactive Peptides Derived from Goatskin Tulum Cheese of the Ereğli Region at Different Stages of Ripening. *Food Research International* **2022**, *162*, 112124.
16. Meram, C.; Wu, J. Anti-Inflammatory Effects of Egg Yolk Livetins (α, β, and γ-Livetin) Fraction and Its Enzymatic Hydrolysates in Lipopolysaccharide-Induced RAW 264.7 Macrophages. *Food Research International* **2017**, *100*, 449–459.

17. Millán-Linares, M.D.C.; Yust, M.D.M.; Alcaide-Hidalgo, J.M.; Millán, F.; Pedroche, J. Lupine Protein Hydrolysates Inhibit Enzymes Involved in the Inflammatory Pathway. *Food Chem* **2014**, *151*, 141–147.
18. Tawalbeh, D.; Al-U'datt, M.H.; Wan Ahmad, W.A.N.; Ahmad, F.; Sarbon, N.M. Recent Advances in In Vitro and In Vivo Studies of Antioxidant, ACE-Inhibitory and Anti-Inflammatory Peptides from Legume Protein Hydrolysates. *Molecules* **2023**, *28*, 2423.
19. Li, M.; Ge, Q.; Du, H.; Lin, S. Tricholoma Matsutake-Derived Peptides Ameliorate Inflammation and Mitochondrial Dysfunction in RAW264.7 Macrophages by Modulating the NF-KB/COX-2 Pathway. *Foods* **2021**, *10*.
20. Chromý, V.; Vinklárková, B.; Šprongl, L.; Bittová, M. The Kjeldahl Method as a Primary Reference Procedure for Total Protein in Certified Reference Materials Used in Clinical Chemistry. I. A Review of Kjeldahl Methods Adopted by Laboratory Medicine. *Crit Rev Anal Chem* **2015**, *45*, 106–111.
21. Cunniff, P. Official Methods of Analysis of AOAC International; *Journal of AOAC International*, **1995**; ISBN 0935584544.
22. Adler-Nissen, J. Determination of the Degree of Hydrolysis of Food Protein Hydrolysates by Trinitrobenzenesulfonic Acid. *J Agric Food Chem* **1979**, 27.
23. Bravo, F.I.; Mas-Capdevila, A.; López-Fernández-Sobrino, R.; Torres-Fuentes, C.; Mulero, M.; Alcaide-Hidalgo, J.M.; Muguerza, B. Identification of Novel Antihypertensive Peptides from Wine Lees Hydrolysate. *Food Chem* **2022**, *366*, 130690.
24. Minkiewicz, P.; Iwaniak, A.; Darewicz, M. BIOPEP-UWM Database of Bioactive Peptides: Current Opportunities. *Int J Mol Sci* **2019**, *20*.
25. Mooney, C.; Haslam, N.J.; Pollastri, G.; Shields, D.C. Towards the Improved Discovery and Design of Functional Peptides: Common Features of Diverse Classes Permit Generalized Prediction of Bioactivity. *PLoS One* **2012**, *7*, e45012.
26. Inkanuwat, A.; Sukaboon, R.; Reamtong, O.; Asawanonda, P.; Pattaratanakun, A.; Saisavoey, T.; Sangtanoo, P.; Karnchanatat, A. Nitric Oxide Synthesis Inhibition and Anti-Inflammatory Effect of Polypeptide Isolated from Chicken Feather Meal in Lipopolysaccharide-Stimulated RAW 264.7 Macrophages. *Food Technol Biotechnol* **2019**, *57*, 200–212.
27. Zheng, Y.; Zhang, X.; Zhang, R.; Wang, Z.; Gan, J.; Gao, Q.; Yang, L.; Xu, P.; Jiang, X. Inflammatory Signaling Pathways in the Treatment of Alzheimer's Disease with Inhibitors, Natural Products and Metabolites (Review). *Int J Mol Med* **2023**, *52*.
28. Fernandes, A.; Rodrigues, P.M.; Pintado, M.; Tavaria, F.K. A Systematic Review of Natural Products for Skin Applications: Targeting Inflammation, Wound Healing, and Photo-Aging. *Phytomedicine* **2023**, *115*, 154824.
29. Stiller, C.; Hjemdahl, P. Lessons from 20 Years with COX-2 Inhibitors: Importance of Dose–Response Considerations and Fair Play in Comparative Trials. *J Intern Med* **2022**, *292*, 557–574.
30. Kemp, D.C.; Kwon, J.Y. Fish and Shellfish-Derived Anti-Inflammatory Protein Products: Properties and Mechanisms. *Molecules* **2021**, *26*, 3225.
31. Santiago-Lopez, L.; F. Gonzalez-Cordova, A.; Hernandez-Mendoza, A.; Vallejo-Cordoba, B. Potential Use of Food Protein-Derived Peptides in the Treatment of Inflammatory Diseases. *Protein Pept Lett* **2017**, *24*, 137–145.
32. Hong, H.; Fan, H.; Chalamaiah, M.; Wu, J. Preparation of Low-Molecular-Weight, Collagen Hydrolysates (Peptides): Current Progress, Challenges, and Future Perspectives. *Food Chem* **2019**, *301*, 125222.

33. Khiari, Z.; Ndagijimana, M.; Betti, M. Low Molecular Weight Bioactive Peptides Derived from the Enzymatic Hydrolysis of Collagen after Isoelectric Solubilization/Precipitation Process of Turkey By-Products. *Poult Sci* **2014**, *93*, 2347–2362.
34. Shen, W.; Matsui, T. Intestinal Absorption of Small Peptides: A Review. *Int J Food Sci Technol* **2019**, *54*, 1942–1948.
35. Matsumura, N.; Fujii, M.; Takeda, Y.; Sugita, K.; Shimizu, T. Angiotensin I-Converting Enzyme Inhibitory Peptides Derived from Bonito Bowels Autolysate. *Biosci Biotechnol Biochem* ***1993***, *57*, 695–697.
36. Zhao, L.; Wang, X.; Zhang, X. L.; Xie, Q. F. Purification and Identification of Anti-Inflammatory Peptides Derived from Simulated Gastrointestinal Digests of Velvet Antler Protein (Cervus Elaphus Linnaeus). *J Food Drug Anal* ***2016***, *24*, 376-384.
37. Gao, R.; Shen, Y.; Shu, W.; Bai, F.; Jin, W.; Wang, J.; Yuan, L. Optimization of Enzymatic Conditions of Sturgeon Muscles and Their Anti-Inflammatory Potential. *J Food Qual* ***2020***.
38. Turini, M.E.; DuBois, R.N. Cyclooxygenase-2: A Therapeutic Target. *Annu Rev Med* ***2002***, *53*, 35–57.
39. Jakubczyk, A.; Szymanowska, U.; Karaś, M.; Złotek, U.; Kowalczyk, D. Potential Anti-Inflammatory and Lipase Inhibitory Peptides Generated by in Vitro Gastrointestinal Hydrolysis of Heat-Treated Millet Grains. *CyTA – J. Food* ***2019***, *17*, 324–333.
40. Hayes, M.; Aluko, R.E.; Aurino, E.; Mora, L. Generation of Bioactive Peptides from Porphyridium Sp. and Assessment of Their Potential for Use in the Prevention of Hypertension, Inflammation and Pain. *Mar Drugs* ***2023***, *21*, 422.
41. Zielińska, E.; Baraniak, B.; Karaś, M. Identification of Antioxidant and Anti-inflammatory Peptides Obtained by Simulated Gastrointestinal Digestion of Three Edible Insects Species (Gryllodes Sigillatus, Tenebrio Molitor, Schistocerca Gragaria). *Int J Food Sci Technol* ***2018***, *53*, 2542–2551.
42. Förstermann, U.; Schmidt, H.H.; Pollock, J.S.; Sheng, H.; Mitchell, J.A.; Warner, T.D.; Nakane, M.; Murad, F. Isoforms of Nitric Oxide Synthase. Characterization and Purification from Different Cell Types. *Biochem Pharmacol* **1991**, *42*, 1849–1857.
43. Khatua, S.; Simal-Gandara, J.; Acharya, K. Understanding Immune-Modulatory Efficacy in Vitro. *Chem Biol Interact* **2022**, *352*, 109776.
44. Ahn, C.-B.; Cho, Y.-S.; Je, J.-Y. Purification and Anti-Inflammatory Action of Tripeptide from Salmon Pectoral Fin Byproduct Protein Hydrolysate. *Food Chem* **2015**, *168*, 151–156.
45. Park, Y.R.; Park, C.-I.; Soh, Y. Antioxidant and Anti-Inflammatory Effects of NCW Peptide from Clam Worm (Marphysa Sanguinea). *J Microbiol Biotechnol* **2020**, *30*, 1387–1394.
46. Joshi, I.; Mohideen, H.S.; Nazeer, R.A. A Meretrix Meretrix Visceral Mass Derived Peptide Inhibits Lipopolysaccharide-Stimulated Responses in RAW264.7 Cells and Adult Zebrafish Model. *Int Immunopharmacol* **2021**, *90*, 107140.
47. Li, Y.; Gao, X.; Pan, D.; Liu, Z.; Xiao, C.; Xiong, Y.; Du, L.; Cai, Z.; Lu, W.; Dang, Y.; et al. Identification and Virtual Screening of Novel Anti-Inflammatory Peptides from Broccoli Fermented by Lactobacillus Strains. *Front Nutr* **2023**, *9*, 1118900.

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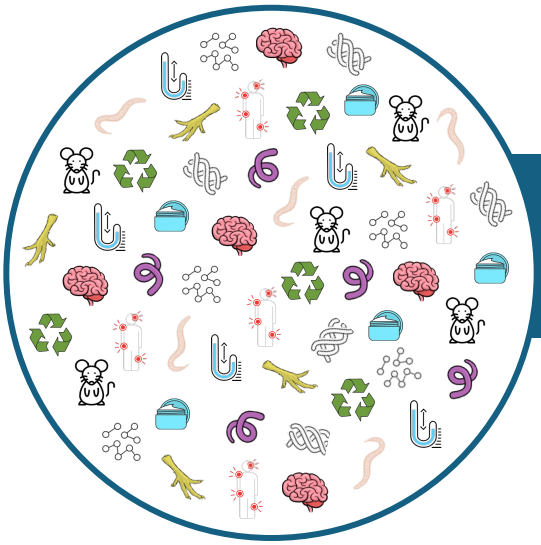
PROTEIN HYDROLYSATES AND PEPTIDES FROM ANIMAL BY-PRODUCTS AS A PREVENTIVE STRATEGY FOR LOCAL INFLAMMATION AND NEUROINFLAMMATION
Néstor Ibarz Blanch



Chapter 2

OBJECTIVE:

To develop a hydrolysate from a chicken by-product that can prevent the development of Alzheimer's disease and identify the peptides that it contains.

UNIVERSITAT ROVIRA I VIRGILI
PROTEIN HYDROLYSATES AND PEPTIDES FROM ANIMAL BY-PRODUCTS AS A PREVENTIVE STRATEGY FOR LOCAL INFLAMMATION AND NEUROINFLAMMATION
Néstor Ibarz Blanch

Manuscript 3

Objective: To review the potential of chicken by-products as sources of bioactive peptides and select the best candidate for generating POPi peptides.

Chicken slaughterhouse by-products: A source of protein hydrolysates to manage non-communicable diseases

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Chicken slaughterhouse by-products: A source of protein hydrolysates to manage non-communicable diseases

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ABSTRACT

Background: The poultry industry is a growing livestock sector, which generates large volumes of by-products with negative environmental impacts that must be revalorized. Modern lifestyles have contributed to a significant increase in the incidence of non-communicable diseases worldwide. Thus, many efforts have been made to identify novel agents with biological activities. In this regard, chicken slaughterhouse by-products are interesting materials because of their protein content, which can be subjected to proteolytic procedures to obtain bioactive peptides.

Scope and approach: This review focuses on the valorization of chicken slaughterhouse by-products as a source of functional protein hydrolysates and peptides; specifically, it reviews the potential of these compounds to manage non-communicable diseases and the mechanisms involved.

Key findings and conclusions: Hydrolysates obtained from chicken slaughterhouse by-products have antioxidant, antidiabetic, anti-inflammatory, anti-coagulant, anti-anaemic, cardioprotective, hepatoprotective, and neuroprotective properties, as well as the ability to manage body weight and lipid metabolism. Antioxidant and antihypertensive effects were the activities most studied in both *in vitro* and *in vivo*; however, only a chicken leg-derived hydrolysate was tested in humans, demonstrating its blood pressure-lowering effects. The obtained results are promising enough to encourage further research to explore the versatility of chicken by-products as a source of bioactive peptides.

1. Introduction

The incidence of chronic diseases has increased worldwide during the last decades, and this growing trend is projected to be maintained during the forthcoming years. These non-communicable conditions are strongly correlated with risk factors, such as hypertension, oxidative stress, and inflammatory processes (Hajat & Stein, 2018). Therefore, there is a huge interest in the development of novel strategies, which could help prevent or ameliorate the onset or progression of these diseases. In this context, there is strong evidence that different compounds present in food or natural sources could exert these desired effects, overcoming some side or adverse effects of existing therapeutic treatments (Rezvankhah, Yarmand, Ghanbarzadeh, & Mirzaee, 2021). In this regard, bioactive peptides, a relevant group of dietary-derived compounds, are promising candidates.

Bioactive peptides are specific protein fragments of small length (2–20 amino acid residues) that, when released from the native protein by intentional methods including fermentation and hydrolysis (enzymatic or chemical), as well as by unintentional methods, such as gastrointestinal digestion or food technological processes, exert

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interesting biological activities (Korhonen & Pihlanto, 2006; Nikhita & Sachindra, 2021). Food of animal origin is rich in proteins and is considered an excellent source of bioactive peptides (Borrajo et al., 2019; Mora, Gallego, & Toldrá, 2018). A recent peptidomic study revealed that *in vitro* gastrointestinal digestion of meat proteins from different sources generated a high number of peptides capable of inhibiting dipeptidyl peptidase IV (DPP-IV) and angiotensin-converting enzyme (ACE), which are crucial for the management of diabetes and hypertension, respectively (Martini, Conte, & Tagliazucchi, 2019). According to this aforementioned study, chicken meat seems to be a better potential protein source for antioxidant peptides than pork meat, and is a source of ACE inhibitory (ACEi) peptides that are more active than the peptides released from turkey and pork meat. Other studies have also obtained peptides with *in vitro* antioxidant, ACEi, alcohol dehydrogenase-activating, bile acid-binding or anti-inflammatory activities from chicken breast muscle or spent hen muscle subjected to different hydrolysis conditions (Hamzeh, Sangsawad, Noisa, Choowongkomon, & Yongsawatdigul, 2022; Saiga et al., 2003; Wu, Lin, et al., 2020; Xiao et al., 2020; Sun et al., 2012; Yu, Field, & Wu, 2018). Moreover, chicken protein hydrolysates showed beneficial effects in different animal models, including the reduction of high blood pressure (BP), decreased oxidative stress, stimulation of interleukin (IL) 1, amelioration of acute alcohol-induced liver injury, and reduction of serum total cholesterol (TC) (Fan et al., 2022; Fu, Therkildsen, Aluko, & Lametsch, 2019; Udenigwe et al., 2017; Wu, Lin, et al., 2020; Xiao, Zhou, Zhao, Su, & Sun, 2018).

In addition, agro-food industry by-products have emerged as an important source of bioactive peptides (Le Gouic, Harnedy, & FitzGerald, 2018). Their use for this purpose provides an excellent opportunity to valorize them, generating value-added products within the principles of a circular economy. In this sense, the meat industry is a relevant sector, the by-products of which have a significant impact, and their management must be improved to ameliorate this growing problem (Li, Arulnathan, Heidari, & Pelletier, 2022). For instance, chicken meat industry, which represented the 36% of global meat production in 2020, generates approximately 25–30% of the live chicken weight in by-products, being the chicken meat production of over 80.2 million metric tons in 2021 (Food and Agriculture Organization of the United Nations, 2013; 2022). Chicken slaughterhouse by-products are rich in proteins, including collagen, and keratin (Bravo et al., 2023). Different studies have confirmed that collagen is an excellent source of peptides with a wide range of bioactivities including lipid-lowering, metal-chelating, antioxidant, antidiabetic, immune modulation, and ACEi (Fu et al., 2019).

Given the importance of reducing the incidence of non-communicable diseases and valorizing food industry by-products, which are rich in proteins containing bioactive peptides, this review focuses on collecting the existing evidence regarding the functional properties of the hydrolysates related to non-communicable diseases and peptides obtained from chicken slaughterhouse wastes.

2. Poultry industry and chicken slaughterhouse by-products

Poultry farming is one of the most potent livestock sectors, because avian foodstuffs constitute a key source of high-quality protein for the human diet. Environmentalist, vegan, and vegetarian currents are being consolidated during the last decades, as well as plant-based diets; however, the global production and consumption of poultry meat are still steadily growing. In fact, more than 100,000 tons of chicken meat and 70 million tons of eggs are annually produced worldwide, followed by an upward trend during recent years, and is projected as the livestock segment with the highest growth rate until 2050 (Kanani, Heidari, Gilroyed, & Pelletier, 2020; Laca, Laca, & Diaz, 2021; Li et al., 2022). Thus, the impact of poultry waste on the environment is growing along with the soaring production numbers and, although in a lower amount than other livestock species, it generates greenhouse gases in form of respiratory CO_2, manure CH_4 and N_2O emissions, being produced 61, 0.97 and 0.57 million metric tons per year, respectively (Seidavi, Zaker-Esteghamati, & Scanes, 2019). In addition to chicken excreta and focusing on chicken meat production, diverse slaughterhouse by-products are released during the production of broiler meat. Thus, reutilization strategies to revalorize these materials have been proposed, developed, and encouraged, because compensation is offered to poultry farmers that implement sustainable activities in several countries (Tao & Wang, 2020).

The principles of a circular economy lead to reduce waste accumulation and pollution, and create value-added products. Normally, the most widespread approaches are related to the reuse of poultry by-products (from farms, slaughterhouses, industrial facilities, etc.) as fertilizers or compost because of their interesting carbon and amino acid contents, but with the disadvantage of high nitrogen concentration, although they can be easily treated and processed to design biostimulators that have proven their efficacy on plants (Chiarelotto, Restrepo, Lorin, & Damaceno, 2021; Izydorczyk et al., 2022). Furthermore, in line with the adoption of renewable energy sources, chicken slaughterhouse leftovers have been subjected to specific operations (including hydrolysis, acidogenesis, acetogenesis, and methanogenesis) to produce biogas, and therefore, electricity (Arshad et al., 2018; Siddiki et al., 2021). Even the textile industry has taken advantage of recycled poultry materials because chicken feather wastes can be processed to produce keratin filaments with enhanced properties such as higher ductility, compared to natural feathers (Mi et al., 2020). Moreover, different poultry by-products, such as blood or bones, are usually used for animal feed, as mentioned in Section 3. In addition, other studies have focused on the valorization of these by-products into high-value products. These by-products are rich in proteins, with the collagen being one of the major proteins in some of these by-products (Bravo et al., 2023). Given the beneficial effects of collagen, some studies have focused on developing methods to extract collagen from chicken feet, bones, skin, and sternal cartilage (Akram & Zhang, 2020; Cao & Xu, 2008; Cliche, Amiot, Avezard, & Gariepy, 2003; Matinong, Chisti, Pickering, & Haverkamp, 2022; Munasinghe, Schwarz, & Nyame, 2014; Potti & Fahad, 2017). Finally, it is worth mentioning that they have been widely used for the production of bioactive peptides (explained in Section 4.2) that could be used in animal feed and human nutrition to prevent non-communicable diseases (Bravo et al., 2023), which could be useful for valorizing these by-products.

3. Chicken slaughterhouse by-products

Diverse slaughterhouse by-products are released during the production of broiler meat, including blood, feathers, bones, legs (feet), blood, crests, beaks, skin, and viscera. Fig. 1 summarizes the main characteristics of these by-products. In this review, egg-derived by-products and spent egg-laying hens were not considered, since they are not directly generated in slaughterhouses (Fan & Wu, 2022; Quina et al., 2014).

3.1. Blood

Chicken blood represents approximately 6–7.5% of the live animal weight (Kelly & Alworth, 2013). Despite its high protein content (80–90% in dried whole blood, with high amounts of Arg, Thr, Tyr, and Ile), it is mostly converted to animal feed or inexpensive food products as 'blood cubes' particularly in Asian countries as a source not only of protein but also iron (Sorapukdee & Narunatsopanon, 2017; Wongngam, Mitani, Katayama, Nakamura, & Yongsawatdigul, 2020). Other minor components are fat (7–8%) and carbohydrates (7–8%) (Huang & Liu, 2010).

By-product	Blood	Bones	Feathers	Feet	Skin	Viscera
% of animal (w/w)	6-7.5	-	10	-	8-20	30
% Protein	80-90	25	90	75	8-12	90
Main protein	Hemoglobin	Collagen	Keratin	Collagen	Collagen	-

Fig. 1. Schematic representation of the main characteristics of chicken slaughterhouse by-products.

3.2. Bones and cartilages

Chicken bones are complex materials that are mainly formed by collagen surrounded by 'bone minerals' (modified forms of calcium apatite or hydroxyapatite) and fat. The organic part of the bone is mostly protein (70% of the organic part and up to 35% of the bone), with mostly collagen (approximately 90%, rich in Pro and Gly) (Cansu & Boran, 2015). Poultry components with higher bone share include carcasses, backs, necks, legs, and wings, among others. Normally, bone residues can be used for animal feed as well as for human diet and are traditionally added to soups and broths (Cansu & Boran, 2015; Polyanskikh, Danyliv, Dubrovina, Ozherelyeva, & Vasilenko, 2020).

Besides bones and related to them, cartilages are interesting as they contain high amounts of type-2 collagen, which could be used for cartilage engineering approaches (Wu, Korntner, Mullen, & Zeugolis, 2021). Thus, different studies have been carried out to extract this collagen from chicken sternal cartilage using a multi-step process, which combines protein hydrolysis with other steps, such as precipitation and purification or ultrasound treatment (Akram & Zhang, 2020; Cao & Xu, 2008). Moreover, they contain bioactive compounds, highlighting glycosaminoglycans, such as hyaluronic acid or sulfated glycosaminoglycans such as chondroitin sulfate (Narayanaswamy, Kanagesan, Pandurangan, & Padmanabhan, 2016; Stiborova et al., 2020). The potent immunomodulatory effects of these molecules have directed research towards many functionalities, including anti-atherosclerosis, anti-osteoarthritis, and anti-aging (Stiborova et al., 2020). In chickens, an interesting cartilage type is the trachea. It contains approximately 70% protein and 16% fat (Pramualkijja, Pirak, & Euston, 2021) and is part of the solid waste from slaughterhouses, although it has a lower impact in terms of volume and weight than other chicken by-products.

3.3. Crest

Although chicken crests are usually consumed and can be found in numerous and varied recipes around the world, they are often discarded on many occasions, especially those from female animals that do not contain a significant amount of edible meat. Therefore, several studies have been conducted to provide additional value to these parts, targeting hyaluronic acid as a target molecule (Rosa et al., 2007; Severo da Rosa, Hoelzel, Viera, Barreto, & Beirão, 2008). This molecule has a potent antioxidant effect and good physicochemical properties which allow its biomedical applications in plastic, ocular, and osteoarthritis surgeries, as well as in tissue engineering (Sudha & Rose, 2014).

3.4. Feathers

Approximately 5 million tons of feathers are generated annually worldwide. Thus, there is a great interest in searching for new methods to valorize this by-product as an alternative to landfilling or incineration (Callegaro, Brandelli, & Daroit, 2019). Feathers represent up to 10% of the chicken's total weight and are composed of over 85–90% protein, with keratin as the major component (Fakhfakh et al., 2011; Taskin & Kurbanoglu, 2011). This structural protein is rich in Gly, Ala, Ser, Cys, Leu, Val, Arg, Ile, Phe and contains smaller proportions of Lys, Met, His and Trp. Due to its interesting protein and amino acidic composition, feather meal is used as feed supplement for animals (Adler, Slizyte, Honkapää, & Løes, 2018).

3.5. Legs and feet

Although they are used in some countries as part of traditional cuisine, in many others, they are discarded and considered as by-products (Potti & Fahad, 2017). The major constituents of this by-product are proteins (>75%), especially collagen (>50%), and fat (approx. 15%). Special attention has been devoted to collagen in terms of its technological and functional properties (Araújo, Lima, Pereira, & Madruga, 2019).

3.6. Skin

One of the chicken slaughterhouse by-products with the highest impact is chicken skin, which represents between 8 and 20% of chicken carcass weight (Heydarpour et al., 2006). It is usually underutilized despite its composition, containing 30–40% fat with high concentrations of n-3 and n-6 fatty acids, and 8–12% protein, which makes it a good source of collagen. Normally, this skin is easily and rapidly defatted, obtaining a protein content over 60% with abundance of hydrophobic amino acids (Fallah-Delavar & Farmani, 2018; Onuh, Girgih, Aluko, & Aliani, 2013). Several studies have focused on developing methods for extracting this collagen from this by-product (Cliche et al., 2003; Matinong et al., 2022; Munasinghe et al., 2014).

3.7. Viscera

More than 30% of poultry by-products are referred to as chicken viscera, which are the internal organs and materials that are not usually commercialized and are therefore discarded along the production chain. Although hearts, livers, and gizzards can be found in offal shops in some countries (Spain, Italy, and some African and Asian regions) (Babicz, Kropiwiec-Domańska, Skrzypczak, Szyndler-Nędza, & Szulc, 2020), they are less frequent and have a great impact on waste. Nevertheless, these chicken parts contain a relevant amount of high-value protein with a balanced amino acid profile and low carbohydrate content. Because of this composition, they have been widely used for animal feeding, particularly in aquaculture, to replace fish flour. Interestingly, after processing and defatting viscera, the meal contains more than 90% protein and 22% lipids (Giri, Sahoo, & Mohanty, 2010; Oké, Olaniran Odountan, Abou, & Odountan, 2016).

4. Production and bioactivity of chicken slaughterhouse by-products

4.1. Production of chicken slaughterhouse by-products

Chicken slaughterhouse by-products, such as blood, bone, cartilage, comb, feather, feet, gizzard, liver, residues from mechanical chicken

deboning, skin, trachea, viscera, and wattle, were used as sources of bioactive peptides (Alahyaribeik, Nazarpour, Tabandeh, Honarbakhsh, & Sharifi, 2022; Bezerra et al., 2020; Chen et al., 2017; Gonçalves dos Santos Aguilar, Santos de Souza, & Soares de Castro, 2020; Lima et al., 2019; Mas-Capdevila, Pons, Aleixandre, Bravo, & Muguerza, 2018; Nie, Xu, Zhao, & Meng, 2017; Onuh et al., 2013; Yang et al., 2019; Zheng, Si, Ahmad, Li, & Zhang, 2018). These peptides were intentionally released from proteins contained in chicken by-products, mainly via enzymatic hydrolysis. Chemical hydrolysis using hydrochloric or sulfuric acid was also used for blood red cells and feathers, respectively (Ben Hamad Bouhamed, Krichen, & Kechaou, 2020; Nikhita & Sachindra, 2021).

Regarding the enzymatic process, different steps compromised the production of these bioactive peptides, which are contained in the elaborated protein hydrolysates (Fig. 2). Initially, chicken by-products were usually milled or ground to facilitate protein solubilization (Alahyaribeik et al., 2022; Mas-Capdevila et al., 2018; Yang, Lin, Liu, & Chen, 2014). Moreover, some were subjected to a lipid removal process using organic solvents (Alahyaribeik & Ullah, 2020; Onuh, Girgih, Aluko, & Aliani, 2014). Regarding blood, it was used directly to elaborate protein hydrolysates or subjected to centrifugation to obtain corpuscle and plasma fractions (Carrera-Alvarado, Toldrá, & Mora, 2022; Cheng, Lai, Lin, & Sakata, 2016). The next step was protein solubilization, which was performed by mixing the chicken by-products with water, and the resultant solution was heated or boiled (Carrera-Alvarado et al., 2022; Mas-Capdevila et al., 2018; Xiong et al., 2020). In this step, a particular by-product was the feather, as its proteins (mainly keratin) are difficult to solubilize. Thus, feathers were subjected to steam or temperature-pressure processes (Adler et al., 2018; Qin et al., 2022) or heated in a solution containing hydroxymethyl aminomethane, urea, ethylene diamine tetraacetic acid, and sodium sulfite to increase keratin solubility (Alahyaribeik et al., 2022; Alahyaribeik & Ullah, 2020). Finally, these protein solutions, with or without non-soluble residues, were hydrolyzed to obtain bioactive peptides. The most common method was using commercial enzymes, although some studies isolated proteases from feathers (Fakhfakh et al., 2011; Fontoura et al., 2014) and different *Bromelia* species (Romero-Garay et al., 2020). The most common enzymes used for this issue and in a variable concentration enzyme/substrate were Alcalase® and pepsin (Bjørndal et al., 2020; Chakka, Ramanatikara, Zituji, Pedda, & Narayan, 2021; Pramualkijja et al., 2021; Safar Razavizadeh, Farmani, & Motamedzadegan, 2022b; Yang et al., 2014), although enzymes such as trypsin, papain, Flavourzyme®, Protamex®, neutral Protease, Neutrase®, elastase, and collagenase, among others, were also used alone or in combination (Casanova-Martí et al., 2019; Mas-Capdevila et al., 2018; Nadalian, Kamaruzaman, Yusop, Babji, & Yusop, 2019; J. Yang et al., 2019; Zheng et al., 2018). The hydrolysis time varied between 30 min and 24 h (Casanova-Martí et al., 2019; Romero-Garay, Becerra-Verdín, Soto-Domínguez, Montalvo-González, & García-Magaña, 2022), with shorter times (1 and 2 h) being the most used (Aloysius et al., 2018; Bjørndal et al., 2020; Mas-Capdevila et al., 2018; K. T. Yang et al., 2014). In addition, fermentation using bacteria species, such as *Pedobacter* sp. *3.14.7, Bacillus pumilus strain A1,* or *Chryseobacteriumsp.kr6,* was the most used method for generating protein hydrolysates from feathers (Bezus et al., 2021; Fakhfakh et al., 2011; Fontoura et al., 2014). This is because these bacteria contain alkaline keratinases that allow the hydrolysis of feather keratin. However, this process appeared to be slower than hydrolysis with commercial enzymes, as it required hydrolysis times of 48 h (Alahyaribeik et al., 2022; Fontoura et al., 2014). Temperature and pH (less common) were mainly adjusted based on the manufacturer's description using the conditions in which enzymes are the most active or optimal bacterial growth conditions. For ending the hydrolysis/fermentative step, enzymes or bacteria were heat-inactivated at temperatures between 80 °C and 95 °C for 15–30 min. Finally, it is common to include a filtering or centrifugation step to remove the insoluble solids or bacteria (Mas-Capdevila et al., 2018). In some cases, an ultracentrifugation step is also included to obtain the 3–10 KDa fraction to purify and concentrate the bioactive peptides, obtaining peptide solutions with improved bioactivity compared to the initial hydrolysate ((Alahyaribeik et al., 2022; Inkanuwat et al., 2019; Zhang, Kouguchi, Shimizu, Ohmori, et al., 2010).

Once obtained, bioactivities of the hydrolysates were usually assessed *in vitro* and/or *in vivo* in its liquid format *or* by its resuspension

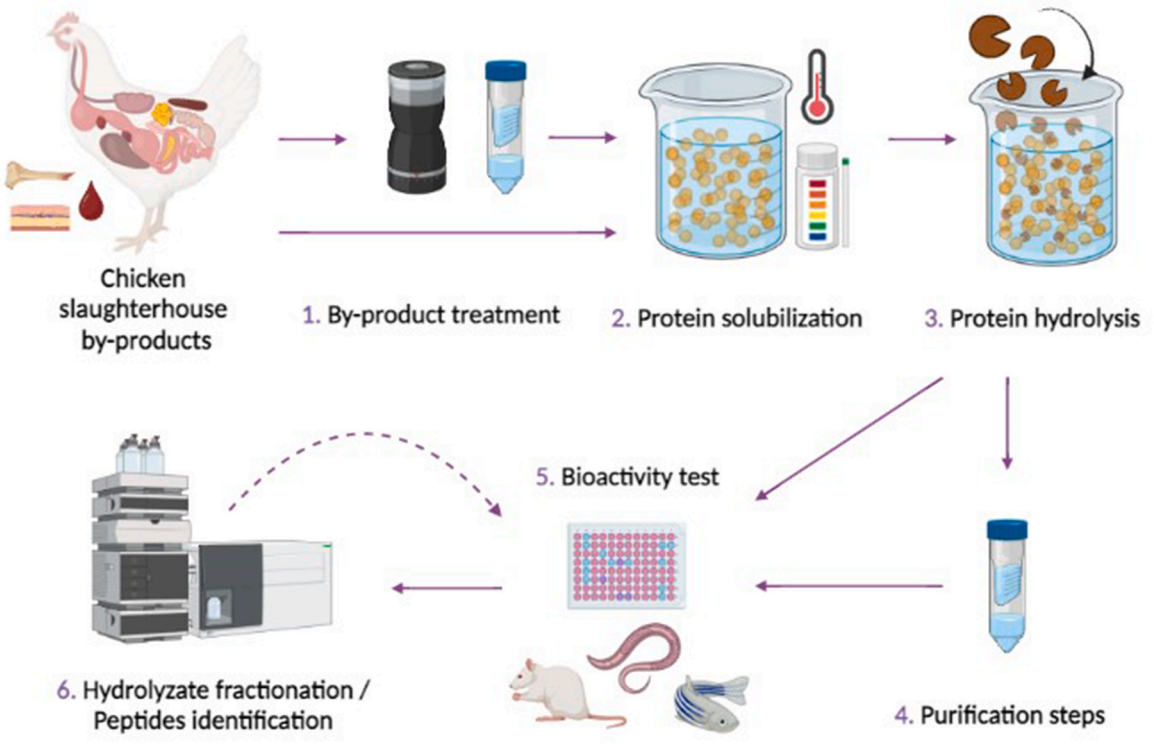

Fig. 2. Schematic representation of the main steps to prepare bioactive hydrolysates from chicken slaughterhouse by-products.Created with BioRender.com.

in water at the desired concentration after freeze-drying. Once the bioactivity was determined, subsequent purification steps, such as ultrafiltration with different molecular weight cutoff membranes followed by reverse-phase HPLC fractionation, were used for the isolation and identification of the bioactive peptides responsible for the bioactivity of the hydrolysate (Bravo, Mas-Capdevila, Margalef, Arola-Arnal, & Muguerza, 2019; Inkanuwat et al., 2019).

4.2. Bioactivity of chicken slaughterhouse by-products

4.2.1. Antioxidant effects

Oxidative stress plays an important role in the onset and/or progression of various non-communicable diseases, including cardiovascular, neurodegenerative, chronic kidney, and chronic obstructive pulmonary diseases, as well as atherosclerosis, cancer, and diabetes (Liguori et al., 2018). Oxidative stress refers to an imbalance between the generation of reactive oxygen and nitrogen species (ROS and RNS, respectively) in cells and the ability of biological systems to eliminate them. These radical and non-radical molecules are by-products generated through different metabolic processes such as the mitochondrial chain, phagocytosis, prostaglandin synthesis, and the cytochrome P450 system, as well as exogenous sources such as tobacco, pollution, radiation, heavy metals, or alcohol (Halliwell & Gutteridge, 2015; Phaniendra, Jestadi, & Periyasamy, 2015). Some examples of biological ROS or compound generators of ROS are the radical superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and hypochlorous acid (HOCl), which can damage proteins, lipids, and DNA (Martemucci et al., 2022; Pérez de la Lastra, Juan, Plou, & Pérez-Lebeña, 2022). Moreover, nitric oxide ($NO^{\bullet}$), a RNS, is generated by inducible, endothelial, and neural nitric oxide synthases (iNOS, eNOS and nNOS, respectively), which catalyze the oxidation of L-arginine to L-citrulline, as well as by S-nitrosothiol decomposition or the reaction of L-arginine with H_2O_2 (Forstermann & Sessa, 2012; Nagase et al., 1997; Singh, Hogg, Joseph, & Kalyanaraman, 1996). It is well known that NO is a potent vasodilator; however, when it reacts with $O_2^{\bullet -}$ to produce peroxynitrites ($ONOO^{\bullet}$) (Ibarz-Blanch et al., 2022), the beneficial effect of this molecule disappears. Moreover, peroxynitrites are involved in the oxidation of low-density lipoprotein and amino acids residues, such as tyrosine and methionine and DNA (Bartesaghi & Radi, 2018; Douki & Cadet, 1996; Pérez de la Lastra et al., 2022).

As mentioned above, organisms have endogenous antioxidant mechanisms (enzymatic and non-enzymatic) that contribute to the neutralization of ROS and RNS, thereby avoiding in consequence their prejudicial effects. Thus, $O_2^{\bullet}$ is removed by the enzymes superoxide dismutase (SOD), H_2O_2 by catalase (CAT), glutathione peroxidase (GPx), and peroxiredoxin, whereas peroxide radicals are eliminated by glutathione peroxidases (GPx) (Aoyama & Nakaki, 2015; Wang, Branicky, Noë, & Hekimi, 2018). Moreover, it is worth mentioning the role of reduced glutathione (GSH) against oxidative stress because it contains sulfhydryl groups that can be easily oxidized and reduced back, and is considered as the main endogenous antioxidant. In this regard, $GSSG^{\bullet -}$ (the oxidized form of GSH) is produced when some ROS or GSH-protein mixed disulfides are reduced via GPx or glutathione reductases. The reduction of $GSSG^{\bullet -}$ is catalyzed by GSH reductase (GR) in a NADPH-dependent manner (Aoyama & Nakaki, 2015).

In addition to these endogenous antioxidant mechanisms, other important sources of antioxidant molecules are food compounds such as vitamin C, vitamin E, phenolic compounds, carotenoids, trace elements (Se, Cu, Zn, and Mn), and peptides. They can directly reduce the oxidation of cellular molecules or increase the production and/or activity of endogenous antioxidants (Medina-Vera et al., 2021; Tadesse & Emire, 2020; Wołonciej, Milewska, & Roszkowska-Jakimiec, 2016; Xu et al., 2017). Therefore, given the importance of antioxidant compounds in the prevention of oxidative stress, there is a huge interest in searching new dietary-derived compounds with antioxidant properties. Consequently, different techniques have been also developed to evaluate the *in vitro* antioxidant effects of these compounds. The most commonly used techniques are based on the assessment of the reducing ability or radical-trapping activity of a compound when it reacts with an oxidized metal ion or a known radical, respectively (Zhong & Shahidi, 2015). Related to this, some of the most common antioxidant assays are $DPPH^{\bullet}$ (2,2-diphenyl-1-picryl-hydrazyl-hydrate) radical-scavenging activity, $ABTS^{\bullet +}$ (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical-scavenging activity, nitric oxide scavenging activity, total reactive antioxidant potential (TRAP), ferric reducing antioxidant power (FRAP) or cupric ion reducing antioxidant capacity (CUPRAC). Another common assay is the oxygen radical absorbance capacity (ORAC) assay, which measures the ability of a compound to quench peroxyl radicals produced by AAPH (2,2′-azobis(2-methylpropionamidine) dihydrochloride) in presence of oxygen and heat (see Amorati & Valgimigli, 2018; Zhong & Shahidi, 2015 for more detailed information about antioxidant assays).

Antioxidant capacity is one of the main activities studied in hydrolysates obtained from chicken by-products. Specifically, blood, bones, cartilage, a mixture of combs and wattles, feathers, gizzard, liver, mechanically separated meat, skin, viscera, and trachea have been used to generate hydrolysates with antioxidant properties, which were determined by a wide range of assays (Table 1). These bioactive hydrolysates were generated using different methodologies such as fermentation and enzymatic and chemical hydrolysis. The most studied chicken by-products were feathers, which were fermented with different bacterial species (*Pedobacter* sp. *3.14.7, Bacillus pumilus strain A1,* or *Chryseobacteriumsp.kr6*), some of which contain alkaline keratinases, or directly treated with different proteases, including enzymes with keratinolytic activity. For example, the solution obtained from feather fermentation by means of the feather-extracted bacteria *Pedobacter* sp. *3.14.7,* exerted both ABTS and nitric oxide radical-scavenging activities (Bezus et al., 2021). Moreover, KER102 keratinase, a serine protease isolated from *Bacillus* sp.RCM-SSR-102, was used to generate a feather hydrolysate with DPPH and ABTS radical-scavenging activities (Kshetri et al., 2020). In addition, this peptic solution protected the DNA from oxidative damage.

Interestingly, the antioxidant activity of hydrolysates from chicken by-products varied depending on different factors, such as the type of chicken source, the method used to determine the antioxidant capacity, or the hydrolysis conditions (type of enzyme, enzyme/substrate ratio, hydrolysis time, and pH). Moreover, the same by-products from different origins produced hydrolysates with different antioxidant capacities, although the same hydrolysis conditions were applied. In this regard, Onuh et al. (2014) observed that the type of skin (thigh or breast) and enzyme concentration in both DPPH radical-scavenging activity and metal chelation ability, as well as the enzyme type (Alcalase® or Pepsin + Pancreatin) for the metal chelation ability, were key in the generation of antioxidant hydrolysates from chicken skin (Onuh et al., 2014). Furthermore, hydrolysates obtained from thigh (3% Alcalase®) and breast (1% Pepsin + Pancreatin) skins showed strong DPPH radical-scavenging activity and metal chelation activity, and weak hydroxyl and superoxide radical-scavenging activities. Another example of the effects of enzymes and hydrolysis conditions on the generation of antioxidant peptides is the study by Jeampakdee et al. (2020). In this study, Neutrase® was more efficient in generating hydrolysates with DPPH radical-scavenging activity from feather meal or viscera than those generated using Alcalase® or Flavourzyme® (Gonçalves dos Santos Aguilar et al., 2020; Jeampakdee et al., 2020). Furthermore, an enzyme-concentration response for DPPH radical-scavenging activity was observed in feather hydrolysates (Jeampakdee et al., 2020). In contrast, Flavourzyme® was the best enzyme in generating viscera-derived hydrolysates with the highest total antioxidant activity compared to those elaborated with Neutrase® or Alcalase® (Gonçalves dos Santos Aguilar et al., 2020); while Alcalase® was the best enzyme in generating trachea-derived antioxidant hydrolysates in comparison with Flavourzyme®, Protamex® or Papain (Pramualkijja et al., 2021). The hydrolysis time is also an important

Table 1
In vitro antioxidant effects of chicken by-product hydrolysates.

By-product		Enzyme	Amino acid sequence/s	ABTS$^{\bullet+}$-SA	ORAC	DPPH$^{\bullet}$-SA	FRAP/reducing power	Iron/metal chelating activity	$O2^{-}$-SA	•OH-SA	•NO-SA	TEAC	Mo (VI) reduction	β-carotene bleaching inhibition	References
Blood	Meal	Alcalase®		33.0–58.4 μmol TE/g		84.0–86.0 μmol TE/g	2.26–6.2 μmol TE/g								(da Silva Bambirra Alves et al., 2021)
	Red cells	Chlorhydric acid (0.05%)				57% (n.e)									Nikhita and Sachindra (2021)(
	Red cells	Alcalase®				77% (n.e)									Nikhita and Sachindra, 2021)
	Red cells	Pepsin				~1.4 mg/mL									Hamzeh et al. (2019)(Hamzeh et al., 2019)
	Plasma	Pepsin	AMADCCS, YPKAPFS, LLINLIK, DMVECMD, YEGLSKLVK, SPGSLCA, PTATVLG, CGLAGREE, FTVTFENTD, YRDSIVA, HLAVGAS, IEGTSYA, MIGMTPTV, FSYLISKD, DVKDTCM, VWGLASDL, YGTAITS, MQPAMAAI, LSVPLVL, AGSSPI, KIPLNLV, ISVCNSLL, EELPLPL, ALPTGG, DTKSDFH, TKSDFHL, MGTAPMW, ISRDWRGV, GKLGAVL, LRCQCIS, LIVKALMAK			~1.0 mg/mL								
	Corpuscle	Papain + Flavourzyme®	AEDKKLIQ			95% (n.e)	1.8%		53%						Zheng et al. (2018)
	Plasma	Alcalase®				77% (n.e)	0.6%	91%							Cheng et al. (2016)
	Cells	Alcalase®				78% (n.e)	0.3%	86%							(Cheng et al., 2016)
Bones	Carcass	Alcalase®		6988 μmol TE/L	11,683 μmol TE/L										Londoño-Zapata et al. (2022)
		Papain + Neutral protease + Trypsin				~60% (6 mg/mL)	~0.4 (AU) (6 mg/mL)			~25% (10 mg/mL)					Nie et al. (2017)
Cartilage		Trypsin		55.5 mg TE/g		9.8 mg/mL	0.5								Yang et al. (2019)
Combs and Wattles		Alcalase®	GADGAP, GKDLR	~58%		~42% (n.e)	~95%	94%							Bezerra et al. (2020)
Feather		*Bacillus licheniformis.* Fraction <3 kDa				5.0 mg/mL									Alahyaribeik et al. (2021)

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Table 1 (*continued*)

By-product		Enzyme	Amino acid sequence/s	ABTS$^{\bullet+}$-SA	ORAC	DPPH$^{\bullet}$-SA	FRAP/reducing power	Iron/metal chelating activity	$O2^{-}$-SA	•OH-SA	•NO-SA	TEAC	Mo (VI) reduction	β-carotene bleaching inhibition	References
		Pedobacter sp		0.6 g/L			2.2 AU (2 mg/mL)				0.5 g/L	3.6 mTE/g protein			Bezus et al. (2021)
		Bacillus pumilus A1				0.3 mg/mL	~1.6 AU (3 mg/mL)								Fakhfakh et al. (2011)
		Chryseobacteriumsp. kr6		16 mg/mL		83% (n.e)									Fontoura et al. (2014)
		Chryseobacteriumsp. kr6	LPGPILSSFPQ	~10% (3 mg/mL)											Fontoura et al. (2019)
		Bacillus subtilis S1–4.	SNLCRPCG	0.4 mg/mL		0.4 mg/mL		1.9 mg/mL							Wan et al. (2016)
		Sulfuric acid	–			0.5 mg/mL	1.1 AU (5 mg/mL)						60.6 μmol α-TE/mL	~80% (10 mg/mL)	Ben Hamad Bouhamed et al. (2020)
		KER102 keratinase	–	20 μg/mL		1.0 mg/mL									Kshetri et al. (2020)
		Alcalase®	–			90.43% (5 mg/mL)									Alahyaribeik and Ullah (2020)
	Meal	Neutrase®	FDDRGRX, VTLAVTKH, VSEIXSIPIS	9.3 μg/mL		16.5 μg/mL									Jeampakdee et al. (2020)
		Bacillus sp. P45 (fraction <3000 Da)		46.8 μmol TE/g	808.1 μmol TE/g										da Cunha et al. (2023)
Gizzard		*Pediococcus acidilactici* ATCC 8042		19.5 μM TE/g protein		37.5 μM TE/g protein	~40 μM TE/g protein								Ali et al. (2021)
		Bifidobacterium longum B379M				76% (n.e)									Zinina et al. (2022)
Liver		Trypsin				~75% (2 mg/mL)	~0.5 AU (2 mg/mL)			55% (2 mg/mL)					Xiong et al. (2020)
		Alcalase®		7.3 mg		1.74 mg	~0.5 AU (100 μL)		1.4 mg						Chakka, Elias, Jini, Sakhare, and Bhaskar (2015)
Mechanical deboning residues		Alcalase®				4.2 mg/mL									Sbeghen et al. (2022)
Skin		Alcalase® + Freeze dried				47% (n.e)	0.3% (n.e)	92% (n.e)		7% (n.e)					Wan Omar and Sarbon (2016)(
		Alcalase®.+ Vacuum oven dried				38% (n.e)	0.4% (n.e)	86% (n.e)		29% (n.e)					Wan Omar and Sarbon, 2016)
		Alcalase®+ pronase E + collagenase (Fraction)				16.7 mg/mL	~0.03 AU (20 mg/mL)	1.1 mg/mL	8.7 mg/mL						Sarbon, Badii, and Howell (2018)
		Elastase	GAHTGPRKPFKPR, GMPGFDVR, ADASVLPK	1.1 mg/mL		2.8 mg/mL		1.2 mg/mL							Nadalian et al. (2019)
		Alcalase®				61% (n.e)									Safar Razavizadeh, Farmani, and

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Table 1 (*continued*)

By-product		Enzyme	Amino acid sequence/s	ABTS$^{\bullet+}$-SA	ORAC	DPPH$^{\bullet}$-SA	FRAP/reducing power	Iron/metal chelating activity	$O2^{-}$-SA	•OH-SA	•NO-SA	TEAC	Mo (VI) reduction	β-carotene bleaching inhibition	References
															Motamedzadegan (2022a)
	Breast	Pepsin + pancreatin			3180 μM TE/g protein	~32% (1 mg/mL)		~80% (1 mg/mL)							Onuh et al. (2014) (Onuh et al., 2014)
	Thigh	Alcalase®			3497 μM TE/g protein	~34% (1 mg/mL)		~95% (1 mg/mL)							
Trachea		Alcalase®		134.3 μmol TE/mg		4.4 μmol TE/mL	22.5 μmol TE/mg								(Pramualkijja et al., 2021, 2022)
		Alcalase® + Whey proteins + NaCl (1.5%)		181.0 μM/mL		40.0 μM/mL	46.0 μM/mL								Pramualkijja et al. (2022)
Viscera		Flavourzyme® + Alcalase®				71% (n.e)									Gonçalves dos Santos Aguilar et al. (2020)
		Alcalase®				188.1 μmol TE/g	1057.6 μmol TE/g								da Silva and de Castro (2020)

Table shows the most active hydrolysate obtained in each study. ~Data calculated from reported figures (real value were not specified by authors). Numbers in brackets indicate the concentration of protein for testing the antioxidant activity.
Abbreviations: ABTS$^{\bullet+}$ -SA (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid radical scavenging activity), DPPH$^{\bullet}$-SA(2,2-diphenyl-1-picryl-hydrazyl-hydrate radical scavenging activity), FRAP (ferric reducing antioxidant power), n.e (not specified), •NO-SA (nitric oxide scavenging activity), $O2^{-}$-SA(superoxide radical scavenging activity), •OH-SA (hydroxyl radical scavenging activity), ORAC (oxygen radical absorbance capacity), α-TE (α-tocopherol equivalents), TE (Trolox equivalents), TEAC (Trolox equivalent antioxidant activity).

factor in the generation of antioxidant hydrolysates. Thus, the maximum values of activity were found in the aforementioned trachea-derived hydrolysate in the range of 1 and 3 h of hydrolysis for the FRAP method, in the range of 2 and 6 h for the DPPH method, and 6 h for the ABTS method (Pramualkijja et al., 2021). Short hydrolysis times were also better for obtaining the maximum ABTS radical-scavenging activity of red blood cell hydrolysates (1–4 h) (Hamzeh, Wongngam, Kiatsongchai, & Yongsawatdigul, 2019). However, longer hydrolysis periods (18 h) were necessary to obtain the highest ABTS and DPPH radical-scavenging activities in hydrolysates obtained from gizzard fermentation by means of *Pediococcus acidilactici* ATCC 8042 (Ali, Gullo, Rai, & Bavisetty, 2021). The hydrolysate elaborated under these conditions showed strong ABTS and DPPH radical-scavenging activities of 22.8 and 26.3 times, respectively, which were higher than the initial gizzard.

Additionally, technological processes, such as drying, heating, or the matrix conditions in which hydrolysates are incorporated, might change the antioxidant activity of hydrolysates (Table 1). In this regard, a study carried out using an antioxidant skin hydrolysate showed that freeze-dried method was better in conserving its DPPH radical-scavenging and metal-chelating activities than the dried in a vacuum oven; however, hydroxyl radical-scavenging properties were better retained after the vacuum oven drying process than the freeze-drying method (Wan Omar & Sarbon, 2016). Furthermore, the antioxidant stability of thermic processes appears to depend on the hydrolysate itself. Thus, the DPPH radical-scavenging activity of a peptic fraction (<3000 Da) obtained from feather fermentation with *Bacillus licheniformis* was stable after a 30 min of heat treatment (20–100 °C); however, the DPPH radical-scavenging activity of a blood corpuscle hydrolysate was diminished after a 30 min of heat treatment at 80 and 100 °C (Zheng et al., 2018). Moreover, the antioxidant activity of these chicken by-product hydrolysates appears to be affected by pH. In this regard, the aforementioned feather fermented solution was very sensitive to pH, losing its activity at all evaluated pH values (2, 4, 6, and 10) (Alahyaribeik, Seyed, Tabandeh, Honarbakhsh, & Ghazanfari, 2021), whereas the blood corpuscle hydrolysate only lost DPPH radical-scavenging activity at pH higher than 7.0 (Zheng et al., 2018). In addition, another affecting parameter is the protein glycation, which might be produced in the formulation of functional foods using these hydrolysates. There is evidence that the glycation process might increase the antioxidant activity of hydrolysates (Nie et al., 2017; Xiong et al., 2020), although the results depend on the antioxidant assay used (Londoño-Zapata et al., 2022). Londoño-Zapata et al. (2022) observed that glycation with lactose increased the ORAC activity of a bone-derived hydrolysate by 6.57%; however, it decreased the ABTS radical-scavenging activity in the same hydrolysate by 3.73% (Londoño-Zapata et al., 2022). Another example was a bone-protein hydrolysate generated by a mixture of enzymes Papain, Neutral protease and Trypsin. This hydrolysate and its peptic-derived fractions (< and >3000 Da) showed strong DPPH radical-scavenging activity and moderate reducing power; however, both activities, as well as, its hydroxyl radical-scavenging activity significantly increased when proteins and peptides were glycated with galactose (3:1 galactose: hydrolysate, 100 °C, 90 min) (Nie et al., 2017). Moreover, the addition of an Alcalase®-digested trachea hydrolysate to a NaCl solution containing other proteins such as egg white and whey proteins produced changes in the DPPH and ABTS radical-scavenging activities, and ferric reducing ability of the hydrolysate, which depended on the used protein type and concentration. The most active mixture was a combination of 0.5% trachea hydrolysate and whey protein dissolved in 1.5% NaCl (Pramualkijja, Pirak, & Euston, 2022). Furthermore, Sbeghen et al. (2022) observed that ultrafiltration (4000 Da membrane, 2.5 bar at 45 °C) can be a useful technique to improve antioxidant activity in an Alcalase®-digested hydrolysate obtained from mechanically separated chicken meat, as the hydrolysate retentate showed increased DPPH radical-scavenging activity compared to the original hydrolysate (Sbeghen et al., 2022).

Finally, the influence of gastrointestinal digestion on the antioxidant activity of chicken by-product hydrolysates must be considered. In this regard, DPPH radical-scavenging activity seems to be quite stable in this biological process, with an increase in the bioactivity of hydrolysates obtained from blood and isolated red blood cells, and only a reduction of 5% in blood corpuscle hydrolysates after being subjected to Pepsin-Pancreatin digestion (Hamzeh et al., 2019; Zheng et al., 2018).

Even though chicken by-products have been the source of a huge number of hydrolysates with *in vitro* antioxidant effects, the scarce number of hydrolysates that have been evaluated *in vivo* is striking (Table 2). One of the more studied hydrolysates is a Pepsin-digested liver hydrolysate, which has shown *in vitro* DPPH radical-scavenging and ferrous ion chelating abilities (Chou, Wang, Lin, & Chen, 2014). Its antioxidant effects were observed in different oxidative stress animal models, such as mice with D-galactose-induced oxidative stress (Chou et al., 2014), thioacetamide (TAA)-induced fibrotic rat (Chen et al., 2017), streptozotocin (STZ)-induced hyperglycemic mouse (Yeh et al., 2022), mouse with alcoholic liver disease (Lin et al., 2017), and hamsters and mice consuming a high-fat diet (Wu, Lin, et al., 2020; Wu, Lin, Yang, Wang, & Chen, 2021; Yang et al., 2014). For example, the administration of 250 mg/kg body weight (BW) of the Pepsin-derived liver hydrolysate to the D-galactose ROS-induced mouse model for 6 weeks produced a strong antioxidant effect (Chou et al., 2014). Specifically, these animals showed a reduction in thiobarbituric acid reactive species (TBARS) levels and an increase in Trolox equivalent antioxidant capacity (TEAC), GSH levels, and the activity of the antioxidant enzymes SOD, CAT, and GPx in different animal organs, compared to the control D-galactose group (Chou et al., 2014). TBARS assay is an indicator of malondialdehyde (MDA) levels, which is a lipid peroxidation marker (Gaweł, Wardas, Niedworok, & Wardas, 2004), and TEAC assay is commonly used for determining antioxidant capacity in animal tissues. Its effects were attributed to (*i*) the high content of Gly, Cys, Glu, present in the liver-derived hydrolysate, which can be used to produce GSH (Samarasinghe, Munkanatta Godage, VanHecke, & Ahn, 2014); as well as due to the content of the metals Mn and Se, which are cofactors for SOD and GPx, respectively, and (*ii*) its peptic profile (Li & Zhou, 2011; Zoidis, Seremelis, Kontopoulos, & Danezis, 2018). Moreover, chronic administration of this hydrolysate to rats with thioacetamide (TAA)-induced fibrosis (600 mg/kg BW) and to alcoholic mice (320 and 1280 mg/kg BW) also ameliorated the hepatic oxidative state of the animals compared to TAA-induced fibrotic or alcoholic animals, respectively (Chen et al., 2017; Lin et al., 2017). The results were important as the liver-derived hydrolysate counteracted some of the effects of TAA on the liver, such as the activity of SOD and CAT, as well as the TEAC levels, which were similar to those determined in healthy animals. Similarly, oxidative stress induced by the alcoholic diet was counteracted by the consumption of this hydrolysate (TBARS, TEAC, SOD activity, and GPx activity) (Lin et al., 2017).

Moreover, other chicken by-product-derived hydrolysates showed antioxidant effects *in vivo,* although they were not selected based on their *in vitro* antioxidant capacity (Table 2). In this regard, a Protamex®-digested foot hydrolysate with antihypertensive effects, increased hepatic GSH levels in spontaneously hypertensive rats (SHR) compared to the control SHR group (Mas-Capdevila et al., 2019). Moreover, an Alcalase®-digested liver hydrolysate improved serum total antioxidant activity and DPPH radical-scavenging activity in cyclophosphamide-induced anemic mice, although these effects were dependent on the administered concentration and the method used to obtain the hydrolysate (fermentation or enzymatic hydrolysis with Alcalase®) (Chakka et al., 2021).

Antioxidant activity of hydrolysates is attributed to the peptide composition, with the best antioxidant activity for peptides included in fractions of 1 and 3 kDa (Sbeghen et al., 2022). However, contradictory results were found for antioxidant chicken hydrolysates. In this regard, the <3 kDa fraction of a feather meal hydrolysate obtained using *Bacillus* sp. P45 was the most active for peroxyl and ABTS radical-scavenging

Table 2
In vivo antioxidant effects of chicken by-product hydrolysates.

By-product	Hydrolysis conditions	Amino acid sequence/s*	Animal model	Treatment dose and period	Antioxidant effect	Reference
Feather	*Bacillus licheniformis* 5% (v/v), 48 h, 40 °C (F < 3 kDa)		Broiler chicks	50 mg/L for 36 days	↓MDA (thigh muscle)	Alahyaribeik et al. (2021, 2022)
Foot	Protamex® 0.4 AU/g prot, 2 h, 50 °C, pH 7.0		SHR	55 mg/kg BW, 1 day	↑hepatic GSH	Mas-Capdevila et al. (2019)
Liver	Pepsin 1:400, 2 h, 37 °C		57BL/6 mice + d-galactose (1.2 g/BW) subcutaneous injection	50 mg/kg BW for 6 weeks	↓brain and liver TBARS ↑ serum and brain GSH ↑liver and kidney SOD activity ↑heart CAT activity and GPx activity	Chou et al. (2014)
			57BL/6 mice + d-galactose (1.2 g/BW) subcutaneous injection	250 mg/kg BW for 6 weeks	↑liver size ↓TBARS (serum, brain, liver) ↑ brain GSH, TEAC, SOD activity, and CAT activity (↑hepatic GSH, TEAC, SOD activity, and CAT activity ↑kidney GSH, CAT activity and GPx activity ↑heart GPx activity	
			Wistar rats + 100 mg TAA/kg BW (intraperitoneal injection)	200 mg/kg BW for 10 weeks	↓hepatic TBARS ↑hepatic GSH and SOD activity	Chen et al. (2017)
				600 mg/kg bw for 10 weeks	↓hepatic TBARS ↑hepatic TEAC, SOD activity, and CAT activity	
			C57BL/6J (B6) mice + alcoholic diet	80 mg/kg BW for 8 weeks	↑hepatic TEAC and GPx activity	Lin et al. (2017)
				320 mg/kg bw for 8 weeks	↓hepatic TBARS ↑hepatic TEAC, SOD activity, and GPx activity	
				1280 mg/kg BW for 8 weeks	↓hepatic TBARS ↑hepatic TEAC, SOD activity, GPx activity	
			Golden Syrian hamsters + high-fat diet	100 mg/kg bw for 8 weeks	↓serum and hepatic TBARS ↑hepatic GSH, CAT activity, and GPx activity	Yang et al. (2014)
				200 mg/kg BW for 8 weeks	↓serum and hepatic TBARS ↑serum TEAC ↑ hepatic GSH, SOD activity, CAT activity, and GPx activity	
				400 mg/kg BW for 8 weeks (oral)	↓serum and liver TBARS ↑serum TEAC ↑ hepatic GSH, SOD activity, CAT activity, and GPx activity	
			C57BL/6 mice + basal diet	20 weeks	↑hepatic SOD activity	Wu, Korntner, Mullen, and Zeugolis (2021)
			C57BL/6 mice + high-fat diet	~170 mg/kg BW for 20 weeks	↓hepatic TBARS ↑hepatic SOD activity, CAT activity, and GPx activity	
				~510 mg/kg BW for 20 weeks	↓hepatic TBARS ↑hepatic SOD activity and GPx activity	
			C57BL/6 mice + high-fat diet	~170 mg/kg BW for 20 weeks	↓serum TBARS ↑serum TEAC and GSH	Wu, Lau, et al. (2020) Wu, Lau, et al. (2020)
				~510 mg/kg BW for 20 weeks	↓serum TBARS ↑serum TEAC and GSH	

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Table 2 (*continued*)

By-product	Hydrolysis conditions	Amino acid sequence/s*	Animal model	Treatment dose and period	Antioxidant effect	Reference
			Institute of Cancer Research(ICR) mice + STZ (50 mg/kg BW) with nicotinamide (120 mg/kg BW)	409.46 mg mg/kg BW for 9 weeks	↑brain GSH, TEAC, SOD activity, CAT activity, GPx activity	Yeh et al. (2022)
				818.92 mg/kg BW for 9 weeks	↓brain TBARS ↑brain and hepatic TEAC, GSH, SOD activity, CAT activity, and GPx activity	
				1223.38 mg/kg BW for 9 weeks	↓brain TBARS ↑brain and hepatic TEAC, GSH, SOD activity, CAT activity, and GPx activity	
	Alcalase® 1.5%, 150 min, 45 °C		Swiss-albino female mice + basal diet without iron + cyclophosphamide (100 mg/kg BW)	1.5% for 28 days	↑total antioxidant activity in serum. Similar to healthy group	Chakka et al. (2021)
				3% for 28 days	↑DPPH scavenging activity in serum, > healthy group	
				4.5% for 28 days	↓total antioxidant activity in serum ↑DPPH scavenging activity in serum, > healthy group	
	Pediococcus acidilactici N-CIM5368, 10%, 24 h, 37 °C			1.5% for 28 days	↓Total antioxidant activity in serum. ↑DPPH scavenging activity in serum, ≥ healthy group	
				3% for 28 days	↑Total antioxidant activity in serum. Similar to healthy group. ↑DPPH scavenging activity in serum, ≥ healthy group	
				4.5% for 28 days	↑DPPH scavenging activity in serum, ≥ healthy group	

Table shows the most active hydrolysate obtained in each study. ↑ and ↓ indicate an increase or decrease of a parameter, respectively, in comparison with their respective non-treated ill animals.
Abbreviations: BW (body weight), CAT (catalase), DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate), GPx (glutathione peroxidase), GSH (reduced glutathione), MDA (malondialdehide), SOD (superoxide dismutase), SHR (spontaneously hypertensive rats), STZ (streptozotocin), TBARS (thiobarbituric acid reactive species), TEAC (trolox equivalent antioxidant capacity).
(*)Amino acid sequences were not reported by the authors in the cited works.

activities (da Cunha, Brandelli, Braga, Sala, & Kalil, 2023), whereas the >4 kDa fraction of an Alcalase®-digested hydrolysate obtained from mechanically separated chicken meat exhibited higher DPPH radical-scavenging activity than the permeate obtained from the hydrolysate ultrafiltration (Sbeghen et al., 2022). Moreover, the highest DPPH and hydroxyl radical-scavenging activities, and ORAC of two chicken skin hydrolysates corresponded to the smallest peptide fraction (<1 kDa) (Onuh et al., 2014). A higher TEAC and reducing power was also observed in the <10 kDa fraction of a Pepsin-digested cartilage hydrolysate in comparison with the original hydrolysate and its >10 kDa fraction (J. Yang et al., 2019). Different antioxidant amino acid sequences have been identified in the antioxidant chicken by-product hydrolysates. In this regard, the amino acid sequence EDKKLIQ was identified in an antioxidant hydrolysate obtained from blood corpuscles, which showed similar radical-scavenging activity (hydroxyl, superoxide, and DPPH) and reducing power as GSH (Zheng et al., 2018). In addition, the peptides LPGPILSSFPQ and SNLCRPCG were also identified in antioxidant feather hydrolysates obtained by fermentation with *Chryseobacteriumsp.kr6* and *Bacillus subtilis* S1–4, respectively, which were capable to scavenge different free radicals or show reducing power (Fontoura et al., 2019; Wan, Dong, Yang, & Feng, 2016). Moreover, peptides from different collagen chains and elastin were identified in an antioxidant hydrolysate obtained from combs and wattles (Bezerra et al., 2020). Some of these peptides were the amino acid sequences GADGAP and GKDGLR, which might exert antioxidant activity according to their specific amino acid sequence (Bezerra et al., 2020). In this regard, the presence of Phe, Ala, Val, Pro and His in peptides have been linked to good radical-scavenging activity (Samaranayaka & Li-Chan, 2011). Moreover, Trp (mainly when it is located at the extreme of a peptide) and Pro seem to act as potent hydrogen donors because of the indole and pyrrolidine rings that they contain, respectively (Fan, He, Zhuang, & Sun, 2012; Girgih et al., 2014). Moreover, the presence of Lys, Glu or Gln in a peptide have been also linked to hydroxyl radical-scavenging and metal binding activities (Shazly et al., 2017). Additionally, Ala and Ile have been associated with the lipid peroxidation inhibitory activity exerted by different dietary peptides (Luo et al., 2020; Zhao et al., 2018) and the presence of polar or charged amino acids (Arg, His or Asp) in a peptide has been associated with iron-binding activity (Sun et al., 2017).

4.2.2. *Effects on cardiovascular related diseases*

Antihypertensive effects have been one of the most researched effects of chicken by-product hydrolysates. The selection of antihypertensive hydrolysates was based on their ability to inhibit ACE *in vitro*. This enzyme is key to BP regulation within the Renin-Angiotensin-Aldosterone system. Its importance lies in the fact that it catalyzes the inactivation of the vasodilator bradykinin, and more importantly, the generation of angiotensin (Ang) II from Ang I. Ang II contributes to increase BP via different pathways. Some of them are i) stimulation of vascular smooth muscle cell contraction, ii) release of endothelial vasoconstrictor factors such as endothelin 1 (ET-1) or ROS, and iii) stimulation of aldosterone release by adrenal glands, producing renal water and sodium reabsorption (Patel, Rauf, Khan, & Abu-Izneid, 2017; Pueyo & Michel, 1997; Touyz et al., 2002). Further details regarding the RAAS can be found in López-Fernández-Sobrino, Torres-Fuentes, Bravo, & Muguerza, 2022.

ACEi activity was observed in hydrolysates derived from a wide range of chicken by-products, including foot, blood, feather, skin, bone, viscera, trachea, comb, and wattle (Bravo et al., 2023). The IC_{50} values of the best ACEi hydrolysates obtained in different studies ranged

between 2.7-2650 μg/mL, although most of them ranged between 130 and 550 μg/mL (Bravo et al., 2023). IC_{50} indicates the minimum amount of hydrolysate required to inhibit 50% of the enzyme. Thus, the lowest value indicates the most active hydrolysate. Alcalase® was the most commonly used cocktail enzyme for generating these bioactive hydrolysates (Chen et al., 2002; Cheng et al., 2008a; Cheng et al., 2008b; Cheng, Liu, Wan, Lin, & Sakata, 2008; Gonçalves dos Santos Aguilar et al., 2020; Huang & Liu, 2010; Nikhita & Sachindra, 2021; Onuh et al., 2013; Onuh, Girgih, Malomo, Aluko, & Aliani, 2015; Pramualkijja et al., 2021; Wongngam et al., 2020). Moreover, studies comparing the effects of different proteases on the generation of ACEi peptides showed that Alcalase® was also the enzyme responsible for releasing the most active peptides in chicken whole blood, blood corpuscle, bone, skin, trachea, and residues, considering the specific test conditions (Chen et al., 2002; Cheng et al., 2008; Huang & Liu, 2010; Onuh et al., 2013; Pramualkijja et al., 2021; Wongngam et al., 2020). However, the best ACEi activity was found in a Protamex®-digested foot hydrolysate (IC_{50} = 27 μg/mL) (Mas-Capdevila et al., 2018), where Alcalase® digestion was not assayed. Furthermore, it was found that the ACEi activity of a specific hydrolysate could be improved through different processes, such as by i) performing gastrointestinal *in vitro* digestion (Saiga et al., 2008); ii) further hydrolysis with other enzymes (Saiga et al., 2008; Yuliatmo, Fitriyanto, Bachruddin, & Erwanto, 2017); and iii) isolating the fraction containing the smallest peptides (Huang & Liu, 2010; Onuh et al., 2013, 2015; Saiga et al., 2008; Wongngam et al., 2020) and iv) by using plastein reaction (Gao et al., 2020). More detailed information about the effects of chicken by-product hydrolysates on the ACEi can be found in Bravo et al. (2023).

In addition to *in vitro* assays, the antihypertensive effects of many of these hydrolysates have been evaluated in hypertensive rats after chronic and/or acute administration (Bravo et al., 2023). Acute administration of these hydrolysates (doses ranging between 50 and 3000 mg/kg BW) to hypertensive rats produced strong reductions in the systolic BP (SBP) (Bravo et al., 2023). The maximum SBP drop ranged from 26 to 60 mm Hg (Chen et al., 2002; Mas-Capdevila et al., 2018; Onuh et al., 2013; Saiga et al., 2008; Wongngam et al., 2020; Zhang, Kouguchi, Shimizu, Ohmori, et al., 2010). Moreover, some of these hydrolysates, such as those obtained from foot, blood, bone, skin, and chicken residues, also produced antihypertensive effects after their long-term administration to hypertensive animals (Chen et al., 2002; Cheng et al., 2008; Cheng et al., 2008b; Mas-Capdevila et al., 2019; Onuh et al., 2016; Saiga et al., 2008; Wongngam et al., 2020; Zhang, Kouguchi, Shimizu, Ohmori, et al., 2010). In addition, these BP-lowering effects could be produced by the action of some of these hydrolysates on ACE, resulting in its partial inhibition (Chen et al., 2002; Mas-Capdevila et al., 2018; Onuh et al., 2016). Consequently, this would lead to a decrease in Ang II production and a reduction in BP levels. Moreover, it was also demonstrated that they could also act on vessels improving hypertension-induced wall thickness (Cheng et al., 2008b), aorta hypertrophy (Zhang, Kouguchi, Shimizu, Ohmori, et al., 2010), and endothelial dysfunction (Mas-Capdevila et al., 2019; Zhang, Kouguchi, Shimizu, Ohmori, et al., 2010). This last hypertension-associated effect is characterized by an imbalance in the proportion of endothelial-derived vasoconstrictor (endothelin 1) and vasodilator factors (NO and prostaglandin), and a reduction in the bioavailability of endothelial NO (Hadi, Carr, & Al Suwaidi, 2005; Yanai et al., 2008), among others. For example, the administration of a Protamex®-digested chicken foot hydrolysate to diet-induced hypertensive rats for 3 weeks resulted in the downregulation of aortic *Edn1* (endothelin 1 gene), upregulation of aortic *Sirt1* (sirtuin 1 gene), as well as an increase in hepatic levels of GSH compared to hypertensive control animals (Mas-Capdevila et al., 2019). Moreover, an improvement in the antioxidant status together with a decrease in BP were observed in cyclophosphamide-induced anemia mice consuming a chicken liver hydrolysate (Chakka et al., 2021). Sirtuin 1 is crucial for the activation of eNOS, stimulates *eNos* transcription (ENOS is responsible for the production of the vasodilator NO), and inhibits NADPH oxidase activity, reducing the production of endothelial ROS by this enzyme (Ibarz-Blanch et al., 2022; Mattagajasingh et al., 2007; Zarzuelo et al., 2013). ROS are involved in the reduction of NO bioavailability, as excessive superoxide anions can scavenge NO, forming peroxynitrite (Rubanyi & Vanhoutte, 1986). Consequently, it would reduce the bioavailability of NO. Another example is the <3 kDa fraction of a chicken leg-derived hydrolysate obtained using *Aspergillus oryzae* proteases and Protease FP, which produced an increase in serum NO levels or a reduction in plasma intercellular adhesion molecule-1 (iCAM-1) levels in NG-nitro-L-arginine-methyl ester (L-NAME)-induced hypertensive rats after its acute or chronic administration, respectively (Zhang, Kouguchi, Shimizu, Ohmori, et al., 2010). iCAM-1 levels, involved in the atherosclerotic-lesions formation and development, have been linked to different cardiovascular diseases and endothelial dysfunction (Gross et al., 2012; Lawson & Wolf, 2009; Springer, 1995). Finally, it is worth mentioning that chicken leg hydrolysates obtained by means of *Aspergillus oryzae* proteases and Protease FP also showed antihypertensive effects in mildly and prehypertensive volunteers after 4 or 12 weeks of consumption (Kouguchi et al., 2013; Saiga-Egusa, Koji, Hayakawa, Takahata, & Morimatsu, 2009). These volunteers, depending on the study, also showed a reduction in plasma renin activity (Saiga-Egusa et al., 2009), which catalyzes the formation of Ang I from angiotensinogen, in brachial-ankle pulse wave velocity (Kouguchi et al., 2013), which is an indicator of arterial stiffness (Tomiyama et al., 2019), and an increase in endothelial progenitor cells colonies (Saiga-Egusa et al., 2009), which are involved in vascular repair (Zhang, Malik, & Rehman, 2014). More detailed information on the antihypertensive effects of chicken by-product hydrolysates can be found in Bravo et al., 2023; Bravo et al., 2023).

In addition to their antihypertensive effects, a mixture of chicken combs and wattles was recently used to generate an Alcalase®-digested hydrolysate with anticoagulant activity, which exerted its effects on the intrinsic pathway (Alencar-Bezerra et al., 2019). Anticoagulants are used to manage several cardiovascular diseases, including thrombotic disorders, acute myocardial infarction, and atrial fibrillation (Larson, German, Shatzel, & DeLoughery, 2019). In addition, liver hydrolysates have shown anti-anemia effects in cyclophosphamide-induced anemia mice (Chakka et al., 2021). In this regard, liver proteins fermented with *Pediococcus acidilactici* N-CIM5368 or hydrolyzed using Alcalase® were administered daily to healthy Swiss-albino female mice fed an iron-deficient diet. After anemia induction for 2 weeks, animals lost BW and their hemoglobin levels decreased; however, both parameters were restored after one week of consuming the hydrolysates in comparison with the control group. Anemia is a published health problem that occurs in 42% of children aged <5 years and 40% of pregnant women (World Health Organization (WHO), n.d.). It is frequent in patients with cardiovascular diseases and can be considered the fifth cardiovascular risk factor (Kaiafa et al., 2015). In addition, other liver hydrolysate administered to C57BL/6 mice consuming a high-fat diet exerted cardioprotective effects (Wu, Lin, et al., 2020). The long-term consumption of this diet by rodents simulates the development of characteristics of human metabolic syndrome, including diabetes, obesity, renal dysfunction, and alterations in lipid homeostasis (Buettner, Schölmerich, & Bollheimer, 2007; Sun et al., 2020). Thus, this Pepsin-digested liver hydrolysate improved the lipid profile and decreased renal lipid deposition, fibrosis, cardiac fibrosis, and inflammation in the treated animals compared with the non-supplemeted group (Wu, Lin, et al., 2020). Finally, an anti-inflammatory Corolase PP® hydrolysate obtained from chicken rest materials, derived from mechanical deboning, showed anti-atherosclerotic effects in apolipoprotein E (ApoE)-deficient ($Apoe^{-/-}$) mice after being consumed for 12 weeks, and this effect was not related to plasma cholesterol-lowering activity (Bjørndal et al., 2020). ApoE is a structural component of most lipoproteins (Hauser, Narayanaswami, & Ryan, 2011). It is a critical ligand for targeting plasmatic apolipoproteins to the liver for metabolism (Mahley & Rall,

2000). Therefore, Apoe$^{-/-}$ mice are unable to properly metabolize lipids, accumulate them in the plasma, and facilitate the atherosclerotic plaque formation via inflammatory cell migration (Lee et al., 2017). Furthermore, a reduction in plasma monocyte chemoattractant protein-1 (MCP-1) protein was also observed in animals consuming the hydrolysate, suggesting that atherosclerosis plaque was reduced because of the decreased chemoattraction of monocytes (Bjørndal et al., 2020).

4.2.3. Antidiabetic effects

Some chicken by-products, such as blood, mechanical chicken deboning residues, feet, and feathers, have also been used to obtain hydrolysates capable of inhibiting DPP-IV enzymes (Table 3) (Casanova-Martí et al., 2019; Fontoura et al., 2014; Hatanaka, Kawakami, & Uraji, 2014; Lima et al., 2019). This protease is an interesting target for the treatment and/or prevention of type 2 Diabetes Mellitus (Green, Flatt, & Bailey, 2006). This chronic disease is the most common type of diabetes and is characterized by impaired insulin secretion and insulin resistance (Shaw, Sicree, & Zimmet, 2010). DPP-IV can hydrolyse different neuropeptides, chemokines, and regulatory peptides, including two incretin hormones: glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). This serine peptidase/prolyl oligopeptidase acts mainly on the amino acids Pro and Ala located at position 2 of the N-terminus (Mulvihill & Drucker, 2014). Some of the actions of these two incretins are to increase the survival and expansion of mass of pancreatic β-cells and the biosynthesis and the glucose-dependent secretion of insulin as well as to reduce insulin clearance in liver and to exert "insulin-like" effects on hepatic, adipose and skeletal tissues (Green et al., 2006). GLP-1 also inhibits glucagon secretion (Holst, 2007). These functions would result in a reduction in plasma glucose and an increase in cell glucose uptake and metabolism. Thus, inhibition of DPP-IV activity would lengthen the half-life of the active form of these two gut-derived hormones and improve the management of glucose homeostasis in patients with type 2 Diabetes Mellitus.

As mentioned above, DPP-IV inhibitory activity has been observed in hydrolysates obtained from the blood, feet, feathers, and mechanical chicken deboning residues. Table 3 shows the IC_{50} values for these hydrolysates, which ranged from 297.4 to 3570 μg/mL. Studies carried out with mechanical deboning residue hydrolysates using Corolase® and Flavourzyme® showed that DPP-IV inhibitory properties depended on the enzyme (Corolase®>Flavourzyme®) and hydrolysis time (80 min > 240 min) (Lima et al., 2019). The most active DPPV-I inhibitory hydrolysates were those elaborated from chicken feet (Casanova-Martí et al., 2019). Moreover, it is worth mentioning that the antihyperglycemic effect of one of these foot hydrolysates was demonstrated in two animal models of diet- and age-induced glucose intolerance. Specifically, during an oral glucose tolerance test, diet-induced glucose intolerant animals administered an acute dose of a Neutrase®-derived foot hydrolysate showed a lower peak plasma glucose than that observed in untreated rats. Moreover, this hydrolysate tended to normalize the plasma glucose levels of the animals after 120 min of being administered an intragastric glucose load (Casanova-Martí et al., 2019). Additional *in vitro* studies also showed that STC-1 enteroendocrine cells and ileum tissues stimulated by this foot hydrolysate increased active GLP-1 secretion, indicating the potential role of GLP-1 in the antihyperglycemic effect of this hydrolysate (Casanova-Martí et al., 2019). In addition, a Pepsin-digested liver hydrolysate also showed antihyperglycemic effects in STZ-induced diabetes and high-fat diet-induced insulin resistance in mice (Wu, Korntner, et al., 2021; Yeh et al., 2022). Moreover, this hydrolysate also decreased insulin resistance in both animal models as a decrease in the HOMA-IR value (insulin resistance indicator) and the insulin receptor β in the liver and skeletal muscle were observed in STZ-induced diabetes and high-fat diet-induced insulin resistance mice, respectively (Wu, Korntner, et al., 2021; Yeh et al., 2022). In addition, an increase in the protein level of GLUT4, an insulin-stimulated glucose transporter in muscle and fat, was found in different tissues after the consumption of hydrolysate (Wang, Wang, Hu, Huang, & Chen, 2020), indicating that it could be one of the mechanisms underlying its beneficial effects.

Regarding peptides, only five amino acid sequences were identified in different hydrolysates, which corresponded to tri- and dipeptides (Hatanaka et al., 2014; Lima et al., 2019). The IC_{50} values for DPP-IV inhibitory activity ranged from 0.091 mM for LA to >20 mM for GAO (Table 4). Moreover, Carrera-Alvarado et al., 2022 identified three DPP-IV inhibitory peptides (GPF, GGGW, and IGL) resulting from *in silico* gastrointestinal digestion of peptides identified in chicken blood hydrolysates (Carrera-Alvarado et al., 2022). The inhibition of DPP-IV by peptides is strongly influenced by the amino acid profile and position in a manner similar to that of other enzymes. Different studies carried out on peptides from other protein origins have shown that the presence of hydrophobic amino acids in the N-terminal extreme of the peptide exerts a better DPP-IV inhibitory activity (Nongonierma & FitzGerald, 2016; Tulipano, Faggi, Nardone, Cocchi, & Caroli, 2015). Moreover, an increase in this activity was observed when the peptides contained in their N-Terminal extreme: Trp or Met on the last position, Ala or Pro on the second position, and Pro on the second position and it is flanked on both sides by Leu and or Ile (Kęska & Stadnik, 2022; Nongonierma & FitzGerald, 2016; Power, Nongonierma, Jakeman, & FitzGerald, 2014; Tulipano et al., 2015). Good DPP-IV inhibitory activity has been demonstrated when Pro appears at the N-terminal end between the first four amino acid residues and is flanked by Phe, Gly, Leu, Ala and Val (Boots, 2012; Carrera-Alvarado et al., 2022). Moreover, peptides containing Pro and or Hyp have shown a strong inhibitory effect on the DPP-IV activity (Harnedy, O'Keeffe, & FitzGerald, 2015; Lacroix & Li-Chan, 2012). Chicken by-product-derived peptides have not yet been tested in animal models; however, other studies carried out on peptides identified in hydrolysates from different origins have shown that dipeptides containing Leu increased glucose uptake by skeletal muscle cells (Morifuji, Koga, Kawanaka, & Higuchi, 2009).

4.2.4. Effects on biometric parameters and lipid homeostasis

Chicken by-product hydrolysates have also been evaluated for their impact on different biometric parameters, including BW, BW gain, food intake, and tissue weight (Table 5). In addition, the administration of these hydrolysates has shown positive results in the modulation of lipid homeostasis in different animal models (Table 5). In this regard, the administration of the <3 kDa fraction of a feather hydrolysate, obtained by fermentation with *Bacillus licheniformis*, to broiler chicks positively enhanced serum lipidic parameters. Specifically, it decreased the levels of circulating TC, triglycerides (TG), low-density lipoprotein-cholesterol (LDL-C), and very-low-density lipoprotein-cholesterol (vLDL-C) after 10 days of treatment with this hydrolysate. However, these changes were not seen after 26 or 36 days of treatment (Alahyaribeik et al., 2022). Interestingly, consumption of this hydrolysate significantly increased BW gain, which was the desired outcome for this animal model. This increase may be due to an enhancement in the microbiota, as well as in the small intestinal morphology and mucosal immune system due to the ingested peptides (Alahyaribeik et al., 2022). Similar results enhancing the circulating lipid profile were also observed by the consumption of a Pepsin-digested liver hydrolysate by Golden Syrian hamsters and mice fed a high-fat diet for 8 and 20 weeks, respectively (Wu, Lin, et al., 2020, 2021; Yang et al., 2014). In hamsters, decreased levels of serum TG, TC, LDL-C and the LDL-C/HDL-C ratio were observed at all evaluated doses (100, 200 and 400 mg/kg BW). Additionally, an increase in serum HDL-C levels was observed at 200 and 400 mg/kg BW (Yang et al., 2014). All these changes contributed to a better cardiometabolic profile related to the circulating lipid species in the context of an obesogenic diet. In addition, this hydrolysate also decreased BW, BW gain, and liver weight, as well as in the levels of key enzymes, such as alanine transaminase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) in mice fed a high-fat diet (Wu, Korntner, et al., 2021). In the same line, hydrolysates from mixed slaughterhouse by-products

Table 3
Antidiabetic effects of chicken by-product hydrolysates.

By-product	Hydrolysis conditions	Amino acid sequence/s	*In vitro* DPP-IV inhibitory activity		Animal model	Doses	Period	Effects	Mechanisms	References
			%*	Ic_{50} (μg/mL)						
Blood	Alcalase® 2%, 2 h, 55 °C + Protana® Prime 5%, 16 h, 55 °C	GPF, IGL, GGGW	61 (10 mg/mL)							Carrera-Alvarado et al. (2022)
	Alcalase® 2%, 2 h, 55 °C + Protana Prime 5%, Protana UBoost 3%, 16 h, 55 °C	GPF, IGL, GGGW	54 (10 mg/mL)							
Feather	*Chryseobacterium* sp. kr 6, 24 and 48 h, 30 °C, pH 8		42-45 (n.e)							Fontoura et al. (2014)
Foot	Neutrase® 0.4 AU/g prot, 24 h, 50 °C, pH 7.0		100 (20 mg/mL)	297						Casanova-Martí et al. (2019)
	Protamex® 0.4 AU/g prot, 2 h, 50 °C, pH 7		93 (20 mg/mL)	300						
	Neutrase 0.4 AU/g prot, 24 h, 25 °C, pH 7		83 (20 mg/mL)	303	Diet-induced glucose intolerance (Wistar rats)	471.71 mg/kg BW	Acute	↓peak plasma glucose after OGTT		
					STC1 enteroendocrine cells	5 mg/mL		↑active GLP1 secretion		
					Ileum tissue	15 mg/mL		↑active GLP1 secretion		
	Streptomyces collagenase 1%, 17 h, 45 °C, pH 7.5	GAO*, GPA, GPO*		3570						Hatanaka et al. (2014)
Liver	Pepsin 1:400, 2 h, 37 °C				High fat-induced insulin resistance mice	~170 mg/kg BW	Daily 20 weeks	↓fasted glucose ↓peak plasma glucose after OGTT	↑GLUT4 (liver, skeletal muscle and perirenal fat) ↓Irβ (skeletal muscle	Wu, Korntner, et al. (2021)
						~510 mg/kg BW	Daily 20 weeks	↓peak plasma glucose after OGTT	↑GLUT4 (liver, skeletal muscle and peri-renal fat) ↓Irβ (liver and skeletal muscle	
					Streptozotocin-induced diabetic mice	409.46, 818.92, and 1223.38 mg/kg	Daily for 9 weeks	↓HOMA-IR ↓serum glucose, and insulin ↓peak plasma glucose after OGTT	↑GLUT4 (liver, skeletal muscle and brain)	Yeh et al. (2022)
Mechanical deboning residues	Corolase® 80 min, 50 °C	TL, 5-HT, DF, TL, LA, LAD, VEVD, LL, ETGKGEDGE, FL, LFFSMLLML, LF	60 (1 mg/mL)	919						Lima et al. (2019)
	Corolase® 80 min, 50 °C (Fraction 5)		54 (1 mg/mL)	155	Skeletal muscle cells	1 mg/mL		↑ Glucose uptake		

Table shows the most active hydrolysate obtained in each study.* Numbers in brackets indicate the concentration of protein/dry hydrolysate for testing the DPP-IV inhibitory activity. ↑ and ↓ indicate an increase or decrease of a parameter, respectively, in comparison with their respective non-treated ill animals.
Abbreviations: GLUT4 (insulin-regulated glucose transporter), GLP-1 (glucagon-like peptide 1), Irβ (insulin receptor β), n.e (not specified), OGTT (glucose tolerance test), STC1 (intestinal secretin tumor cell line).(*)'O' indicates 'hydroxyproline'.

Table 4
Dipeptidyl peptidase IV (DPP-IV) inhibitory activity of peptides identified in chicken by-product hydrolysates.

By-product	Hydrolysis conditions	Amino acid sequence*	DPP-IV inhibitory activity (IC_{50})	References
Blood	Alcalase® 2%, 2 h, 55 °C + Protana® Prime 5%, 16 h, 55 °C or hydrolysate using Alcalase® 2%, 2 h, 55 °C + Protana® Prime 5%, Protana® UBoost 3%, 16 h, 55 °C	GPF	0.94 mM	Carrera-Alvarado et al. (2022)
		GGGW	2.73 mM	
		IGL	2.22 mM	
Feet	*Streptomyces* collagenase 1%, 17 h, 45 °C, pH 7.5	GAO	>20 mM	Hatanaka et al. (2014)
		GPA	5.03 mM	
		GPO	2.51 mM	
Mechanical deboning residues	Corolase® 80 min, 50 °C	LA	0.09 mM	Lan et al. (2015); Lima et al. (2019)
		FL	0.40 mM	Lima et al. (2019); Nongonierma and FitzGerald (2013)

(*)'O' indicates 'hydroxyproline'.

(including viscera, heart and blood), obtained by the action of *Bromelia pinguin* and *Bromelia karatas* enzymes, exerted an enhancement in the lipid circulating species and reduced the levels of ALT and AST in Wistar rats fed an obesogenic diet (Romero-Garay et al., 2022). Based on these studies, chicken hydrolysates seems to have potential to ameliorate lipid disorders in the context of an obesogenic diet.

Regarding the mechanisms underlying these effects, an increase in hepatic mitochondrial fatty acid β-oxidation in the liver was observed in high-fat diet-induced obese C57BL/6 mice supplemented for 12 weeks with an Alcalase®- or Corolase PP-derived hydrolysate from chicken meat rest raw materials, resulting from mechanical deboning (Aloysius et al., 2018). In addition, these same hydrolysates administered to ApoE-deficient mice ($Apoe^{-/-}$), which develop hyperlipidemia, increased plasma fatty acid C20:5 n-3 levels and delta-5 desaturase index, while no changes in plasma and hepatic TG and TC were observed (Bjørndal et al., 2020). Moreover, a foot hydrolysate administered to ApoE-deficient C57BL/6.KOR-$ApoE^{shl}$ mice reduced plasma and hepatic TC levels, as well as TG levels, compared to untreated animals (Zhang, Kouguchi, Shimizu, Sato, et al., 2010). Thus, although more studies are needed to elucidate the mechanisms of action, it seems clear that the consumption of these chicken by-product hydrolysates has a direct metabolic effect on the organism.

4.2.5. Anti-inflammatory effects

Inflammation is a natural response of an organism to nocuous stimuli or conditions, such as infection, tissue damage, and stress. The inflammatory response can be triggered by both endogenous and exogenous (microbial or non-microbial) inducers (Medzhitov, 2008). The inflammatory process can be regulated by numerous mediators such as cytokines, IL, and enzymes. Due to this fact, inflammation plays a crucial role in the majority of diseases or disorders in organisms, and therefore, the search for compunds capable of counteracting inflammation has gained importance in recent years (Chen et al., 2018). However, chicken by-products have been poorly studied as sources of anti-inflammatory compounds (Table 6). The study of NO levels in lipopolysaccharide (LPS)-activated murine macrophages (RAW 264.7) is frequently used as a preliminary screening method for anti-inflammatory compounds (Han et al., 2022; Liu, Chen, Zheng, Yu, & Wei, 2022; Wang et al., 2018). In addition to the vasodilatory effect of endothelial NO, this molecule is also considered a pro-inflammatory mediator when it is overproduced, with iNOS being one the main NO-producer enzymes. This enzyme is associated with injury-induced NO production and is expressed in many cell types, including macrophages and neutrophils (Kobayashi, 2010).

Regarding chicken by-product hydrolysates, a potent reduction in NO production in LPS-activated murine macrophages was observed when they were treated with a peptidic fraction isolated from a Flavourzyme®-digested feather meal hydrolysate (Inkanuwat et al., 2019). This hydrolysate was previously selected for its good NO scavenging activity *in vitro*. This anti-inflammatory effect was linked to the down-regulation of ciclooxigenase-2 (*Cox2)* and tumor necrosis factor alpha *(Tnf-α)* expression, and a decrease in the mRNA expression and protein levels of iNOS and interleukin (IL)-6 (Inkanuwat et al., 2019). COX-2 is a master regulator enzyme of one of the main inflammatory pathways. More concisely, it synthesizes the pro-inflammatory oxylipin prostaglandine-2 from arachidonic acid and is the target of non-steroideal anti-inflammatory drugs (Ju et al., 2022). Moroever, *Cox2* is an inducible early response gene that can be activated by different factors, including IL-1, TNF-α, or LPS (Gandhi, Khera, Gaur, Paul, & Kaul, 2017). TNF-α and IL-6, together with IL-1 and interferon-γ (IFN-γ), are the most important pro-inflammatory cytokines (Kany, Vollrath, & Relja, 2019). Moreover, the peptide SNPSVAGVR was identified in the hydrolysate fraction, showing a decrease in NO production in the same cell inflammatory model at 30 and 60 mM, which was linked to the downregulation of *Cox2, Tnf-α, iNos,* and *Il6* gene expression (Inkanuwat et al., 2019). In addition to this feather hydrolysate, the *in vitro* anti-inflammatory effect of a chicken feet hydrolysate was also demonstrated in TNF-α-induced inflamed human umbilical vein endothelial cells (HUVEC). A decrease in the levels of several pro-inflammatory proteins, such as soluble (s) ICAM-1 and soluble vascular cell adhesion molecule-1 (sVCAM-1) (Kouguchi et al., 2012); however, anti-inflammatory effects were more potent when the collagen-derived dipeptides PO and PG were tested in TNF-α-induced inflamed HUVECs because these peptides downregulated *iNos* expression, as well as reduced sICAM-1, sVCAM-1 (only for PO sequence), and IL-8 levels compared to the inflamed control HUVECs (Kouguchi et al., 2012). IL-8 is a neutrophil chemotactic factor that exerts proinflammatory effects. Their synthesis by cells such as fibroblasts, epithelial cells, monocytes, neutrophils, tumor cells, endothelial cells, and mesothelial cells, can be stimulated by LPS, TNF-α, and IL-1β (Qazi, Tang, & Qazi, 2011). Circulating ICAM-1 and VCAM-1 are involved in leukocyte-endothelial cell adhesion in different inflammatory diseases, and their expression can be stimulated by IL-1β or TNF-α (Sans et al., 1999). Moreover, some of these anti-inflammatory effects have been confirmed in animals, as plasma sICAM-1 levels, as well as plasma sICAM-1, IL-6, and TNF-α levels were reduced (after the long-term consumption of the fraction <3 kDa of the aforementioned foot hydrolysate) in L-NAME-induced hypertensive rats and ApoE-deficient C57BL/6.KOR-$ApoE^{shl}$ mice, respectively (Y. Zhang, Kouguchi, Shimizu, Sato, et al., 2010; Y. Zhang, Kouguchi, Shimizu, Ohmori, et al., 2010).

Moreover, the *in vivo* anti-inflammatory activity of different hydrolysates obtained from chicken rest raw materials, coming from mechanical meat deboning residues, has been observed in high-fat diet/high sucrose-induced obese C57BL/6 mice. In this sense, obese animals consuming the hydrolysates for 12 weeks showed a reduction in the plasma levels of several pro-inflammatory markers, such as IL-1α, IL-1β, IL-2, IL-6, IL-17, TNF-α, IFN-γ, and MCP-1, when compared with the control group, despite the fact that the levels of the anti-inflammatory cytokine IL-10 were also reduced (Aloysius et al., 2018). Remarkably, MCP-1 facilitates monocytes migration and colonization (Deshmane,

Table 5
Effects of chicken by-product hydrolysates on biometric parameters and lipid metabolism.

By-product	Hydrolysis conditions	Amino acid sequence/s*	Model	Dose	Administration period	Effects	References
Feather	*Bacillus licheniformis* (Fraction <3 kDa)		Broiler chicks	50 mg/L in drinking water	36 days	Treatment of 10 days: ↓serum TC, TG, LDL-C, vLDL-C Treatment of 26 days: ↑ADWG	Alahyaribeik et al. (2022)
Feet	*Aspergillus oryzae* protease (Fraction <3 kDa)		ApoE-deficient C57BL/6. KOR-ApoE^{shl} mice	10%	Daily for 12 weeks	↓plasma and hepatic TC ↓hepatic TG	Zhang, Kouguchi, Shimizu, Sato, et al. (2010)
Liver	Pepsin, 2 h, 37 °C		57BL/6 mice + d-galactose (1.2 g/BW) with subcutaneous injection	250 mg/kg BW	Daily for 6 weeks	↓serum TC	Chou et al. (2014)
			Golden Syrian hamsters + high-fat diet	100 mg/kg BW	Daily for 8 weeks	↓heart, liver and kidney weight ↓serum TG, TC, LDL-C, LDL-C/HDL-C	Yang et al. (2014)
				200 mg/kg BW		↓heart and kidney weight ↓serum TG, TC, LDL-C, LDL-C/HDL-C ↑serum HDL-C	
				400 mg/kg BW		↓heart, liver and kidney weight ↓serum TG, TC, LDL-C, LDL-C/HDL-C ↑serum HDL-C	
			Wistar rats + 100 mg TAA/kg BW (intraperitoneal injection)	200 mg/kg BW	Daily for 10 weeks	↓food intake and serum ALT	Chen et al. (2017)
				600 mg/kg BW		↓food intake, serum AST, and ALT	
			C57BL/6J (B6) mice + alcoholic diet	80 mg/kg BW	Daily for 8 weeks	↓spleen weight ↓perirenal and epididymal fat ↓serum AST, ALP, TG	Lin et al. (2017)
				320 mg/kg BW		↓spleen weight ↓perirenal and epididymal fat ↓serum AST, ALP, TG	
				1280 mg/kg BW		↓spleen weight ↓perirenal and epididymal fat ↓serum AST, ALP, TG	
			C57BL/6 mice + high-fat diet	~170 mg/kg BW	Daily for 20 weeks	↓heart and liver weight ↓blood AST, LDL-C/HDL-C ↑blood HDL-C	Wu, Lau, et al. (2020)
				~510 mg/kg BW		↓heart and liver weight ↓blood AST, LDL-C/HDL-C ↑blood HDL-C	
			C57BL/6 mice + high-fat diet	~170 mg/kg BW	Daily for 20 weeks	↓final BW, BW gain, and feed efficiency ↓blood TG, ALP, free fatty acids	Wu, Korntner, et al. (2021)
				~510 mg/kg BW		↓final BW, BW gain, feed efficiency ↓liver weight ↓blood TG, ALP, AST, ALT, free fatty acids	
			Institute of Cancer Research(ICR) mice + STZ (50 mg/kg BW) with nicotin-amide (120 mg/kg BW)	409.46 mg mg/kg BW	Daily for 9 weeks	↑hindlimb muscle ↓serum TG, TC, AST, ALT	Yeh et al. (2022)
				818.92 mg/kg BW		↑hindlimb muscle ↓serum TG, TC, AST, ALT	
				1223.38 mg/kg BW		↑hindlimb muscle ↓serum TG, TC, AST, ALT	
Mechanical deboning residues	Alcalase® 0.1%, 1 h, 50 °C		High fat/high sucrose diet-induced obese C57BL/6 mice	9.5 E%	Daily for 12 weeks	↑liver β-oxidation of palmitoyl-Co-A ↑plasma TG ↓plasma NEFA	Aloysius et al. (2018)

(*continued on next page*)

Table 5 (*continued*)

By-product	Hydrolysis conditions	Amino acid sequence/s*	Model	Dose	Administration period	Effects	References
				Casein was replaced by 62.5% of hydrolsate	Daily for 12 weeks	↑plasma long-chain SFAs and MUFAs, C20:5n-3, C18:3n-6, delta 5-desaturase index, C20:5n-3/C18:3n-3 ratio ↓plasma total trans fatty acids	Bjørndal et al. (2020)
	Protamex®, 0.1%, 1 h, 50 °C		High fat/high sucrose diet-induced obese C57BL/6 mice	9.5 E%	Daily for 12 weeks	↓WAT depot	Aloysius et al. (2018)Aloysius et al. (2018)
	Corolase® PP, 0.1%, 1 h, 50 °C		High fat/high sucrose diet-induced obese C57BL/6 mice	9.5 E %	Daily for 12 weeks	↑liver β-oxidation of palmitoyl-Co-A ↓plasma NEFA	
				Casein was replaced by 62.5% of hydrolsate	Daily for 12 weeks	↑plasma long-chain SFAs and MUFAs, C20:5n-3, C18:3n-6, delta 5-desaturase index, C20:5n-3/C18:3n-3 ratio ↓plasma C22:4n-6	Bjørndal et al. (2020)
	Papain + Bromelain, 0.1% (1:1, w/w), 1 h, 50 °C		High fat/high sucrose diet-induced obese C57BL/6 mice	9.5 E%	Daily for 12 weeks	↓WAT depot ↑plasma TG ↓plasma NEFA	Aloysius et al. (2018)
Residues (viscera: 44.5% (w/w), heart: 44.5% (w/w), and blood: 11% (w/w))	*Bromelia pinguin* enzymes, 30 min, 40 °C, pH 6.5 *Bromelia karatas* enzymes, 4 h, 25 °C, pH 6.5 Bromelain, 4 h, 37 °C, pH 7.0.		Wistar rats + hypercaloric diet + STZ	200 mg/kg BW	Daily for 5 weeks	↓serum AL, AST, TC, LDL-C, vLDL-C, TG ↑serum HDL-C ↓serum ALT, AST, TC, LDL-C ↑serum VLDL-C, TG ↓serum ALT, AST, TC, LDL-C	Romero-Garay et al. (2022)

Table shows the most active hydrolysate obtained in each study. ↑ and ↓ indicate an increase or decrease of a parameter, respectively, in comparison with their respective non-treated ill animals.
Abbreviations: ADWG (average daily weight gain), ALP (alkaline phosphatase), ALT (alanine transaminase), Apo E (apolipoprotein E), AST (aspartate aminotransferase), BW (body weight), E (energy), HDL-C (high-density lipoprotein cholesterol), LDL-C (low-density lipoprotein cholesterol), MUFA (monounsaturated fatty acid), NEFA (non-esterified fatty acids), SFA (saturated fatty acid), STZ (streptozotocin), TAA (thioacetamide), TC (total cholesterol), TG (total triglycerides), VLDL-C (very low-density lipoprotein cholesterol), WAT (white adipose tissue). (*)Amino acid sequences were not reported by the authors in the cited works.

Kremlev, Amini, & Sawaya, 2009), and early inflammation is dominated by neutrophils; however in later stages, monocytes become more relevant (Kany et al., 2019). In this sense, the decrease in MCP-1 prevents the progression of the inflammatory process, which combined with the reduction in pro-inflammatory cytokines, suggests an anti-inflammatory potential of these hydrolysates. Their anti-inflammatory effects dependend on the type of hydrolysate consumed, with the most potent of those obtained using Protamex® or Corolase PP® (Aloysius et al., 2018). In addition, an antioxidant chicken liver hydrolysate was also able to decrease several inflammatory markers in high-fat diet animal models (Wu, Lin, et al., 2020, 2021; Yang et al., 2014). Interestingly, supplementation with this liver hydrolysate not only decreased the levels of inflammatory and fibrotic proteins in myocardia of high-fat diet-fed mice, but also attenuated matrix metalloproteinases (MMP2 and MMP9) protein expression (Wu, Lin, et al., 2020). Moreover, the supplementation with the liver-derived hydrolysate attenuated high-fat diet-induced autophagosome accumulation in animals (Wu, Lin, et al., 2020), thereby helping to maintain autophagy homeostasis. Autophagy plays an essential role in the myocardial fibrosis process. In fact, cardiac hypertrophy and lipotoxicicty induced by high-fat diet were associated with an impaired autophagic response, where the accumulation of autophagosomes and an excess of lipids promoted endoplasmic reticulum stress, as well as apoptosis in high-fat diet-fed mice (Che et al., 2018; He et al., 2018). The antiinflammatory effects of the aforementioned liver hydrolysate was also observed in animal models of thioacetamide-induced liver damage (Chen et al., 2017) and in an alcoholic liver disease (Lin et al., 2017) being the lowest tested dose the most effective (80 > 320>1280 mg/kg BW) as it was found in the 80 mg/kg BW dose more pro-inflammatory parameters modified (IL-1β, IL-6 and TNF-α) (Lin et al., 2017). Finally, anti-inflammatory effects were also observed in rats suffering papain-induced osteoarthritis after being administered a Pepsin-digested cartilage hydrolysate for 4 weeks (Yang et al., 2019). These animals showed lower blood levels of TNF-α and IL-1β than control group.

4.2.6. *Neuroprotective effects*

Neurodegenerative diseases, characterized by progressive loss of functional neurons, are another growing disease in our society, especially in the elderly population. It is affected by approximately 50 million people and is expected to double in the next few decades (Hansson, 2021). The prevalence of neurodegenerative diseases is increasing with an increase in the lifespan of the global population. Therefore, new therapies are being investigated to prevent neurological disorders. Recently, it was observed in STZ-induced diabetic mice that the administration of a Pepsin-digested liver hydrolysate improved cognitive dysfunction and behavioral performance of the animals (Yeh et al., 2022). The authors attributed its effects to the reduction of apoptosis in brain cells and amyloid-beta (Aβ) plaques accumulation in the brain with respect to STZ-induced diabetic mice. Apoptosis is characterized by the symptoms of many neurodegenerative diseases and neurological disorders, such Alzheimer's disease or Huntington's disease, and amyotrophic lateral sclerosis (Moujalled, Strasser, & Liddell, 2021). In addition, Aβ plaque accumulation is one of the main histopathological hallmarks of Alzheimer's disease and has been widely

Table 6
Anti-inflammatory effects of chicken by-product hydrolysates.

By-product	Hydrolisis conditions	Amino acid sequence/s	Model	Doses	Administration period	Effect***	References
Cartilage	Trypsin, 10 g/kg by-product, 3 h, 55 °C, pH 7.0	–	Specific pathogen free-grade rats +5% papain solution injected to articular cavity	100 mg/kg BW	Daily for 30 days	↓blood TFN-α, IL-1β, IL-10	Yang et al. (2019)
Feather	Flavourzyme® 10 mg/mL, 4 h, 50 °C, pH 7.0 (F < 0.65 kDa)	SNPSVAGVR, SLFLHTHSIVADK, AVLKKKVTSTFGR, LSPWPVKGV	LPS-activated RAW 264.7	40 μg/mL		↓IL-6 protein and expression ↓iNOS protein and expression ↓*Cox2, Tnf-α*	Inkanuwat et al. (2019)
Feet	*Aspergillus oryzae* protease	PO**, OG**	TNF-α-induced inflamed HUVEC	0.1%		↓sICAM-1 and sVCAM-1	Kouguchi et al. (2012)
	Fraction <3000 Da: *Aspergillus oryzae* protease	–	Hypertensive rats (Wistar Kyoto rats + l-NAME)	2.0 g/kg BW	Daily for 8 weeks	↓plasma siCAM-1 levels	Zhang, Kouguchi, Shimizu, Ohmori, et al. (2010)
	Fraction <3000 Da: *Aspergillus oryzae* protease	–	ApoE-deficient C57BL/6. KOR-ApoE^{shl} mice	10%	Daily for 12 weeks	↓plasma IL-6, sICAM-1, and TNF-α	
Intestine*	Neutrase® +Tripsin (2:1) 0.2%, 4 h, 48.4 °C, pH 7.0	–	Healthy Common carp	25% protein replace	Daily for 8 weeks	↑intestinal *Il-10 and Nf-kB*	Wu et al. (2022)
		–		50% protein replace	Daily for 8 weeks	↓intestinal *Il-1β, Tnf-α* ↑intestinal *Il-10, TGF-β2, and MyD88*	
		–		75% protein replace	Daily for 8 weeks	↓intestinal *Il-8* ↑intestinal *Il-10, TGF-β2, and MyD88*	
		–		100% protein replace	Daily for 8 weeks	↑intestinal *Il-1β, Il-6α, Il-10, Nf-kB, and MyD88*	
Liver	Pepsin 1:400, 2 h, 37 °C	–	Golden Syrian hamsters + high-fat diet	100 mg/kg BW	Daily for 8 weeks	↓liver IL-1β and TNF-α	Yang et al. (2014)
		–		200 mg/kg BW		↓liver TNF-α	
		–		400 mg/kg BW		↓liver TNF-α	
		–	Wistar rats + 100 mg TAA/kg BW (intraperitoneal injection)	200 mg/kg BW	Daily for 10 weeks	↓serum IL-6 and TGF-β	Chen et al. (2017)
		–		600 mg/kg BW	Daily for 10 weeks	↓serum IL-6, TGF-β, and TNF-α	
		–	C57BL/6J (B6) mice + alcoholic diet	80 mg/kg BW		↓liver IL-1β, IL-6, and TNF-α	Lin et al. (2017)
		–		320 mg/kg BW	Daily for 8 weeks	↓liver IL-1β and TNF-α	
		–		1280 mg/kg BW	Daily for 8 weeks	↓liver IL-1β	
		–	C57BL/6 mice + high-fat diet	~170 mg/kg BW	Daily for 20 weeks	↓myocardic IL-1β, IL-6, TNF-α, TGF-β, COX-2, CD36, αSMA, MMP-9, andLC3BII/LC3BI ↑myocardic P62	Wu, Lau, et al. (2020)
		–		~510 mg/kg BW	Daily for 20 weeks	↓myocardic IL-1β, IL-6, TNF-α, TGF-β, COX-2, CD36, αSMA, MMP9, MMP2, LC3BII/LC3BI ↑myocardic P62 and Rab7	
		–	C57BL/6 mice + high-fat diet	~170 mg/kg BW	Daily for 20 weeks	↓liver IL-1β and TNF-α	Wu, Korntner, et al. (2021)
		–		~510 mg/kg BW	Daily for 20 weeks	↓liver IL-1β and TNF-α	
Mechanical deboning residues	Alcalase® 0.1%, 1 h, 50 °C	–	High fat/high sucrose diet-induced obese C57BL/6 mice	9.5 E%	Daily for 12 weeks	↓plasma IL-1β, IL-2, IL-6,IL-10, IFN-γ, TFN-α, MCP-1, and RANTES	Aloysius et al. (2018)
	Alcalase® 0.1%, 1 h, 50 °C	–	ApoE-deficient (Apoe$^{-/-}$) mice + high fat and high sucrose diet	10.6 E%	Daily for 12 weeks	↑plasma IL-2	Bjørndal et al. (2020)
	Protamex®, 0.1%, 1 h, 50 °C	–	High fat/high sucrose diet-induced obese C57BL/6 mice	9.5 E%	Daily for 12 weeks	↓plasma IL-1β, IL-1α, IL-2, IL-6, IL-10,IL-17,	Aloysius et al. (2018)

(*continued on next page*)

Table 6 (*continued*)

By-product	Hydrolisis conditions	Amino acid sequence/s	Model	Doses	Administration period	Effect***	References
						IFN-γ, TFN-α, MCP-1, and GM-CSF	
	Corolase® PP, 0.1%, 1 h, 50 °C	–	High fat/high sucrose diet-induced obese C57BL/6 mice	9.5 E%	Daily for 12 weeks	↓plasma IL-1β, IL-1α, IL-2, IL-6, IL-10, IFN-γ, TFN-α, MCP-1, and GM-CSF	Aloysius et al. (2018)
	Corolase® PP, 0.1%, 1 h, 50 °C	–	ApoE-deficient (Apoe$^{-/-}$) mice + high fat and high sucrose diet	10.6 E%	Daily for 12 weeks	↓plasma MCP-1	Bjørndal et al. (2020)
	Papain + Bromelain, 0.1% (1:1, w/w), 1 h, 50 °C	–	High fat/high sucrose diet-induced obese C57BL/6 mice	9.5 E%	Daily for 12 weeks	↓plasma IL-1β, IFN-γ, TFN-α, MCP-1, and GM-CFS	Aloysius et al. (2018)

Table shows the most active hydrolysate obtained in each study. ↑ and ↓ indicate an increase or decrease of a parameter, respectively, in comparison with their respective non-treated ill animals, except for intestine hydrolysate (*). In this last case, results were compared with those of healthy animals consuming a basic fishmeal.

Abbreviations: αSMA (smooth muscle alpha-actin), Apo E (apolipoprotein E), COX2 (cyclooxigenase-2), E (energy), GM-CSF (granulocyte-macrophage colony-stimulating factor), HUVEC (Human umbilical vein endothelial cells), IL (interleukin), IFN-γ (interferon-gamma), iNOS (inducible nitric oxide synthase), LC3 (microtubule-associated protein 1A/1B-light chain 3), L-NAME (N-nitro L-arginine methyl ester), LPS (lipopolysaccharide), MCP-1 (Monocyte chemoattractant protein-1), MMP-9 (matrix metallopeptidase 9), NF-kB (nuclear factor kB), RANTES (chemokine (C–C motif) ligand 5), sICAM-1 (soluble intercellular adhesion molecule 1), sVCAM-1 (soluble vascular adhesion molecule 1), TAA (thioacetamide), TGF-β (transforming growth factor beta), TNFα (tumor necrosis factor alpha). (**)'O' indicates 'hydroxyproline'. (***) genes are represented by a cursive letter.

studied as a therapeutic target (Ashrafian, Zadeh, & Khan, 2021).

4.2.7. Other effects

Recently, the potential of chicken by-products for osteoarthritis prevention has been highlighted. In this regard, an unspecified hydrolysate obtained from chicken sternal cartilage was administered to female rats with osteoarthritis induced by medial meniscus resection (MMx) and anterior to the medial collateral ligament (ACL) on the left knees, for two weeks (50, 200 and 500 mg/kg BW) before the surgery (Ma et al., 2021). After 12 weeks, in which animals exercised daily for 30 min to induce osteoarthritis, the consumption of the hydrolysate (500 mg/kg BW) prevented the formation of osteophytes in the treated animals and increased the cartilage matrix and collagen content in comparison to control osteoarthritis-induced rats. Moreover, it was observed that hydrolysate-treated animals had repaired the cartilage defects, and the meshwork of collagen fibers was almost intact (Ma et al., 2021). Moreover, a Trypsin-digested cartilage hydrolysate alleviated the effects of osteoarthritis induced by papain injection in male rats, with the <10 kDa fraction being the most effective (Yang et al., 2019).

Using a completely different framework, Lin et al. (2017) observed that pepsin-digested chicken liver hydrolysates ameliorated the development of alcoholic fatty livers in mice. Male C57BL/6J (B6) mice were fed a Lieber-DeCarli regular alcoholic diet containing 5% (v/v) ethanol (35% of the daily caloric intake) and supplemented with chicken hydrolysate for 8 weeks (Lin et al., 2017). The results showed an amelioration of the enlargement of liver and spleen sizes, as well as of the serum AST, ALT, and ALP levels in mice supplemented with the hydrolysate compared to the non-supplemented mice. Furthermore, the antioxidant profile, lipid content, and inflammation status were also reversed by the chicken hydrolysate. The authors suggested that these effects could be attributed to the upregulation of fatty acid β-oxidation, downregulation of fatty acid synthesis, increased antioxidant defenses, and increased alcohol metabolic enzymatic activities (acetaldehyde dehydrogenase).

Finally, the effects of the two chicken by-product hydrolysates on the intestinal function were studied. In this regard, upregulation of intestinal tight junction protein-1 (*Zo-1*) and *Mlck* gene expression was found in common carp fed a chicken intestine hydrolysate (the usual protein fishmeal was replaced by 25, 50, 75, or 100% of the hydrolysate) (Wu et al., 2022). However, their beneficial effects on intestinal barrier function depended on the consumed dose, being the most active when fishmeal protein was replaced by 50% hydrolysate. Moreover, broiler chicks fed a chicken feather hydrolysate had higher muscle layer thickness in several parts of the intestine and lower epithelial thickness in the duodenum and ileum than non-treated animals (Alahyaribeik et al., 2022). These effects suggested that the hydrolysate could increase the intestinal absorptive capacity.

5. Conclusion, current limitations and future perspectives

The study of chicken by-products as a source of bioactive peptides is a very recent activity, with the majority of the studies being carried out in the last decade. Different chicken by-products, including blood, bones, cartilage, combs, feathers, feet, livers, skin, viscera, and wattles have been used to prepare hydrolysates with a wide range of activities. In this regard, the two most searched *in vitro* activities were antioxidant and ACEi activities, although hydrolysates also showed potential effects as DPP-IV inhibitors. Moreover, chicken by-product derived hydrolysates have shown to exert antioxidant, antihypertensive, antidiabetic, anti-hyperglycemic, anti-hypercholesterolemic, anti-inflammatory, anti-coagulant, anti-anemic, hepatoprotective, and neuroprotective effects. Blood, bones, cartilage, combs and wattles, feather, gizzard, liver, skin, trachea, viscera, and residues from mechanical chicken deboning have been useful to obtain antioxidant hydrolysates; blood, feather, foot, liver, and residues from mechanical chicken deboning for antidiabetic hydrolysates; feather, foot, liver, residues from mechanical chicken deboning, viscera, and blood for lipid metabolism management hydrolysates, and cartilage, feather, feet, intestine, liver, residues from mechanical chicken deboning for obtaining anti-inflammatory peptides. Chicken feet stood out as source of antihypertensive peptides and chicken liver as a source of hydrolysates able to exert multiple bioactivities such as antidiabetic, anti-inflammatory and modulator of biometric parameters and lipid metabolism. Although many of the developed hydrolysates have been *in vivo* tested, only one of them has been evaluated in humans, showing antihypertensive effects.

Considering the evidence, chicken by-product hydrolysates have potential applications as functional ingredients; however, many studies are still necessary to achieve this purpose; for instance, studies focus on evaluating the effects of these hydrolysates in clinical trials. Moreover, the design of novel foods or nutraceuticals with specific uses in human health that utilize wastes from poultry farming is still a challenge, since chicken by-products utilization entails several difficulties, such as the limited access to these materials or the lack of environmental education and culture, particularly in developing areas. Thus, efforts must be

carried out to create awareness and motivate industries and workers on poultry leftovers revalorization.

Furthermore, further research is encouraged considering the promising results that have been obtained to date, and some gaps must be addressed, such as analysis of the peptide sequence of the bioactive fractions and other parameters such as molecular weights, as well as allergenicity and immune-reactivity aspects, since novel peptide regions can be exposed after hydrolysis. Besides, most of these hydrolysates are designed to be finally consumed as functional food, so sensory analysis must be an essential part of the future research, since chicken by-products may provide some odd flavors that requires to be eliminated or masked.

In conclusion, the obtaining of bioactive peptides from these by-products could be useful to the valorization of these materials and contribute to drive the development of circular economy principles.

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Declaration of competing interest

The authors declare no conflict of interest.

Data availability

No data was used for the research described in the article.

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References

Adler, S. A., Slizyte, R., Honkapää, K., & Løes, A. K. (2018). In vitro pepsin digestibility and amino acid composition in soluble and residual fractions of hydrolyzed chicken feathers. *Poultry Science, 97*(9), 3343–3357. https://doi.org/10.3382/ps/pey175

Akram, A. N., & Zhang, C. (2020). Extraction of collagen-II with pepsin and ultrasound treatment from chicken sternal cartilage; physicochemical and functional properties. *Ultrasonics Sonochemistry, 64*, Article 105053. https://doi.org/10.1016/j.ultsonch.2020.105053

Alahyaribeik, S., Nazarpour, M., Tabandeh, F., Honarbakhsh, S., & Sharifi, S. davood (2022). Effects of bioactive peptides derived from feather keratin on plasma cholesterol level, lipid oxidation of meat, and performance of broiler chicks. *Tropical Animal Health and Production, 54*(5), 271. https://doi.org/10.1007/s11250-022-03244-1

Alahyaribeik, S., Seyed, D. S., Tabandeh, F., Honarbakhsh, S., & Ghazanfari, S. (2021). Stability and cytotoxicity of DPPH inhibitory peptides derived from biodegradation of chicken feather. *Protein Expression and Purification, 177*, Article 105748. https://doi.org/10.1016/j.pep.2020.105748. August 2020.

Alahyaribeik, S., & Ullah, A. (2020). Effects of ultrasound-assisted alkaline extraction on antioxidant activity and functional characteristics of chicken feather keratin peptides. *ChemistrySelect, 5*(44), 13788–13794. https://doi.org/10.1002/slct.202002887

Alencar-Bezerra, T. K., Gomes de Lacerda, J. T. J., Ramos-Salu, B., Vilela-Oliva, M. L., Juliano, M. A., Bertoldo-Pacheco, M. T., et al. (2019). Identification of angiotensin I-converting enzyme-inhibitory and anticoagulant peptides from enzymatic hydrolysates of chicken combs and wattles. *Journal of Medicinal Food, 22*(12), 1294–1300. https://doi.org/10.1089/jmf.2019.0066

Ali, A. M. M., Gullo, M., Rai, A. K., & Bavisetty, S. C. B. (2021). Bioconservation of iron and enhancement of antioxidant and antibacterial properties of chicken gizzard protein hydrolysate fermented by Pediococcus acidilactici ATTC 8042. *Journal of the Science of Food and Agriculture, 101*(7), 2718–2726. https://doi.org/10.1002/jsfa.10898

Aloysius, T., Carvajal, A., Slizyte, R., Skorve, J., Berge, R., & Bjørndal, B. (2018). Chicken protein hydrolysates have anti-inflammatory effects on high-fat diet induced obesity in mice. *Medicines, 6*(1), 5. https://doi.org/10.3390/medicines6010005

Amorati, R., & Valgimigli, L. (2018). Methods to measure the antioxidant activity of phytochemicals and plant extracts. *Journal of Agricultural and Food Chemistry, 66*(13), 3324–3329. https://doi.org/10.1021/acs.jafc.8b01079

Aoyama, K., & Nakaki, T. (2015). Glutathione in cellular redox homeostasis: Association with the excitatory amino acid aarrier 1 (EAAC1). *Molecules, 20*(5), 8742–8758. https://doi.org/10.3390/molecules20058742

Araújo, Í. B. S., Lima, D. A. S., Pereira, S. F., & Madruga, M. S. (2019). Quality of low-fat chicken sausages with added chicken feet collagen. *Poultry Science, 98*(2), 1064–1074. https://doi.org/10.3382/ps/pey397

Arshad, M., Bano, I., Khan, N., Shahzad, M. I., Younus, M., Abbas, M., et al. (2018). Electricity generation from biogas of poultry waste: An assessment of potential and feasibility in Pakistan. *Renewable and Sustainable Energy Reviews, 81*, 1241–1246. https://doi.org/10.1016/j.rser.2017.09.007

Ashrafian, H., Zadeh, E. H., & Khan, R. H. (2021). Review on Alzheimer's disease: Inhibition of amyloid beta and tau tangle formation. *International Journal of Biological Macromolecules, 167*, 382–394. https://doi.org/10.1016/j.ijbiomac.2020.11.192

Babicz, M., Kropiwiec-Domańska, K., Skrzypczak, E., Szyndler-Nędza, M., & Szulc, K. (2020). Analysis of technological and consumption quality of offal and offal products obtained from Pulawska and Polish landrace pigs. *Animals, 10*(6), 964. https://doi.org/10.3390/ani10060964

Bartesaghi, S., & Radi, R. (2018). Fundamentals on the biochemistry of peroxynitrite and protein tyrosine nitration. *Redox Biology, 14*, 618–625. https://doi.org/10.1016/j.redox.2017.09.009

Ben Hamad Bouhamed, S., Krichen, F., & Kechaou, N. (2020). Feather protein hydrolysates: A study of physicochemical, functional properties and antioxidant activity. *Waste and Biomass Valorization, 11*(1), 51–62. https://doi.org/10.1007/s12649-018-0451-2

Bezerra, T., Estévez, M., Lacerda, J. T., Dias, M., Juliano, M., Mendes, M. A., et al. (2020). Chicken combs and wattles as sources of bioactive peptides: Optimization of hydrolysis, identification by LC-ESI-MS2 and bioactivity assessment. *Molecules, 25*(7), 1698. https://doi.org/10.3390/molecules25071698

Bezus, B., Ruscasso, F., Garmendia, G., Vero, S., Cavello, I., & Cavalitto, S. (2021). Revalorization of chicken feather waste into a high antioxidant activity feather protein hydrolysate using a novel psychrotolerant bacterium. *Biocatalysis and Agricultural Biotechnology, 32*, Article 101925. https://doi.org/10.1016/j.bcab.2021.101925

Bjørndal, B., Aloysius, T. A., Lund, A., Slizyte, R., Bohov, P., Carvajal, A. K., et al. (2020). A chicken protein hydrolysate exerts anti-atherosclerotic effect beyond plasma cholesterol-lowering activity in Apoe−/− mice. *Food Science and Nutrition, 8*(7), 3052–3060. https://doi.org/10.1002/fsn3.1300

Boots, J.-W. P. (2012). EP1831361B1.

Borrajo, P., Pateiro, M., Barba, F. J., Mora, L., Franco, D., Toldrá, F., et al. (2019). Antioxidant and antimicrobial activity of peptides extracted from meat by-products: A review. *Food Analytical Methods, 12*(11), 2401–2415. https://doi.org/10.1007/S12161-019-01595-4

Bravo, F. I., Calvo, E., López-Villalba, R. A., Torres-Fuentes, C., Muguerza, B., García-Ruiz, A., et al. (2023). Valorization of chicken slaughterhouse byproducts to obtain antihypertensive peptides. *Nutrients, 15*(2), 457. https://doi.org/10.3390/nu15020457

Bravo, F. I., Mas-Capdevila, A., Margalef, M., Arola-Arnal, A., & Muguerza, B. (2019). Novel antihypertensive peptides derived from chicken foot proteins. *Molecular Nutrition & Food Research, 63*(12), Article 1801176. https://doi.org/10.1002/mnfr.201801176

Buettner, R., Schölmerich, J., & Bollheimer, L. C. (2007). High-fat diets: Modeling the metabolic disorders of human obesity in rodents. *Obesity, 15*(4), 798–808. https://doi.org/10.1038/oby.2007.608

Callegaro, K., Brandelli, A., & Daroit, D. J. (2019). Beyond plucking: Feathers bioprocessing into valuable protein hydrolysates. *Waste Management, 95*, 399–415. https://doi.org/10.1016/j.wasman.2019.06.040

Cansu, Ü., & Boran, G. (2015). Optimization of a multi-step procedure for isolation of chicken bone collagen. *Korean Journal for Food Science of Animal Resources, 35*(4), 431–440. https://doi.org/10.5851/kosfa.2015.35.4.431

Cao, H., & Xu, S.-Y. (2008). Purification and characterization of type II collagen from chick sternal cartilage. *Food Chemistry, 108*(2), 439–445. https://doi.org/10.1016/j.foodchem.2007.09.022

Carrera-Alvarado, G., Toldrá, F., & Mora, L. (2022). DPP-IV Inhibitory peptides GPF, IGL, and GGGW obtained from chicken blood hydrolysates. *International Journal of Molecular Sciences, 23*(22), Article 14140. https://doi.org/10.3390/ijms232214140

Casanova-Martí, À., Bravo, F. I., Serrano, J., Ardévol, A., Pinent, M., & Muguerza, B. (2019). Antihyperglycemic effect of a chicken feet hydrolysate: Via the incretin system: DPP-IV-inhibitory activity and GLP-1 release stimulation. *Food & Function, 10*(7), 4062–4070. https://doi.org/10.1039/c9fo00695h

Chakka, A. K., Elias, M., Jini, R., Sakhare, P. Z., & Bhaskar, N. (2015). In-vitro antioxidant and antibacterial properties of fermentatively and enzymatically prepared chicken liver protein hydrolysates. *Journal of Food Science and Technology, 52*(12), 8059–8067. https://doi.org/10.1007/s13197-015-1920-2

Chakka, A. K., Ramanatikara, J., Zituji, S. P., Pedda, M. S., & Narayan, B. (2021). In-vivo anti-anaemic effects of bioactive compounds prepared from chicken liver using biotechnological tools. *Waste and Biomass Valorization, 12*(12), 6699–6708. https://doi.org/10.1007/s12649-021-01464-4

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., et al. (2018). Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget, 9*(6), 7204. https://doi.org/10.18632/ONCOTARGET.23208

Cheng, F. Y., Lai, I. C., Lin, L. C., & Sakata, R. (2016). The in vitro antioxidant properties of alcalase hydrolysate prepared from silkie fowl (Gallus gallus) blood protein. *Animal Science Journal, 87*(7), 921–928. https://doi.org/10.1111/asj.12509

Cheng, F.-Y., Liu, Y.-T., Wan, T.-C., Lin, L.-C., & Sakata, R. (2008). The development of angiotensin I-converting enzyme inhibitor derived from chicken bone protein. *Animal Science Journal, 79*, 122–128. https://doi.org/10.1111/j.1740-0929.2007.00507.x

Cheng, F. Y., Wan, T. C., Liu, Y. T., Lai, K. M., Lin, L. C., & Sakata, R. (2008a). A study of in vivo antihypertensive properties of enzymatic hydrolysate from chicken leg bone protein. *Animal Science Journal, 79*(5), 614–619. https://doi.org/10.1111/j.1740-0929.2008.00571.x

Cheng, F. Y., Wan, T. C., Liu, Y. T., Lai, K. M., Lin, L. C., & Sakata, R. (2008b). Attenuating development of cardiovascular hypertrophy with hydrolysate of chicken leg bone protein in spontaneously hypertensive rats. *Asian-Australasian Journal of Animal Sciences, 21*(5), 732–737. https://doi.org/10.5713/ajas.2008.70599

Chen, Y. H., Liu, Y. H., Yang, Y. H., Feng, H. H., Chang, C. T., & Chen, C. C. (2002). Antihypertensive effect of an enzymatic hydrolysate of chicken essence residues. *Food Science and Technology Research, 8*(2), 144–147. https://doi.org/10.3136/fstr.8.144

Chen, P. J., Tseng, J. K., Lin, Y. L., Wu, Y. H. S., Hsiao, Y. T., Chen, J. W., et al. (2017). Protective effects of functional chicken liver hydrolysates against liver fibrogenesis: Antioxidation, anti-inflammation, and antifibrosis. *Journal of Agricultural and Food Chemistry, 65*(24), 4961–4969. https://doi.org/10.1021/acs.jafc.7b01403

Che, Y., Wang, Z., Yuan, Y., Zhang, N., Jin, Y., Wan, C., et al. (2018). Role of autophagy in a model of obesity: A long-term high fat diet induces cardiac dysfunction. *Molecular Medicine Reports, 18*(3), 3251–3261. https://doi.org/10.3892/mmr.2018.9301

Chiarelotto, M., Restrepo, J. C. P. S., Lorin, H. E. F., & Damaceno, F. M. (2021). Composting organic waste from the broiler production chain: A perspective for the circular economy. *Journal of Cleaner Production, 329*, Article 129717. https://doi.org/10.1016/j.jclepro.2021.129717

Chou, C. H., Wang, S. Y., Lin, Y. T., & Chen, Y. C. (2014). Antioxidant activities of chicken liver hydrolysates by pepsin treatment. *International Journal of Food Science and Technology, 49*(7), 1654–1662. https://doi.org/10.1111/ijfs.12471

Cliche, S., Amiot, J., Avezard, C., & Gariepy, C. (2003). Extraction and characterization of collagen with or without telopeptides from chicken skin. *Poultry Science, 82*(3), 503–509. https://doi.org/10.1093/ps/82.3.503

da Cunha, I. C., Brandelli, A., Braga, A. R. C., Sala, L., & Kalil, S. J. (2023). Feather meal as a source of peptides with antioxidant activity from enzymatic hydrolysis. *Waste and Biomass Valorization, 14*, 421–430. https://doi.org/10.1007/s12649-022-01886-8

Deshmane, S. L., Kremlev, S., Amini, S., & Sawaya, B. E. (2009). Monocyte chemoattractant protein-1 (MCP-1): An overview. *Journal of Interferon and Cytokine Research, 29*(6), 313–326. https://doi.org/10.1089/jir.2008.0027

Douki, T., & Cadet, J. (1996). Peroxynitrite mediated oxidation of purine bases of nucleosides and isolated DNA. *Free Radical Research, 24*(5), 369–380. https://doi.org/10.3109/10715769609088035

Fakhfakh, N., Ktari, N., Haddar, A., Mnif, I. H., Dahmen, I., & Nasri, M. (2011). Total solubilisation of the chicken feathers by fermentation with a keratinolytic bacterium, Bacillus pumilus A1, and the production of protein hydrolysate with high antioxidative activity. *Process Biochemistry, 46*(9), 1731–1737. https://doi.org/10.1016/j.procbio.2011.05.023

Fallah-Delavar, M., & Farmani, J. (2018). Recovery and characterization of enzymatic protein hydrolyzates and fat from chicken skin. *JAOCS, Journal of the American Oil Chemists' Society, 95*(9), 1151–1161. https://doi.org/10.1002/aocs.12131

Fan, J., He, J., Zhuang, Y., & Sun, L. (2012). Purification and identification of antioxidant peptides from enzymatic hydrolysates of Tilapia (Oreochromis niloticus) frame protein. *Molecules, 17*(11), 12836–12850. https://doi.org/10.3390/molecules171112836

Fan, H., Liao, W., Spaans, F., Pasha, M., Davidge, S. T., & Wu, J. (2022). Chicken muscle hydrolysate reduces blood pressure in spontaneously hypertensive rats, upregulates ACE2, and ameliorates vascular inflammation, fibrosis, and oxidative stress. *Journal of Food Science, 87*(3), 1292–1305. https://doi.org/10.1111/1750-3841.16077

Fan, H., & Wu, J. (2022). Conventional use and sustainable valorization of spent egg-laying hens as functional foods and biomaterials: A review. *Bioresources and Bioprocessing, 9*(1), 43. https://doi.org/10.1186/s40643-022-00529-z

Fontoura, R., Daroit, D. J., Correa, A. P. F., Meira, S. M. M., Mosquera, M., & Brandelli, A. (2014). Production of feather hydrolysates with antioxidant, angiotensin-I converting enzyme- and dipeptidyl peptidase-IV-inhibitory activities. *New Biotech, 31*(5), 506–513. https://doi.org/10.1016/j.nbt.2014.07.002

Fontoura, R., Daroit, D. J., Corrêa, A. P. F., Moresco, K. S., Santi, L., Beys-da-Silva, W. O., et al. (2019). Characterization of a novel antioxidant peptide from feather keratin hydrolysates. *New Biotech, 49*, 71–76. https://doi.org/10.1016/j.nbt.2018.09.003. March 2018.

Food and Agriculture Organization of the United Nations. (2013). Poultry development review. Retrieved March 26, 2023, from https://www.fao.org/publications/card/en/c/90c86c8d-96e8-5db6-914f-2bd9fa24e421/.

Food and Agriculture Organization of the United Nations. (2022). Gateway to poultry production and products. Retrieved March 26, 2023, from https://www.fao.org/poultry-production-products/en/.

Forstermann, U., & Sessa, W. C. (2012). Nitric oxide synthases: Regulation and function. *European Heart Journal, 33*(7), 829–837. https://doi.org/10.1093/eurheartj/ehr304

Fu, Y., Therkildsen, M., Aluko, R. E., & Lametsch, R. (2019). Exploration of collagen recovered from animal by-products as a precursor of bioactive peptides: Successes and challenges. *Critical Reviews in Food Science and Nutrition, 59*(13), 2011–2027. https://doi.org/10.1080/10408398.2018.1436038

Gandhi, J., Khera, L., Gaur, N., Paul, C., & Kaul, R. (2017). Role of modulator of inflammation cyclooxygenase-2 in gammaherpesvirus mediated tumorigenesis. *Frontiers in Microbiology, 8*, 538. https://doi.org/10.3389/fmicb.2017.00538

Gao, D., Guo, P., Cao, X., Ge, L., Ma, H., Cheng, H., et al. (2020). Improvement of chicken plasma protein hydrolysate angiotensin I-converting enzyme inhibitory activity by optimizing plastein reaction. *Food Science and Nutrition, 8*(6), 2798–2808. https://doi.org/10.1002/fsn3.1572

Gaweł, S., Wardas, M., Niedworok, E., & Wardas, P. (2004). Malondialdehyde (MDA) as a lipid peroxidation marker. *Wiadomosci Lekarskie (Warsaw, Poland, 57*(9–10), 453–455, 1960.

Girgih, A. T., He, R., Malomo, S., Offengenden, M., Wu, J., & Aluko, R. E. (2014). Structural and functional characterization of hemp seed (Cannabis sativa L.) protein-derived antioxidant and antihypertensive peptides. *Journal of Functional Foods, 6*, 384–394. https://doi.org/10.1016/j.jff.2013.11.005

Giri, S. S., Sahoo, S. K., & Mohanty, S. N. (2010). Replacement of by-catch fishmeal with dried chicken viscera meal in extruded feeds: Effect on growth, nutrient utilisation and carcass composition of catfish Clarias batrachus (Linn.) fingerlings. *Aquaculture International, 18*(4), 539–544. https://doi.org/10.1007/s10499-009-9265-3

Gonçalves dos Santos Aguilar, J., Santos de Souza, A. K., & Soares de Castro, R. J. (2020). Enzymatic hydrolysis of chicken viscera to obtain added-value protein hydrolysates with antioxidant and antihypertensive properties. *International Journal of Peptide Research and Therapeutics, 26*(2), 717–725. https://doi.org/10.1007/s10989-019-09879-3

Green, B. D., Flatt, P. R., & Bailey, C. J. (2006). Dipeptidyl peptidase IV (DPP IV) inhibitors: A newly emerging drug class for the treatment of type 2 diabetes. *Diabetes and Vascular Disease Research, 3*(3), 159–165. https://doi.org/10.3132/dvdr.2006.024

Gross, M. D., Bielinski, S. J., Suarez-Lopez, J. R., Reiner, A. P., Bailey, K., Thyagarajan, B., et al. (2012). Circulating soluble intercellular adhesion molecule 1 and subclinical atherosclerosis: The coronarya artery risk development in young adults study. *Clinical Chemistry, 58*(2), 411–420. https://doi.org/10.1373/clinchem.2011.168559

Hadi, H. A. R., Carr, C. S., & Al Suwaidi, J. (2005). Endothelial dysfunction: Cardiovascular risk factors, therapy, and outcome. *Vascular Health and Risk Management, 1*(3), 183–198.

Hajat, C., & Stein, E. (2018). The global burden of multiple chronic conditions: A narrative review. *Preventive Medicine Reports, 12*, 284–293. https://doi.org/10.1016/j.pmedr.2018.10.008

Halliwell, B., & Gutteridge, J. M. C. (2015). In B. Halliwell, & J. M. C. Gutteridge (Eds.), *Free radicals in biology and medicine*. Oxford, United Kingdom: Oxford University Press. fifth.

Hamzeh, A., Sangsawad, P., Noisa, P., Choowongkomon, K., & Yongsawatdigul, J. (2022). Bioactivities of in vitro transepithelial transported peptides from cooked chicken breast. *International Journal of Peptide Research and Therapeutics, 28*(1), 31. https://doi.org/10.1007/s10989-021-10326-5

Hamzeh, A., Wongngam, W., Kiatsongchai, R., & Yongsawatdigul, J. (2019). Cellular and chemical antioxidant activities of chicken blood hydrolysates as affected by in vitro gastrointestinal digestion. *Poultry Science, 98*(11), 6138–6148. https://doi.org/10.3382/ps/pez283

Han, R., Hernández Álvarez, A. J., Maycock, J., Murray, B. S., & Boesch, C. (2022). Differential effects of oilseed protein hydrolysates in attenuating inflammation in murine macrophages. *Food Bioscience, 49*, Article 101860. https://doi.org/10.1016/j.fbio.2022.101860

Hansson, O. (2021). Biomarkers for neurodegenerative diseases. *Nature Medicine, 27*(6), 954–963. https://doi.org/10.1038/s41591-021-01382-x

Harnedy, P. A., O'Keeffe, M. B., & FitzGerald, R. J. (2015). Purification and identification of dipeptidyl peptidase (DPP) IV inhibitory peptides from the macroalga Palmaria palmata. *Food Chemistry, 172*, 400–406. https://doi.org/10.1016/j.foodchem.2014.09.083

Hatanaka, T., Kawakami, K., & Uraji, M. (2014). Inhibitory effect of collagen-derived tripeptides on dipeptidylpeptidase-IV activity. *Journal of Enzyme Inhibition and Medicinal Chemistry, 29*(6), 823–828. https://doi.org/10.3109/14756366.2013.858143

Hauser, P. S., Narayanaswami, V., & Ryan, R. O. (2011). Apolipoprotein E: From lipid transport to neurobiology. *Progress in Lipid Research, 50*(1), 62–74. https://doi.org/10.1016/j.plipres.2010.09.001

Heydarpour, F., Amini, B., Kalantari, S., Sadraddin, Akbari, A., & Heydarpour, P. (2006). Mean percentage of skin and visible fat in 10 chicken carcass weight. *International Journal of Poultry Science, 6*(1), 43–47. https://doi.org/10.3923/ijps.2007.43.47

He, L., Zhang, J., Zhao, J., Ma, N., Kim, S. W., Qiao, S., et al. (2018). Autophagy: The last defense against cellular nutritional stress. *Advances in Nutrition, 9*(4), 493–504. https://doi.org/10.1093/advances/nmy011

Holst, J. J. (2007). The physiology of glucagon-like peptide 1. *Physiological Reviews, 87*(4), 1409–1439. https://doi.org/10.1152/physrev.00034.2006

Huang, S.-C., & Liu, P.-J. (2010). Inhibition of angiotensin I-Converting enzymes by enzymatic hydrolysates from chicken blood. *Journal of Food and Drug Analysis, 18*(6), 459–463. https://doi.org/10.38212/2224-6614.2239

Ibarz-Blanch, N., Morales, D., Calvo, E., Ros-Medina, L., Muguerza, B., Bravo, F. I., et al. (2022). Role of chrononutrition in the antihypertensive effects of natural bioactive compounds. *Nutrients, 14*(9), 1920. https://doi.org/10.3390/nu14091920

Inkanuwat, A., Sukaboon, R., Reamtong, O., Asawanonda, P., Pattaratanakun, A., Saisavoey, T., et al. (2019). Nitric oxide synthesis inhibition and anti-inflammatory

effect of polypeptide isolated from chicken feather meal in lipopolysaccharide-stimulated RAW 264.7 macrophages. *Food Technology and Biotechnology, 57*(2), 200–211. https://doi.org/10.17113/ftb.57.02.19.5964

Izydorczyk, G., Mikula, K., Skrzypczak, D., Witek-Krowiak, A., Mironiuk, M., Furman, K., et al. (2022). Valorization of poultry slaughterhouse waste for fertilizer purposes as an alternative for thermal utilization methods. *Journal of Hazardous Materials, 424*, Article 127328. https://doi.org/10.1016/j.jhazmat.2021.127328

Jeampakdee, P., Puthong, S., Srimongkol, P., Sangtanoo, P., Saisavoey, T., & Karnchanatat, A. (2020). The apoptotic and free radical–scavenging abilities of the protein hydrolysate obtained from chicken feather meal. *Poultry Science, 99*(3), 1693–1704. https://doi.org/10.1016/j.psj.2019.10.050

Ju, Z., Li, M., Xu, J., Howell, D. C., Li, Z., & Chen, F.-E. (2022). Recent development on COX-2 inhibitors as promising anti-inflammatory agents: The past 10 years. *Acta Pharmaceutica Sinica B, 12*(6), 2790–2807. https://doi.org/10.1016/j.apsb.2022.01.002

Kaiafa, G., Kanellos, I., Savopoulos, C., Kakaletsis, N., Giannakoulas, G., & Hatzitolios, A. I. (2015). Is anemia a new cardiovascular risk factor? *International Journal of Cardiology, 186*, 117–124. https://doi.org/10.1016/j.ijcard.2015.03.159

Kanani, F., Heidari, M. D., Gilroyed, B. H., & Pelletier, N. (2020). Waste valorization technology options for the egg and broiler industries: A review and recommendations. *Journal of Cleaner Production, 262*, Article 121129. https://doi.org/10.1016/j.jclepro.2020.121129

Kany, S., Vollrath, J. T., & Relja, B. (2019). Cytokines in inflammatory disease. *International Journal of Molecular Sciences, 20*(23), 6008. https://doi.org/10.3390/ijms20236008

Kelly, L. M., & Alworth, L. C. (2013). Techniques for collecting blood from the domestic chicken. *Lab Animal, 42*(10), 359–361. https://doi.org/10.1038/laban.394

Kęska, P., & Stadnik, J. (2022). Dipeptidyl peptidase IV inhibitory peptides generated in dry-cured pork loin during aging and gastrointestinal digestion. *Nutrients, 14*(4), 770. https://doi.org/10.3390/nu14040770

Kobayashi, Y. (2010). The regulatory role of nitric oxide in proinflammatory cytokine expression during the induction and resolution of inflammation. *Journal of Leukocyte Biology, 88*(6), 1157–1162. https://doi.org/10.1189/jlb.0310149

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. *International Dairy Journal, 16*(9), 945–960. https://doi.org/10.1016/j.idairyj.2005.10.012

Kouguchi, T., Ito, A., Iwai, K., Shimizu, M., Takahata, Y., Suzuki, T., et al. (2012). Chicken collagen hydrolysate-derived peptides inhibit tumor necrosis factor-α-induced inflammatory response in endothelial cells. *Food Science and Technology Research, 18*(5), 667–671. https://doi.org/10.3136/fstr.18.667

Kouguchi, T., Ohmori, T., Shimizu, M., Takahata, Y., Maeyama, Y., Suzuki, T., et al. (2013). Effects of a chicken collagen hydrolysate on the circulation system in subjects with mild hypertension or high-normal blood pressure. *Bioscience, Biotechnology and Biochemistry, 77*(4), 691–696. https://doi.org/10.1271/bbb.120718

Kshetri, P., Roy, S. S., Chanu, S. B., Singh, T. S., Tamreihao, K., Sharma, S. K., et al. (2020). Valorization of chicken feather waste into bioactive keratin hydrolysate by a newly purified keratinase from Bacillus sp. RCM-SSR-102. *Journal of Environmental Management, 273*, Article 111195. https://doi.org/10.1016/j.jenvman.2020.111195. August.

Laca, A., Laca, A., & Diaz, M. (2021). Environmental impact of poultry farming and egg production. In C. M. Galanakis (Ed.), *Environmental impact of agro-food industry and food consumption* (pp. 81–100). Academic Press. https://doi.org/10.1016/B978-0-12-821363-6.00010-2.

Lacroix, I. M. E., & Li-Chan, E. C. Y. (2012). Dipeptidyl peptidase-IV inhibitory activity of dairy protein hydrolysates. *International Dairy Journal, 25*(2), 97–102. https://doi.org/10.1016/j.idairyj.2012.01.003

Lan, V. T. T., Ito, K., Ohno, M., Motoyama, T., Ito, S., & Kawarasaki, Y. (2015). Analyzing a dipeptide library to identify human dipeptidyl peptidase IV inhibitor. *Food Chemistry, 175*, 66–73. https://doi.org/10.1016/j.foodchem.2014.11.131

Larson, E. A., German, D. M., Shatzel, J., & DeLoughery, T. G. (2019). Anticoagulation in the cardiac patient: A concise review. *European Journal of Haematology, 102*(1), 3–19. https://doi.org/10.1111/ejh.13171

Lawson, C., & Wolf, S. (2009). ICAM-1 signaling in endothelial cells. *Pharmacological Reports, 61*(1), 22–32. https://doi.org/10.1016/S1734-1140(09)70004-0

Le Gouic, A. V., Harnedy, P. A., & FitzGerald, R. J. (2018). Bioactive peptides from fish protein by-products. In J. Mérillon, & K. Ramawat (Eds.), *Bioactive molecules in food. Reference series in phytochemistry* (pp. 1–35). Cham: Springer. https://doi.org/10.1007/978-3-319-54528-8_29-1.

Lee, Y. T., Lin, H. Y., Chan, Y. W. F., Li, K. H. C., To, O. T. L., Yan, B. P., et al. (2017). Mouse models of atherosclerosis: A historical perspective and recent advances. *Lipids in Health and Disease, 16*(1), 12. https://doi.org/10.1186/s12944-016-0402-5

Li, Y., Arulnathan, V., Heidari, M. D., & Pelletier, N. (2022). Design considerations for net zero energy buildings for intensive, confined poultry production: A review of current insights, knowledge gaps, and future directions. *Renewable and Sustainable Energy Reviews, 154*, Article 111874. https://doi.org/10.1016/j.rser.2021.111874

Liguori, I., Russo, G., Curcio, F., Bulli, G., Aran, L., Della-Morte, D., et al. (2018). Oxidative stress, aging, and diseases. *Clinical Interventions in Aging, 13*, 757–772. https://doi.org/10.2147/CIA.S158513

Lima, R. D. C. L., Berg, R. S., Rønning, S. B., Afseth, N. K., Knutsen, S. H., Staerk, D., et al. (2019). Peptides from chicken processing by-product inhibit DPP-IV and promote cellular glucose uptake: Potential ingredients for T2D management. *Food & Function, 10*(3), 1619–1628. https://doi.org/10.1039/c8fo02450b

Lin, Y. L., Tai, S. Y., Chen, J. W., Chou, C. H., Fu, S. G., & Chen, Y. C. (2017). Ameliorative effects of pepsin-digested chicken liver hydrolysates on development of alcoholic fatty livers in mice. *Food & Function, 8*(5), 1763–1774. https://doi.org/10.1039/c7fo00123a

Liu, Y., Chen, W., Zheng, F., Yu, H., & Wei, K. (2022). Xanthatin alleviates LPS-induced inflammatory response in RAW264.7 macrophages by inhibiting NF-κB, MAPK and STATs activation. *Molecules, 27*(14), 4603. https://doi.org/10.3390/molecules27144603

Li, C., & Zhou, H.-M. (2011). The role of manganese superoxide dismutase in inflammation defense. *Enzyme Research.* , Article 387176. https://doi.org/10.4061/2011/387176, 2011.

Londoño-Zapata, L., Franco-Cardona, S., Restrepo-Manotas, S., Gomez-Narvaez, F., Suarez-Restrepo, L., Nuñez-Andrade, H., et al. (2022). Valorization of the by-products of poultry industry (bones) by enzymatic hydrolysis and glycation to obtain antioxidants compounds. *Waste and Biomass Valorization, 13*, 4469–4480. https://doi.org/10.1007/s12649-022-01801-1

López-Fernández-Sobrino, R., Torres-Fuentes, C., Bravo, F. I., & Muguerza, B. (2022). Winery by-products as a valuable source for natural antihypertensive agents. *Critical Reviews in Food Science and Nutrition*, 1–14. https://doi.org/10.1080/10408398.2022.2049202

Luo, X., Fei, Y., Xu, Q., Lei, T., Mo, X., Wang, Z., et al. (2020). Isolation and identification of antioxidant peptides from tartary buckwheat albumin (*Fagopyrum tataricum Gaertn* .) and their antioxidant activities. *Journal of Food Science, 85*(3), 611–617. https://doi.org/10.1111/1750-3841.15004

Mahley, R. W., & Rall, S. C. (2000). Apolipoprotein E: Far more than a lipid transport protein. *Annual Review of Genomics and Human Genetics, 1*(1), 507–537. https://doi.org/10.1146/annurev.genom.1.1.507

Martemucci, G., Costagliola, C., Mariano, M., D'andrea, L., Napolitano, P., & D'Alessandro, A. G. (2022). Free radical properties, source and targets, antioxidant consumption and Health. *Oxygen, 2*(2), 48–78. https://doi.org/10.3390/oxygen2020006

Martini, S., Conte, A., & Tagliazucchi, D. (2019). Comparative peptidomic profile and bioactivities of cooked beef, pork, chicken and Turkey meat after in vitro gastro-intestinal digestion. *Journal of Proteomics, 208*, Article 103500. https://doi.org/10.1016/j.jprot.2019.103500. March.

Mas-Capdevila, A., Iglesias-Carres, L., Arola-Arnal, A., Suarez, M., Muguerza, B., & Bravo, F. I. (2019). Long-term administration of protein hydrolysate from chicken feet induces antihypertensive effect and confers vasoprotective pattern in diet-induced hypertensive rats. *Journal of Functional Foods, 55*, 28–35. https://doi.org/10.1016/j.jff.2019.02.006

Mas-Capdevila, A., Pons, Z., Aleixandre, A., Bravo, F. I., & Muguerza, B. (2018). Dose-related antihypertensive properties and the corresponding mechanisms of a chicken foot hydrolysate in hypertensive rats. *Nutrients, 10*(9), 1295. https://doi.org/10.3390/nu10091295

Matinong, A. M. E., Chisti, Y., Pickering, K. L., & Haverkamp, R. G. (2022). Collagen extraction from animal skin. *Biology, 11*(6), 905. https://doi.org/10.3390/biology11060905

Mattagajasingh, I., Kim, C.-S., Naqvi, A., Yamamori, T., Hoffman, T. A., Jung, S.-B., et al. (2007). SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase. *Proceedings of the National Academy of Sciences, 104*(37), 14855–14860. https://doi.org/10.1073/pnas.0704329104

Ma, C., Yu, M., Huang, Z., Wang, J., Zhao, X., Kang, C., et al. (2021). Oral administration of hydrolysates of cartilage extract in the prevention of osteoarthritis. *Journal of Functional Foods, 78*, Article 104376. https://doi.org/10.1016/j.jff.2021.104376

Medina-Vera, I., Gómez-de-Regil, L., Gutiérrez-Solis, A. L., Lugo, R., Guevara-Cruz, M., Pedraza-Chaverri, J., et al. (2021). Dietary strategies by foods with antioxidant effect on nutritional management of dyslipidemias: A systematic review. *Antioxidants, 10*(2), 225. https://doi.org/10.3390/antiox10020225

Medzhitov, R. (2008). *Origin and physiological roles of inflammation. Nature*. Nature Publishing Group. https://doi.org/10.1038/nature07201. July 24.

Mi, X., Li, W., Xu, H., Mu, B., Chang, Y., & Yang, Y. (2020). Transferring feather wastes to ductile keratin filaments towards a sustainable poultry industry. *Waste Management, 115*, 65–73. https://doi.org/10.1016/j.wasman.2020.07.022

Mora, L., Gallego, M., & Toldrá, F. (2018). ACEI-inhibitory peptides naturally generated in meat and meat products and their health relevance. *Nutrients, 10*(9), 1259. https://doi.org/10.3390/nu10091259

Morifuji, M., Koga, J., Kawanaka, K., & Higuchi, M. (2009). Branched-chain amino acid-containing dipeptides, identified from whey protein hydrolysates, stimulate glucose uptake rate in L6 myotubes and isolated skeletal muscles. *Journal of Nutritional Science and Vitaminology, 55*(1), 81–86. https://doi.org/10.3177/jnsv.55.81

Moujalled, D., Strasser, A., & Liddell, J. R. (2021). Molecular mechanisms of cell death in neurological diseases. *Cell Death & Differentiation, 28*(7), 2029–2044. https://doi.org/10.1038/s41418-021-00814-y

Mulvihill, E. E., & Drucker, D. J. (2014). Pharmacology, physiology, and mechanisms of action of dipeptidyl peptidase-4 inhibitors. *Endocrine Reviews, 35*(6), 992–1019. https://doi.org/10.1210/er.2014-1035

Munasinghe, K. A., Schwarz, J. G., & Nyame, A. K. (2014). Chicken collagen from law market value by-products as an alternate source. *Journal of Food Processing*, 1–6. https://doi.org/10.1155/2014/298295, 2014.

Nadalian, M., Kamaruzaman, N., Yusop, M. S. M., Babji, A. S., & Yusop, S. M. (2019). Isolation, purification and characterization of antioxidative bioactive elastin peptides from poultry skin. *Food Science of Animal Resources, 39*(6), 966–979. https://doi.org/10.5851/kosfa.2019.e90

Nagase, S., Takemura, K., Ueda, A., Hirayama, A., Aoyagi, K., Kondoh, M., et al. (1997). A novel nonenzymatic pathway for the generation of nitric oxide by the reaction of hydrogen peroxide and D- or L-arginine. *Biochemical and Biophysical Research Communications, 233*(1), 150–153. https://doi.org/10.1006/bbrc.1997.6428

Narayanaswamy, R., Kanagesan, S., Pandurangan, A., & Padmanabhan, P. (2016). Basics to different imaging techniques, different nanobiomaterials for image enhancement. In *Nanobiomaterials in medical imaging* (pp. 101–129). Elsevier. https://doi.org/10.1016/B978-0-323-41736-5.00004-2.

Nie, X., Xu, D., Zhao, L., & Meng, X. (2017). Antioxidant activities of chicken bone peptide fractions and their Maillard reaction products: Effects of different molecular weight distributions. *International Journal of Food Properties, 20*(1), S457–S466. https://doi.org/10.1080/10942912.2017.1299176

Nikhita, R., & Sachindra, N. M. (2021). Optimization of chemical and enzymatic hydrolysis for production of chicken blood protein hydrolysate rich in angiotensin-I converting enzyme inhibitory and antioxidant activity. *Poultry Science, 100*(5), Article 101047. https://doi.org/10.1016/j.psj.2021.101047

Nongonierma, A. B., & FitzGerald, R. J. (2013). Inhibition of dipeptidyl peptidase IV (DPP-IV) by tryptophan containing dipeptides. *Food & Function, 4*(12), 1843. https://doi.org/10.1039/c3fo60262a

Nongonierma, A. B., & FitzGerald, R. J. (2016). Structure activity relationship modelling of milk protein-derived peptides with dipeptidyl peptidase IV (DPP-IV) inhibitory activity. *Peptides, 79*, 1–7. https://doi.org/10.1016/j.peptides.2016.03.005

Oké, V., Olaniran Odountan, H., Abou, Y., & Odountan, H. O. (2016). Chicken viscera meal as a main component in diet for African catfish Clarias gariepinus (Burchell 1822) reared in earthen ponds. *Journal of Food and Nutrition Research, 4*(12), 799–805. https://doi.org/10.12691/jfnr-4-12-6

Onuh, J. O., Girgih, A. T., Aluko, R. E., & Aliani, M. (2013). Inhibitions of renin and angiotensin converting enzyme activities by enzymatic chicken skin protein hydrolysates. *Food Research International, 53*(1), 260–267. https://doi.org/10.1016/j.foodres.2013.05.010

Onuh, J. O., Girgih, A. T., Aluko, R. E., & Aliani, M. (2014). In vitro antioxidant properties of chicken skin enzymatic protein hydrolysates and membrane fractions. *Food Chemistry, 150*, 366–373. https://doi.org/10.1016/j.foodchem.2013.10.107

Onuh, J. O., Girgih, A. T., Malomo, S. A., Aluko, R. E., & Aliani, M. (2015). Kinetics of in vitro renin and angiotensin converting enzyme inhibition by chicken skin protein hydrolysates and their blood pressure lowering effects in spontaneously hypertensive rats. *Journal of Functional Foods, 14*, 133–143. https://doi.org/10.1016/j.jff.2015.01.031

Onuh, J. O., Girgih, A. T., Nwachukwu, I., Ievari-Shariati, S., Raj, P., Netticadan, T., et al. (2016). A metabolomics approach for investigating urinary and plasma changes in spontaneously hypertensive rats (SHR) fed with chicken skin protein hydrolysates diets. *Journal of Functional Foods, 22*, 20–33. https://doi.org/10.1016/j.jff.2016.01.010

Patel, S., Rauf, A., Khan, H., & Abu-Izneid, T. (2017). Renin-angiotensin-aldosterone (RAAS): The ubiquitous system for homeostasis and pathologies. *Biomedicine & Pharmacotherapy, 94*, 317–325. https://doi.org/10.1016/j.biopha.2017.07.091

Pérez de la Lastra, J. M., Juan, C. A., Plou, F. J., & Pérez-Lebeña, E. (2022). The nitration of proteins, lipids and DNA by peroxynitrite derivatives-chemistry involved and biological relevance. *Stresses, 2*(1), 53–64. https://doi.org/10.3390/stresses2010005

Phaniendra, A., Jestadi, D. B., & Periyasamy, L. (2015). Free radicals: Properties, sources, targets, and their implication in various diseases. *Indian Journal of Clinical Biochemistry, 30*(1), 11–26. https://doi.org/10.1007/s12291-014-0446-0

Polyanskikh, S. V., Danyliv, M. M., Dubrovina, U. R., Ozherelyeva, O. N., & Vasilenko, O. A. (2020). The technology of dehydrated soup base from poultry meat and bone residue. In *IOP conference series: Materials science and engineering* (Vol. 941) IOP Publishing Ltd. https://doi.org/10.1088/1757-899X/941/1/012040.

Potti, R., & Fahad, M. (2017). Extraction and characterization of collagen from broiler chicken feet (Gallus gallus domesticus) - biomolecules from poultry waste. *Journal of Pure and Applied Microbiology, 11*(1), 315–322. https://doi.org/10.22207/JPAM.11.1.39

Power, O., Nongonierma, A. B., Jakeman, P., & FitzGerald, R. J. (2014). Food protein hydrolysates as a source of dipeptidyl peptidase IV inhibitory peptides for the management of type 2 diabetes. *Proceedings of the Nutrition Society, 73*(1), 34–46. https://doi.org/10.1017/S0029665113003601

Pramualkijja, T., Pirak, T., & Euston, S. R. (2021). Valorization of chicken slaughterhouse by-products: Production and properties of chicken trachea hydrolysates using commercial proteases. *International Journal of Food Properties, 24*(1), 1642–1657. https://doi.org/10.1080/10942912.2021.1986522

Pramualkijja, T., Pirak, T., & Euston, S. R. (2022). Functional properties of egg white protein and whey protein in the presence of bioactive chicken trachea hydrolysate and sodium chloride. *Sustainability, 14*(24), Article 16782. https://doi.org/10.3390/su142416782

Pueyo, M. E., & Michel, J.-B. (1997). Angiotensin II receptors in endothelial cells. *General Pharmacology: The Vascular System, 29*(5), 691–696. https://doi.org/10.1016/S0306-3623(97)00021-9

Qazi, B. S., Tang, K., & Qazi, A. (2011). Recent advances in underlying pathologies provide insight into interleukin-8 expression-mediated inflammation and angiogenesis. *International Journal of Inflammation*, 1–13. https://doi.org/10.4061/2011/908468, 2011.

Qin, X., Xu, X., Guo, Y., Shen, Q., Liu, J., Yang, C., et al. (2022). A sustainable and efficient recycling strategy of feather waste into keratin peptides with antimicrobial activity. *Waste Management, 144*(April), 421–430. https://doi.org/10.1016/j.wasman.2022.04.017

Quina, M. J., Soares, M. A. R., Ribeiro, A. A., Marques, A. P., Costa, I. H., & Magalhães, M. C. (2014). Feasibility study on windrow co-composting to recycle industrial eggshell waste. *Waste and Biomass Valorization, 5*(1), 87–95. https://doi.org/10.1007/s12649-013-9211-5

Rezvankhah, A., Yarmand, M. S., Ghanbarzadeh, B., & Mirzaee, H. (2021). Generation of bioactive peptides from lentil protein: Degree of hydrolysis, antioxidant activity, phenol content, ACE-inhibitory activity, molecular weight, sensory, and functional properties. *Journal of Food Measurement and Characterization, 15*(6), 5021–5035. https://doi.org/10.1007/s11694-021-01077-4

Romero-Garay, M. G., Becerra-Verdín, E. M., Soto-Domínguez, A., Montalvo-González, E., & García-Magaña, M. L. (2022). Health effects of peptides obtained from hydrolysed chicken by-products by the action of Bromelia pinguin and B. karatas proteases in Wistar rats induced with metabolic syndrome. *International Food Research Journal, 29*(5), 1078–1088. https://doi.org/10.47836/ifrj.29.5.10

Romero-Garay, Guillermina, M., Martínez-Montaño, E., Hernández-Mendoza, A., Vallejo-Cordoba, B., González-Córdova, A. F., et al. (2020). Bromelia karatas and Bromelia pinguin: Sources of plant proteases used for obtaining antioxidant hydrolysates from chicken and fish by-products. *Applied Biological Chemistry, 63*(1). https://doi.org/10.1186/s13765-020-00525-x

Rosa, C., Rotta, J., Barreto, P., Beirao, L., Severo, C., Rotta, J., et al. (2007). Extraction quantification and molar mass determination of hyaluronic acid extracted from chicken crest. *Alimentos ENuturicao, 18*(3), 237–240.

Rubanyi, G. M., & Vanhoutte, P. M. (1986). Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. *American Journal of Physiology - Heart and Circulatory Physiology, 250*(5), H822–H827. https://doi.org/10.1152/ajpheart.1986.250.5.h822

Safar Razavizadeh, R., Farmani, J., & Motamedzadegan, A. (2022a). Enzyme-assisted extraction of chicken skin protein hydrolysates and fat: Degree of hydrolysis affects the physicochemical and functional properties. *JAOCS, Journal of the American Oil Chemists' Society*. https://doi.org/10.1002/aocs.12591

Safar Razavizadeh, R., Farmani, J., & Motamedzadegan, A. (2022b). Enzyme-assisted extraction of chicken skin protein hydrolysates and fat: Degree of hydrolysis affects the physicochemical and functional properties. *Journal of the American Oil Chemists' Society, 99*(7), 621–632. https://doi.org/10.1002/aocs.12591

Saiga-Egusa, A., Koji, I., Hayakawa, T., Takahata, Y., & Morimatsu, F. (2009). Antihypertensive effects and endothelial progenitor cell activation by intake of chicken collagen hydrolysate in pre- and mild-hypertension. *Bioscience, Biotechnology and Biochemistry, 73*(2), 422–424. https://doi.org/10.1271/bbb.80189

Saiga, A., Iwai, K., Hayakawa, T., Takahata, Y., Kitamura, S., Nishimura, T., et al. (2008). Angiotensin I-converting enzyme-inhibitory peptides obtained from chicken collagen hydrolysate. *Journal of Agricultural and Food Chemistry, 56*(20), 9586–9591. https://doi.org/10.1021/jf072669w

Saiga, A., Okumura, T., Makihara, T., Katsuta, S., Shimizu, T., Yamada, R., et al. (2003). Angiotensin I-converting enzyme inhibitory peptides in a hydrolyzed chicken breast muscle extract. *Journal of Agricultural and Food Chemistry, 51*(6), 1741–1745. https://doi.org/10.1021/jf020604h

Samaranayaka, A. G. P., & Li-Chan, E. C. Y. (2011). Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications. *Journal of Functional Foods, 3*(4), 229–254. https://doi.org/10.1016/j.jff.2011.05.006

Samarasinghe, K. T. G., Munkanatta Godage, D. N. P., VanHecke, G. C., & Ahn, Y.-H. (2014). Metabolic synthesis of clickable glutathione for chemoselective detection of glutathionylation. *Journal of the American Chemical Society, 136*(33), 11566–11569. https://doi.org/10.1021/ja503946q

Sans, M., Panés, J., Ardite, E., Elizalde, J. I., Arce, Y., Elena, M., et al. (1999). VCAM-1 and ICAM-1 mediate leukocyte-endothelial cell adhesion in rat experimental colitis. *Gastroenterology, 116*(4), 874–883. https://doi.org/10.1016/S0016-5085(99)70070-3

Sarbon, N. M., Badii, F., & Howell, N. K. (2018). Purification and characterization of antioxidative peptides derived from chicken skin gelatin hydrolysate. *Food Hydrocolloids, 85*, 311–320. https://doi.org/10.1016/j.foodhyd.2018.06.048. February 2016.

Sbeghen, A. L., Lira, A. L., Fernandes, I. A., Steffens, C., Brião, V. B., Zeni, J., et al. (2022). Use of ultrafiltration in the separation of hydrolysates from mechanically separated chicken meat and evaluation of antioxidant activity. *Journal of Food Process Engineering, 45*(11). https://doi.org/10.1111/jfpe.14151

Seidavi, A. R., Zaker-Esteghamati, H., & Scanes, C. G. (2019). *Present and potential impacts of waste from poultry production on the environment*. World's Poultry Science Journal. Cambridge University Press. https://doi.org/10.1017/S0043933918000922. March 1.

Severo da Rosa, C., Hoelzel, S. C., Viera, V. B., Barreto, P. M., & Beirão, L. H. (2008). Atividade antioxidante do ácido hialurônico extraído da crista de frango. *Ciência Rural, 38*(9), 2593–2698. https://doi.org/10.1590/S0103-84782008005000031

Shaw, J. E., Sicree, R. A., & Zimmet, P. Z. (2010). Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Research and Clinical Practice, 87*(1), 4–14. https://doi.org/10.1016/j.diabres.2009.10.007

Shazly, A. B., He, Z., El-Aziz, M. A., Zeng, M., Zhang, S., Qin, F., et al. (2017). Fractionation and identification of novel antioxidant peptides from buffalo and bovine casein hydrolysates. *Food Chemistry, 232*, 753–762. https://doi.org/10.1016/j.foodchem.2017.04.071

Siddiki, S. Y. A., Uddin, M. N., Mofijur, M., Fattah, I. M. R., Ong, H. C., Lam, S. S., et al. (2021). Theoretical calculation of biogas production and greenhouse gas emission reduction potential of livestock, poultry and slaughterhouse waste in Bangladesh. *Journal of Environmental Chemical Engineering, 9*(3), Article 105204. https://doi.org/10.1016/j.jece.2021.105204

da Silva Bambirra Alves, F. E., Carpiné, D., Teixeira, G. L., Goedert, A. C., de Paula Scheer, A., & Ribani, R. H. (2021). Valorization of an abundant slaughterhouse by-product as a source of highly technofunctional and antioxidant protein hydrolysates. *Waste and Biomass Valorization, 12*(1), 263–279. https://doi.org/10.1007/s12649-020-00985-8

da Silva, V. G., & de Castro, R. J. S. (2020). Enzymatic hydrolysis of proteins from chicken viscera in the presence of an ionic liquid enhanced their antioxidant properties. *Waste and Biomass Valorization, 11*(7), 3183–3193. https://doi.org/10.1007/s12649-019-00693-y

Singh, R. J., Hogg, N., Joseph, J., & Kalyanaraman, B. (1996). Mechanism of nitric oxide release from S-nitrosothiols. *Journal of Biological Chemistry, 271*(31), 18596–18603. https://doi.org/10.1074/jbc.271.31.18596

Sorapukdee, S., & Narunatsopanon, S. (2017). Comparative study on compositions and functional properties of porcine, chicken and duck blood. *Korean Journal for Food Science of Animal Resources, 37*(2), 228–241. https://doi.org/10.5851/kosfa.2017.37.2.228

Springer, T. A. (1995). Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. *Annual Review of Physiology, 57*(1), 827–872. https://doi.org/10.1146/annurev.ph.57.030195.004143

Stiborova, H., Kronusova, O., Kastanek, P., Brazdova, L., Lovecka, P., Jiru, M., et al. (2020). Waste products from the poultry industry: A source of high-value dietary supplements. *Journal of Chemical Technology and Biotechnology, 95*(4), 985–992. https://doi.org/10.1002/jctb.6131

Sudha, P. N., & Rose, M. H. (2014). Beneficial effects of hyaluronic acid. *Advances in Food & Nutrition Research, 72*, 137–176. https://doi.org/10.1016/B978-0-12-800269-8.00009-9

Sun, N., Cui, P., Jin, Z., Wu, H., Wang, Y., & Lin, S. (2017). Contributions of molecular size, charge distribution, and specific amino acids to the iron-binding capacity of sea cucumber (Stichopus japonicus) ovum hydrolysates. *Food Chemistry, 230*, 627–636. https://doi.org/10.1016/j.foodchem.2017.03.077

Sun, Y., Ge, X., Li, X., He, J., Wei, X., Du, J., et al. (2020). High-fat diet promotes renal injury by inducing oxidative stress and mitochondrial dysfunction. *Cell Death & Disease, 11*(10), 914. https://doi.org/10.1038/s41419-020-03122-4

Sun, Y., Pan, D., Guo, Y., & Li, J. (2012). Purification of chicken breast protein hydrolysate and analysis of its antioxidant activity. *Food and Chemical Toxicology, 50*(10), 3397–3404. https://doi.org/10.1016/j.fct.2012.07.047

Tadesse, S. A., & Emire, S. A. (2020). Production and processing of antioxidant bioactive peptides: A driving force for the functional food market. *Heliyon, 6*(8), Article e04765. https://doi.org/10.1016/j.heliyon.2020.e04765

Tao, J., & Wang, J. (2020). Farmers' willingness to accept compensation for livestock and poultry waste resource utilization and its determinants. *Chinese Journal of Population Resources and Environment, 18*(2), 144–154. https://doi.org/10.1016/j.cjpre.2021.04.019

Taskin, M., & Kurbanoglu, E. B. (2011). Evaluation of waste chicken feathers as peptone source for bacterial growth. *Journal of Applied Microbiology, 111*(4), 826–834. https://doi.org/10.1111/j.1365-2672.2011.05103.x

Tomiyama, H., Ohkuma, T., Ninomiya, T., Nakano, H., Matsumoto, C., Avolio, A., et al. (2019). Brachial-ankle pulse wave velocity versus its stiffness index β-transformed value as risk marker for cardiovascular disease. *Journal of the American Heart Association, 8*(24), Article e013004. https://doi.org/10.1161/JAHA.119.013004

Touyz, R. M., Chen, X., Tabet, F., Yao, G., He, G., Quinn, M. T., et al. (2002). Expression of a functionally active gp91phox-containing neutrophil-type NAD(P)H oxidase in smooth muscle cells from human resistance arteries. *Circulation Research, 90*(11), 1205–1213. https://doi.org/10.1161/01.RES.0000020404.01971.2F

Tulipano, G., Faggi, L., Nardone, A., Cocchi, D., & Caroli, A. M. (2015). Characterisation of the potential of β-lactoglobulin and α-lactalbumin as sources of bioactive peptides affecting incretin function: In silico and in vitro comparative studies. *International Dairy Journal, 48*, 66–72. https://doi.org/10.1016/j.idairyj.2015.01.008

Udenigwe, C. C., Girgih, A. T., Mohan, A., Gong, M., Malomo, S. A., & Aluko, R. E. (2017). Antihypertensive and bovine plasma oxidation-inhibitory activities of spent hen meat protein hydrolysates. *Journal of Food Biochemistry, 41*(4), Article e12378. https://doi.org/10.1111/jfbc.12378

Wan Omar, W. H., & Sarbon, N. M. (2016). Effect of drying method on functional properties and antioxidant activities of chicken skin gelatin hydrolysate. *Journal of Food Science and Technology, 53*(11), 3928–3938. https://doi.org/10.1007/s13197-016-2379-5

Wan, M. Y., Dong, G., Yang, B. Q., & Feng, H. (2016). Identification and characterization of a novel antioxidant peptide from feather keratin hydrolysate. *Biotechnology Letters, 38*(4), 643–649. https://doi.org/10.1007/s10529-015-2016-9

Wang, Y., Branicky, R., Noë, A., & Hekimi, S. (2018). Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling. *Journal of Cell Biology, 217*(6), 1915–1928. https://doi.org/10.1083/jcb.201708007

Wang, T., Wang, J., Hu, X., Huang, X., & Chen, G.-X. (2020). Current understanding of glucose transporter 4 expression and functional mechanisms. *World Journal of Biological Chemistry, 11*(3), 76–98. https://doi.org/10.4331/wjbc.v11.i3.76

Wołonciej, M., Milewska, E., & Roszkowska-Jakimiec, W. (2016). Trace elements as an activator of antioxidant enzymes. *Postępy Higieny I Medycyny Doświadczalnej, 70*, 1483–1498. https://doi.org/10.5604/17322693.1229074

Wongngam, W., Mitani, T., Katayama, S., Nakamura, S., & Yongsawatdigul, J. (2020). Production and characterization of chicken blood hydrolysate with antihypertensive properties. *Poultry Science, 99*(10), 5163–5174. https://doi.org/10.1016/j.psj.2020.07.006

World Health Organization (WHO). Anaemia. Retrieved September 10, 2022, from https://www.who.int/health-topics/anaemia#tab=tab_1.

Wu, Z., Korntner, S., Mullen, A., & Zeugolis, D. (2021). Collagen type II: From biosynthesis to advanced biomaterials for cartilage engineering. *Biomaterials and Biosystems, 4*, Article 100030. https://doi.org/10.1016/j.bbiosy.2021.100030

Wu, P. T., Lau, Y. Q., Dai, F. J., Lin, J. T., Kao, H. Y., & Chau, C. F. (2020). Ability of chicken protein hydrolysate to lower serum cholesterol through its bile acid binding activity. *CYTA - Journal of Food, 18*(1), 493–499. https://doi.org/10.1080/19476337.2020.1779352

Wu, Y. H. S., Lin, Y. L., Huang, C., Chiu, C. H., Nakthong, S., & Chen, Y. C. (2020). Cardiac protection of functional chicken-liver hydrolysates on the high-fat diet induced cardio-renal damages via sustaining autophagy homeostasis. *Journal of the Science of Food and Agriculture, 100*(6), 2443–2452. https://doi.org/10.1002/jsfa.10261

Wu, Y. H. S., Lin, Y. L., Yang, W. Y., Wang, S. Y., & Chen, Y. C. (2021). Pepsin-digested chicken-liver hydrolysate attenuates hepatosteatosis by relieving hepatic and peripheral insulin resistance in long-term high-fat dietary habit. *Journal of Food and Drug Analysis, 29*(2), 375–388. https://doi.org/10.38212/2224-6614.3351

Wu, D., Zhang, Y., Li, J., Fan, Z., Xu, Q., & Wang, L. (2022). Assessment of chicken intestinal hydrolysates as a new protein source to replace fishmeal on the growth performance, antioxidant capacity and intestinal health of common carp (Cyprinus carpio). *Fish & Shellfish Immunology, 125*, 161–170. https://doi.org/10.1016/j.fsi.2022.05.011

Xiao, C., Zhou, F., Zhao, M., Su, G., & Sun, B. (2018). Chicken breast muscle hydrolysates ameliorate acute alcohol-induced liver injury in mice through alcohol dehydrogenase (ADH) activation and oxidative stress reduction. *Food & Function, 9*(2), 774–784. https://doi.org/10.1039/c7fo01387f

Xiao, C., Zhou, F., Zheng, L., Cai, Y., Su, G., Luo, D., et al. (2020). Chicken breast-derived alcohol dehydrogenase-activating peptides in response to physicochemical changes and digestion simulation: The vital role of hydrophobicity. *Food Research International, 136*, Article 109592. https://doi.org/10.1016/j.foodres.2020.109592

Xiong, G. Y., Chen, X., Zhang, X. X., Miao, Y., Zou, Y., Wang, D. Y., et al. (2020). Process optimization and the relationship between the reaction degree and the antioxidant activity of Maillard reaction products of chicken liver protein hydrolysates. *Poultry Science, 99*(7), 3733–3741. https://doi.org/10.1016/j.psj.2020.03.027

Xu, D.-P., Li, Y., Meng, X., Zhou, T., Zhou, Y., Zheng, J., et al. (2017). Natural antioxidants in doods and medicinal plants: Extraction, assessment and resources. *International Journal of Molecular Sciences, 18*(1), 96. https://doi.org/10.3390/ijms18010096

Yanai, H., Tomono, Y., Ito, K., Furutani, N., Yoshida, H., & Tada, N. (2008). The underlying mechanisms for development of hypertension in the metabolic syndrome. *Nutrition Journal, 7*(1), 10. https://doi.org/10.1186/1475-2891-7-10

Yang, K. T., Lin, C., Liu, C. W., & Chen, Y. C. (2014). Effects of chicken-liver hydrolysates on lipid metabolism in a high-fat diet. *Food Chemistry, 160*, 148–156. https://doi.org/10.1016/j.foodchem.2014.03.052

Yang, J., Sun-Waterhouse, D., Xiao, Y., He, W., Zhao, M., & Su, G. (2019). Osteoarthritis-alleviating effects in papain-induced model rats of chicken cartilage hydrolysate and its peptide fractions. *International Journal of Food Science and Technology, 54*(9), 2711–2717. https://doi.org/10.1111/ijfs.14182

Yeh, W. Y., Lin, Y. L., Yang, W. Y., Chou, C. H., Wu, Y. H. S., & Chen, Y. C. (2022). Functional chicken-liver hydrolysates ameliorate insulin resistance and cognitive decline in streptozotocin-induced diabetic mice. *Poultry Science, 101*(6), Article 101887. https://doi.org/10.1016/j.psj.2022.101887

Yu, W., Field, C. J., & Wu, J. (2018). A spent hen muscle protein hydrolysate: A potential IL-10 stimulator in a murine model. *Food & Function, 9*(9), 4714–4719. https://doi.org/10.1039/c8fo00589c

Yuliatmo, R., Fitriyanto, N. A., Bachruddin, Z., & Erwanto, Y. (2017). Increasing of angiotensin converting enzyme inhibitory derived from Indonesian native chicken leg protein using Bacillus cereus protease enzyme. *International Food Research Journal, 24*, 1799–1804.

Zarzuelo, M. J., López-Sepúlveda, R., Sánchez, M., Romero, M., Gómez-Guzmán, M., Ungvary, Z., et al. (2013). SIRT1 inhibits NADPH oxidase activation and protects endothelial function in the rat aorta: Implications for vascular aging. *Biochemical Pharmacology, 85*(9), 1288–1296. https://doi.org/10.1016/j.bcp.2013.02.015

Zhang, Y., Kouguchi, T., Shimizu, M., Ohmori, T., Takahata, Y., & Morimatsu, F. (2010). Chicken collagen hydrolysate protects rats from hypertension and cardiovascular damage. *Journal of Medicinal Food, 13*(2), 399–405. https://doi.org/10.1089/jmf.2009.1246

Zhang, Y., Kouguchi, T., Shimizu, K., Sato, M., Takahata, Y., & Morimatsu, F. (2010). Chicken collagen hydrolysate reduces proinflammatory cytokine production in C57BL/6.KOR-ApoEsh1 mice. *Journal of Nutritional Science and Vitaminology, 56*(3), 208–210. https://doi.org/10.3177/jnsv.56.208

Zhang, M., Malik, A. B., & Rehman, J. (2014). Endothelial progenitor cells and vascular repair. *Current Opinion in Hematology, 21*(3), 224–228. https://doi.org/10.1097/MOH.0000000000000041

Zhao, W.-H., Luo, Q.-B., Pan, X., Chi, C.-F., Sun, K.-L., & Wang, B. (2018). Preparation, identification, and activity evaluation of ten antioxidant peptides from protein hydrolysate of swim bladders of miiuy croaker (Miichthys miiuy). *Journal of Functional Foods, 47*, 503–511. https://doi.org/10.1016/j.jff.2018.06.014

Zheng, Z., Si, D., Ahmad, B., Li, Z., & Zhang, R. (2018). A novel antioxidative peptide derived from chicken blood corpuscle hydrolysate. *Food Research International, 106*(2), 410–419. https://doi.org/10.1016/j.foodres.2017.12.078

Zhong, Y., & Shahidi, F. (2015). Methods for the assessment of antioxidant activity in foods. In C. Galanakis (Ed.), *Handbook of antioxidants for food preservation* (pp. 287–333). London: Academic Press; Elsevier. https://doi.org/10.1016/B978-1-78242-089-7.00012-9.

Zinina, O., Merenkova, S., Rebezov, M., Galimov, D., Khayrullin, M., & Burkov, P. (2022). Physicochemical, functional, and technological properties of protein hydrolysates obtained by microbial fermentation of broiler chicken gizzards. *Fermentation, 8*(7). https://doi.org/10.3390/fermentation8070317

Zoidis, E., Seremelis, I., Kontopoulos, N., & Danezis, G. (2018). Selenium-dependent antioxidant enzymes: Actions and properties of selenoproteins. *Antioxidants, 7*(5), 66. https://doi.org/10.3390/antiox7050066

Manuscript 4

Objective: To obtain a hydrolysate from chicken feet for the prevention of Alzheimer's disease.

A POP-inhibitory chicken-foot hydrolysate delayed amyloid-β-induced paralysis in *Caenorhabditis elegans* and identification of its peptides

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Abstract

There is increasing interest in the use of bioactive peptides, especially those obtained from agri-food by-products, as a preventive tool for neurodegenerative diseases. Inhibition of prolyl oligopeptidase (POP) enzyme has been suggested as a potential target for anti-neurodegenerative drugs. In this work, twelve hydrolysates obtained from chicken feet with variable POP inhibitory (POPi) activity were obtained under different hydrolysis conditions. One of the prepared hydrolysates (named CFH3) showing remarkable POP inhibition was assayed against ß-amyloid (Aβ)-induced paralysis in the CL4176 strain of *C. elegans*. Treatment with that hydrolysate significantly decreased the percentage of paralyzed worms in a concentration-dependent manner (0.5 - 5 mg/mL). This hydrolysate was further fractionated by RP-HPLC, and the peptides from the fraction with the highest POPi activity were identified by UHPLC-Orbitrap MS/MS. Twenty-three amino acid sequences were selected based on ion abundance and Mascot Ion Score. Nine of them had a PeptideRanker score ≥ 0.5, indicating a good probability of showing some bioactivity. Moreover, eight of these novel nine peptides contained proline in their structure, associated with good POP inhibitory activity.

Keywords: Chicken by-product hydrolysate, neuroprotection, prolyl oligopeptidase inhibitory activity, ß-amyloid toxicity protection, *Caenorhabditis elegans*, CL4176 strain.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder affecting the central nervous system. It is the most prevalent form of dementia globally, affecting 40 million individuals worldwide from approximately 55 million people suffering from dementia diseases [1]. AD manifests with various clinical symptoms such as memory loss, cognitive impairment, or behavioral dysfunction [2]. Moreover, several factors have been identified to contribute to the progression of AD, including the toxicity of amyloid-beta (Aβ) peptide, the main component of extracellular amyloid plaques deposited in the brain, intracellular accumulation of neurofibrillary tangles of hyperphosphorylated tau protein, cholinergic, serotonergic, and noradrenergic loss function, oxidative stress, or synaptic dysfunction [3]. Neuroinflammation also plays a key role in AD and other neurodegenerative disorders [4]. The inflammatory response occurs when there is a failure in the clearance of Aβ aggregates, promoting the progression of neurodegenerative diseases [5]. During neuroinflammation, different cells in the central nervous system, such as microglia and astrocytes, release different pro-inflammatory cytokines and inflammatory mediators into the extracellular matrix [6]. Microglial cells also secrete cytosolic enzymes such as prolyl oligopeptidase (POP) into the extracellular space [7]. POP is a conserved serine protease distributed throughout the body that is highly expressed in the brain [8]. It has both enzymatic and non-enzymatic activities and plays multiple biological roles. For instance, it is involved in the development and breakdown of several neuropeptides and hormones (thyrotropin-releasing hormone, substance P, vasopressin, thymosin β4, angiotensin I, and angiotensin II), which contain Pro in their amino acid sequence [9,10]. Some of these are regulators of cognitive functions. This serine protease can also hydrolyze proteins such as collagen or thymosin β4 to release anti-inflammatory peptides. In addition, the action of this enzyme seems to be linked to the inflammatory process, since the inflammatory response disappears or is reduced when it is not present or active [11,12]. Moreover, the POP enzyme has been co-localized with Aβ aggregates in the post-mortem brains of patients with AD, suggesting a role in the generation of neurotoxic peptides [13]. Thus, POP is considered a biological target for the exploration of multiple therapies aimed at combating neurodegeneration through its inhibition [8].

Currently, there is a lack of effective treatments to reverse AD dementia, so that prevention appears as the main strategy [14]. *The Lancet* Commission on Dementia Prevention estimated that 40% of the global risk of developing dementia is associated

with 12 modifiable risk factors, making it potentially preventable [15]. Improvements in lifestyle, such as stress management, sleep, diet, and regular exercise, could delay or prevent the development of neurodegenerative diseases [14–17]. Ensuring the consumption of a healthy nutrient-rich diet is vital for optimal brain function and well-being. Furthermore, some dietary compounds can also act as neuroprotective and cognitive-enhancing agents [16,17]. One of these dietary compounds are the bioactive peptides, which are small amino acid sequences that exhibit beneficial effects in organisms when liberated from the native protein [3]. In addition to their well-known anti-inflammatory and antioxidant properties, *in vitro* studies have shown that dietary peptides can inhibit several enzymes involved in neurodegenerative diseases, including acetylcholinesterase (which degrades the neurotransmitter acetylcholine), beta-secretase 1 (initiates Aβ production), and POP (see reviews [3,9,18]).

POP peptide inhibitors have been obtained from milk, plant-based (corn, soybean, rice, wine, and cocoa), meat, and fish proteins [9]. Some of these sources are animal agri-food by-products[19,20], making them interesting for valorization through the preparation of functional ingredients that can contribute to the circular economy of the sector [21]. Notably, most of these reported POP-inhibiting peptides contained Pro in their amino acid sequence [9], and some of them also demonstrated neuroprotective effects *in vivo* in murine or *Caenorhabditis elegans* models of AD [22–24], as well as in AD patients [25]. Interestingly, some of these peptides also showed good potential to inhibit proteases involved in the development of hypertension or type 2 Diabetes Mellitus, such as angiotensin-converting enzyme (ACE) and dipeptidyl peptidase-IV (DPP-IV) [19,20,26]. Recently, we demonstrated that chicken feet, an agri-food by-product, can be a good source of *in vitro* ACE and DPP-IV inhibitory peptides when subjected to specific enzymatic hydrolysis conditions [27]. However, the potential of chicken feet as a source of POP inhibitors and neuroprotective peptides has not been explored yet. Therefore, the present study aimed to obtain a protein hydrolysate from chicken feet, selected based on its capacity to inhibit POP, able to delay the Aβ-induced paralysis in a *C. elegans* model of AD. In addition, the peptides contained in the hydrolysate were also identified.

2. Materials and Methods

2.1. Reagents and solvents

Food-grade enzymes E3, E4, and E5 were provided by Novozymes (Lyngby, Denmark). Fluorogenic prolyl oligopeptidase assay kit (ref. 80106) was purchased from BPS Bioscience (San Diego, CA, USA). 2,4,6-Trinitrobenzenesulfonic acid, ampicillin, nystatin, and other agar supplementation reagents were purchased from Sigma-Aldrich (Madrid, Spain). The BCA™ protein assay kit was purchased from Thermo Fisher Scientific (Barcelona, Spain). Amicon® Ultra 15 and Oasis HLB SPE columns were provided by Merck Millipore (Madrid, Spain) and Waters (Milford, MA, USA), respectively. Petri plates Ø 35 and 60 mm were purchased from Brand GMBH (Wertheim, Germany). The solvents used for HPLC were HPLC grade. The remaining solvents and reagents were of analytical grade.

2.2. Elaboration of hydrolysates

Chicken feet (*Gallus gallus*) were obtained from a local chicken slaughterhouse (Granja de Gaià, La Riera de Gaià, Spain). The feet were cleaned and ground using a meat grinder (Moulinex, Barcelona, Spain). After the addition of Milli-Q water (0.25 g/mL, w/v), the solution was boiled at a specific temperature and time. Subsequently, the non-soluble particles were removed by centrifugation at 1,250 x *g* for 5 min. Hydrolysis of chicken feet broth was performed using 3 commercial enzymes (E3, E4, and E5) at two hydrolysis times (t1<t3) and two different enzyme/substrate ratios (E/S; C1<C2). Enzymes were inactivated by heating at 90 ºC for 15 min. Hydrolysates were labeled from CFH1 to CFH12. The specific hydrolysis conditions for each hydrolysate are listed in Table 1.

2.3. Determination of hydrolysis degree

The degree of hydrolysis of the hydrolysates was determined by quantifying the free α-amino groups using the TNBS method [33]. Leucine was used to generate a calibration curve (0-1.5 mM). The samples and leucine concentrations were reconstituted and diluted with 1% sodium dodecyl sulfate. 100% of hydrolysis was achieved by hydrolyzing the dried chicken foot broth with 6N HCl at 110 °C for 24 h. Protein content in the samples was determined by the bicinchoninic acid method using the BCA™ protein assay kit, following the manufacturer's instructions (Thermo Fisher Scientific).

Table 1. Hydrolysis conditions for the preparation of each chicken foot broth hydrolysate.

Hydrolysate	Hydrolysis conditions		
	Enzyme	Enzyme/substrate ratio*	Time
CFH1	E3	C1	t1
CFH2	E3	C1	t3
CFH3	E3	C2	t1
CFH4	E3	C2	t3
CFH5	E4	C1	t1
CFH6	E4	C1	t3
CFH7	E4	C2	t1
CFH8	E4	C2	t3
CFH9	E5	C1	t1
CFH10	E5	C1	t3
CFH11	E5	C2	t1
CFH12	E5	C2	t3

2.4. Determination of POP inhibitory activity

The POP inhibitory activity of samples was determined using the fluorogenic prolyl oligopeptidase assay kit following the manufacturer's instructions (BPS Bioscience). Samples were assayed at a final concentration of 0.2 mg of product/mL (w/v) and 10 µg of protein/mL (w/v) for hydrolysates (reconstituted in assay buffer) and RP-HPLC fractions (reconstituted in Milli-Q water), respectively. The BCA™ protein assay kit was used to quantify the protein content according to the manufacturer's instructions (Thermo Fisher Scientific). Fluorescence was measured at λex=360 nm and λem=460 nm. The percentage of inhibition was calculated as follows:

$$\text{POP } inhibition\ (\%) = \frac{(F_C - F_B) - (F_S - F_{BS})}{F_C - F_B} * 100$$

where F_C is the fluorescence produced by enzyme action in the absence of sample (100% activity), F_B is the fluorescence of the reagents in the absence of enzyme and sample (0% activity), F_S is the fluorescence produced by the enzyme action in the presence of the sample, and F_{BS} is the fluorescence of the reagents and sample in the absence of enzyme. Data were expressed as mean (n=3) ± standard deviation. The IC_{50}

was determined for hydrolysates CFH3 and CFH5 and fraction F15 and expressed as µg of protein (BCA assay kit, Thermo Fisher Scientific)/mL (w/v).

2.5. Experiments with Caenorhabditis elegans CL4176 strain

2.5.1. Worm maintenance

The *C. elegans* mutant strain CL4176, dvIs27[pAF29(myo-3/A-Beta 1-42/let UTR) + pRF4(rol-6(su1006))] and the *E. coli* OP50 strain were obtained from the Caenorhabditis Genetics Centre at the University of Minnesota (Minneapolis, MN, USA). Worms were thawed and propagated at 16 ºC in nematode growth medium (NGM) agar plates supplemented with 0.1% $CaCl_2$ 1 M, 0.1% cholesterol (dissolved in ethanol), 0.1% $MgSO_4$ 1 M, 2.5% phosphate buffer 1 M pH 6, 0.1% sodium ampicillin, and 0.18% Nystatin (dissolved in ethanol), and seeded *E. coli* OP50 as a food source.

2.5.2. Paralysis assay

The worm paralysis assay was carried out according to Ayuda-Durán et al. [28]. Gravid adult worms (6-7 individuals) were transferred to fresh NGM agar plates containing *E. coli* OP50 and either the absence of samples (control group) or different concentrations of the hydrolysates CFH3 or CFH5 (0.5, 1, and 5 mg protein mL (w/v)), or the unhydrolyzed chicken foot broth (5 mg protein/mL, w/v). The protein content of the hydrolysates and broth was estimated by the Kjeldahl method, using 6.25 as the protein conversion factor [29]. Nematodes were removed after 24 h at 16 ºC and eggs were left to hatch in the plates. Once the larvae reached the adult stage (48 h after egg release), plates containing nematodes were transferred from 16 to 25 ºC to induce $A\beta_{1-42}$ expression for 24 h (n=100-150 per group). Subsequently, the number of non-paralysed worms in the distinct growth plates was scored under the microscope at 2-hour intervals up to 12 h. Paralysis was considered if worms did not move their bodies or only bent their head upon contact with stimuli using a platinum wire. The percentage of mobile (non-paralyzed) worms was calculated in three independent experiments for each treatment (mean ± standard deviation) and represented over time. The area under the curve (AUC) was also calculated and normalized to that of the control group.

2.6. CFH3 characterization

The moisture content was determined by gravimetry according to the official AOAC method [30]. Total protein content was determined by the Kjeldahl method

using 6.25 as the protein conversion factor [29]. Data are expressed as mean ± standard deviation (percentage, w/w). All analyses were performed in duplicate. Individual amino acid and Hyp contents were determined in an official certificate laboratory (AGROLAB LUFA GmbH, Kiel, Germany), following the official methods BOE-A-1991-18408 and LFBG §64 06.00-8, respectively [31,32]. Collagen content was calculated by multiplying the Hyp content by a conversion factor of 8. Data were expressed as a percentage (w/w) mean ± standard deviation.

2.7. Peptide identification in CFH3 hydrolysate

2.7.1. RP-HPLC fractioning of the hydrolysate

The hydrolysate was passed through a 3 kDa cut-off membrane using Amicon® Ultra 15 centrifugal filter units to obtain the <3 kDa peptide fraction. The freeze-dried <3 kDa fraction, reconstituted in Milli-Q water (10 mg/mL, w/v)), was subjected to further peptide separation by semi-preparative reversed-phase high-performance liquid chromatography (RP-HPLC), using the conditions and equipment described by Bravo et al. [33], with the following changes: i) elution gradient: 0 to 23.5% B in 59.2 min, 23.5-90% B in 5.8 min, and 90-0% B in 2 min, and ii) injection volume: 750 µL. Different peptide fractions were collected during chromatographic separation, lyophilized, and stored at -20 ºC.

2.7.2. UHPLC-Orbitrap MS/MS analysis

The selected RP-HPLC fraction reconstituted in Milli-Q water containing 0.1% formic acid was desalted and concentrated using an Oasis HLB SPE column and a SpeedVac concentrator (Thermo Fisher Scientific, CA, USA), respectively. The peptides were separated on a Vanquish Flex VF-P20-A (Thermo Fisher Scientific) with an Acquity Premier CSH C18 column (150 mm x 2.1 mm i.d., 1.7 µm particle size; Waters). The separation was performed with a 100 min gradient using Milli-Q water containing 0.1% formic acid (phase A), and acetonitrile containing 0.1% formic acid (phase B) at a flow rate of 0.25 mL/min. Mass spectrometry analysis was performed on an Orbitrap Eclipse (Thermo Fisher Scientific) using an enhanced FT-resolution MS spectrum (R=60,000 FHMW) followed by a data-dependent FT-MS/MS acquisition (R=15,000 FHMW, 30% normalized collision energy HCD) from the ten most intense parent ions with a charge state acquisition from one to seven and dynamic exclusion of 0.7 min.

Protein identification/quantification was performed using Proteome Discoverer v.2.5 (Thermo Fisher Scientific) and the Mascot search engine (v2.8, Matrix Science). For protein identification, the workflow was set up using the Mascot node with the proteome of *Gallus gallus*, assuming non-enzymatic digestion. The fragment ion mass tolerance assumed an error of 20 milli mass unit (mmu) for the FT-MS/MS fragmentation mass and 10 ppm for the FT-MS precursor ion mass. Oxidation of methionine and acetylation of the N-terminus were set as the dynamic modifications. The false discovery rate (FDR) for peptide identification was set to a maximum of 1%. Peptide quantification data was retrieved from the 'Precursor ions quantifier' node from Proteome Discoverer using the area of unique and razor peptides. Only those MS/MS with an average of 10 signal-to-noise were used, and the total peptide amount was used as normalization mode. Visual verification of the fragmentation spectra was performed, and only peptides found in both replicates were considered.

2.8. Bioactivity prediction

Identified amino acid sequences were checked in the BIOPEP-UWM database (https://biochemia.uwm.edu.pl/biopep/start_biopep.php, accessed on 20/03/2024) for previously identified bioactivities [34], and the potential bioactivity was evaluated with the software PeptideRanker (http://distilldeep.ucd.ie/PeptideRanker/, accessed on 20/03/2024) [35].

2.9. Statistical analyses

Outlier values were identified using Grubbs' test and were removed before statistical analysis. Shapiro–Wilk's and Levene's tests were used to evaluate data normality and variance homogeneity, respectively. Statistical analyses and graphics were performed using Prism v.9 software (GraphPad Software, San Diego, CA, USA). Statistical significance was considered at $p < 0.05$. The Student's t-test was used to compare between two groups. One-way ANOVA and Bonferroni post-hoc test were used to evaluate significant differences between more than two groups in the AUC. Two-way ANOVA (Bonferroni post-hoc test) was used to explore significant differences in worm mobility over time between groups.

3. Results

3.1. Hydrolysis degree and POPi activity of the hydrolysates

A total of 12 hydrolysates were obtained from the chicken foot broth by the addition of three enzymes at two different enzyme/substrate ratios and using two different hydrolysis times. The degree of hydrolysis (DH) of the 12 hydrolysates ranged between 8.4 and 20.3% (Table 2). It was found that for hydrolysis at the same E/S ratio, the longer the time the higher the degree of hydrolysis ($p < 0.05$, two-way ANOVA). In the same line, for the same hydrolysis time, higher E/S produced more hydrolyzed samples ($p < 0.05$, two-way ANOVA). POPi bioactivity of the hydrolysates at 0.2 mg/mL ranged between 0 and 76% (Table 2).

Table 2. Degree of hydrolysis (DH) and *in vitro* prolyl oligopeptidase inhibitory (POPi) activity of the 12 hydrolysates obtained from chicken foot broth.

Hydrolysates	**DH (%)**	**POPi activity**		
		%*	**IC_{50} (µg prot/mL)**	**IC_{50} (µg prod/mL)**
CFH1	12.4 ± 0.1	60.6 ± 3.6[a]	n.d	n.d
CFH2	13.8 ± 0.2	7.6 ± 1.0[a]	n.d	n.d
CFH3	15.7 ± 0.1	73.9 ± 7.4[b]	57.9 ± 0.9	73.1± 0.9
CFH4	20.3 ± 0.3	52.6 ± 0.4[a]	n.d	n.d
CFH5	11.5 ± 0.8	76.4 ± 4.3[b]	40.1 ± 0.2	46.3 ± 0.3
CFH6	12.6 ± 0.3	47.7 ± 4.1[a]	n.d	n.d
CFH7	13.5 ± 0.0	59.2 ± 1.5[a]	n.d	n.d
CFH8	16.0 ± 1.5	n.e	n.d	n.d
CFH9	8.4 ± 0.4	55.3 ± 1.3[a]	n.d	n.d
CFH10	12.1 ± 0.2	38.4 ± 0.6[a]	n.d	n.d
CFH11	13.1 ± 0.1	51.0 ± 5.0[a]	n.d	n.d
CFH12	17.8 ± 0.1	51.5 ± 4.9[a]	n.d	n.d

Data are shown as mean (n=3) ± standard deviation * POPi activity was assayed at 0.2 mg/mL and expressed as mean ± standard deviation. Different letters indicate differences between the POPi activity of the hydrolysates ($p < 0.05$, one-way ANOVA; post-hoc: Bonferroni test). Abbreviations: n.e (non-effect), n.d (non-determined).

The hydrolysates with the highest bioactivity were CFH3 and CFH5, with 73.9% ± 7.9 and 76.4% ± 4.3 of POP inhibition, respectively. The IC_{50} values were 57.9 and 40.1 µg protein/mL for CFH3 and CFH5, respectively. Only 4 hydrolysates (CFH2, CFH6,

CFH8, and CFH10) had less than 50% inhibition, and only CFH8 did not exhibit any effect. Two-way ANOVA showed that extending the duration of hydrolysis resulted in hydrolysates with lower POPi activity, except for E5 at the highest E/S ratio. The effect of the E/S ratio was enzyme-dependent when the same hydrolysis time was used. Specifically, increasing the E/S ratio for E3 (CFH1 - CFH4) and E5 (only at the highest hydrolysis time; CFH10 and CFH12) produced hydrolysates with greater POPi activity (i.e. from 61 to 74% for CFH1 and CFH3, from 8 to 53% for CFH2 and CFH4, and from 38 to 52% for CFH10 and CFH12, respectively). On the other hand, an increase in E/S for E4 (CFH5 - CFH8) produced less bioactive hydrolysates (i.e., from 76 to 59% for CFH5 and CFH7, respectively) (Table 2).

3.2. Assays in CL4176 C. elegans strain.

The neuroprotective effects of the two hydrolysates with the highest POPi activities (CFH3 and CFH5) and the unhydrolyzed chicken foot broth were assessed using an Aβ-induced paralysis model in the CL4176 *C. elegans* strain. In all the assayed groups, the first paralyzed worms were observed at 26 h after Aβ temperature induction. The control group (non-treated worms) and worms treated with the unhydrolyzed broth (5 mg protein/mL) showed similar behavior over time, achieving 90.2% and 90.1% of paralyzed worms 36 h after Aβ induction, respectively (Figure 1A). No significant differences were observed when the area under the curve (AUC) was calculated for both groups (Figure 1B; $p = 0.19$).

Treatment with hydrolysate CFH3 at all tested doses significantly delayed worm paralysis caused by Aβ aggregation compared to the non-treated group ($p < 0.05$; two-way ANOVA; post-hoc: Bonferroni test) (Figure 2).

The greatest effect was observed at 30 h after paralysis induction, reaching 12.3, 28.2, and 32.6% of less paralyzed worms at 0.5, 1, and 5 mg of protein/mL of CFH3, respectively, compared to that of the control group (Figure 2A). At the end of the experiment (36 h), the two highest doses of CFH3 decreased the content in paralyzed worms by 20% compared with the control group. A dose-dependent effect for CFH3 was observed in the AUC ($p < 0.05$; one-way ANOVA; posthoc: Bonferroni test) (Figure 2B).

The treatment with CFH5 at doses of 0.5 and 1 mg of protein/mL was not able to delay paralysis in the worms (Figure 3A, B). The behavior observed in the group submitted to the concentration of 5 mg protein/mL was significantly different from that

of the control group at 28 h after Aβ induction (18.2% less paralyzed worms) (Figure 3C). However, the percentage of paralyzed worms at the end of the experiment in this group was similar to that in the control group. No significant differences were observed in the analysis of the AUC at the three doses of CFH5, compared to the control group (Figure 3D).

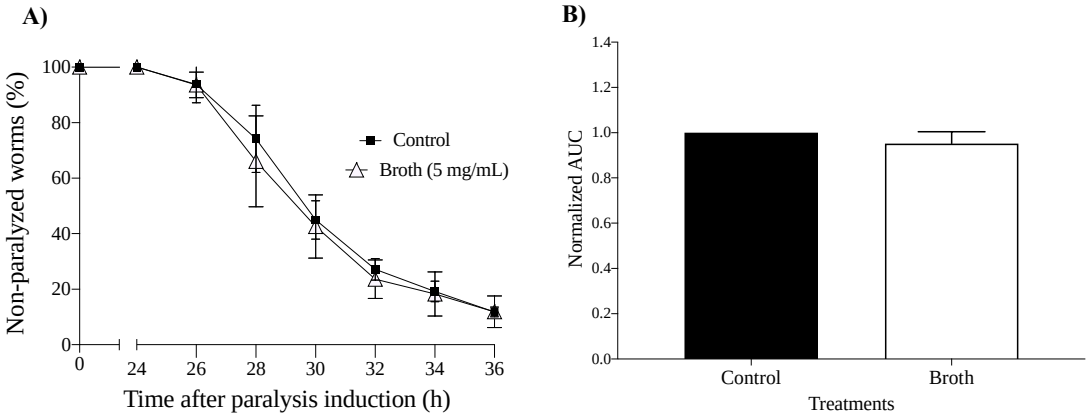

Figure 1. Effect of unhydrolyzed chicken foot broth on amyloid-β (Aβ)-induced paralysis in the CL4176 *Caenorhabditis elegans* mutant strain. Egg-synchronized worms were grown on NGM plates in the absence (control) and presence of unhydrolyzed chicken foot proteins (5 mg/mL), and seeded with OP50 *E. coli* at 16 ºC for 48 h. Subsequently, worms were incubated at 25 ºC for 24 h to induce Aβ expression. Non-paralyzed worms were scored at 2-hour intervals for the next 12 hours. **(A)** Obtained Aβ-induced paralysis curves in the absence (control) and presence of unhydrolyzed chicken foot broth (0.5 mg/mL). No significant differences between the groups were observed ($p > 0.05$; two-way ANOVA; post-hoc Bonferroni test). **(B)** The area under the curve (AUC) of *C. elegans* Aβ-induced paralysis curve, normalized by the control group AUC. No differences were observed between groups ($p > 0.05$, Student's t-test). Data are expressed as the mean of three independent experiments (n = 100 - 150 worms per experiment and group) ± standard deviation.

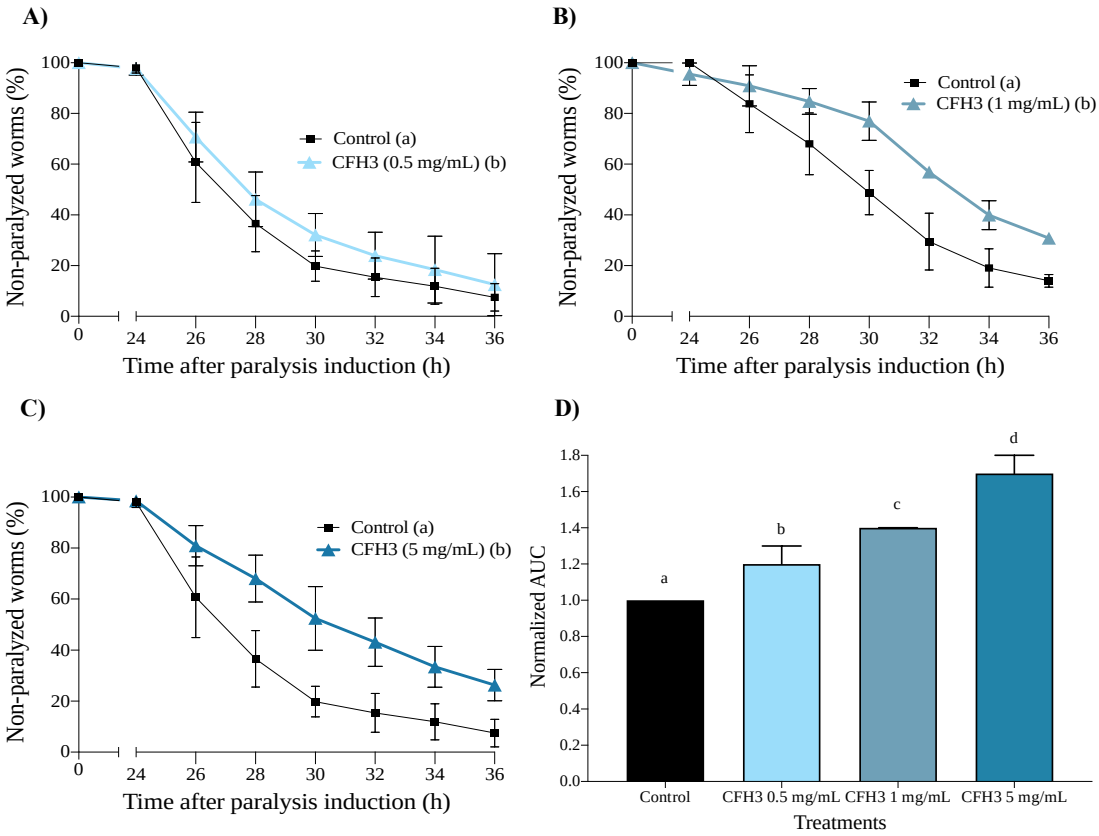

Figure 2. Effect of chicken foot hydrolysate CFH3 on β-amyloid (Aβ)-induced paralysis in the CL4176 *Caenorhabditis elegans* mutant strain. Egg-synchronized worms were grown on NGM plates in the absence (control) and presence of different doses of CFH3 (0.5, 1, and 5 mg protein/mL), and seeded with OP50 *E. coli* at 16 ºC for 48 h. Subsequently, worms were incubated at 25 ºC for 24 h to induce Aβ expression. Non-paralyzed worms were scored at 120-minute intervals for the next 12 h. **(A - C)** Obtained Aβ-induced paralysis curves in the absence (control) and presence of CFH3 at 0.5 mg protein /mL (A), 1 mg protein/mL (B), and 5 mg protein/mL (C). Different letters indicate significant differences between groups ($p < 0.05$; two-way ANOVA; post-hoc Bonferroni test). **(D)** The area under the curve (AUC) of *C. elegans* Aβ-induced paralysis curve, normalized by the control group AUC. Different letters indicate differences between groups ($p < 0.05$; one-way ANOVA; post-hoc Bonferroni test). Data expressed as the mean of three independent experiments (n = 100 - 150 worms per experiment and group) ± standard deviation.

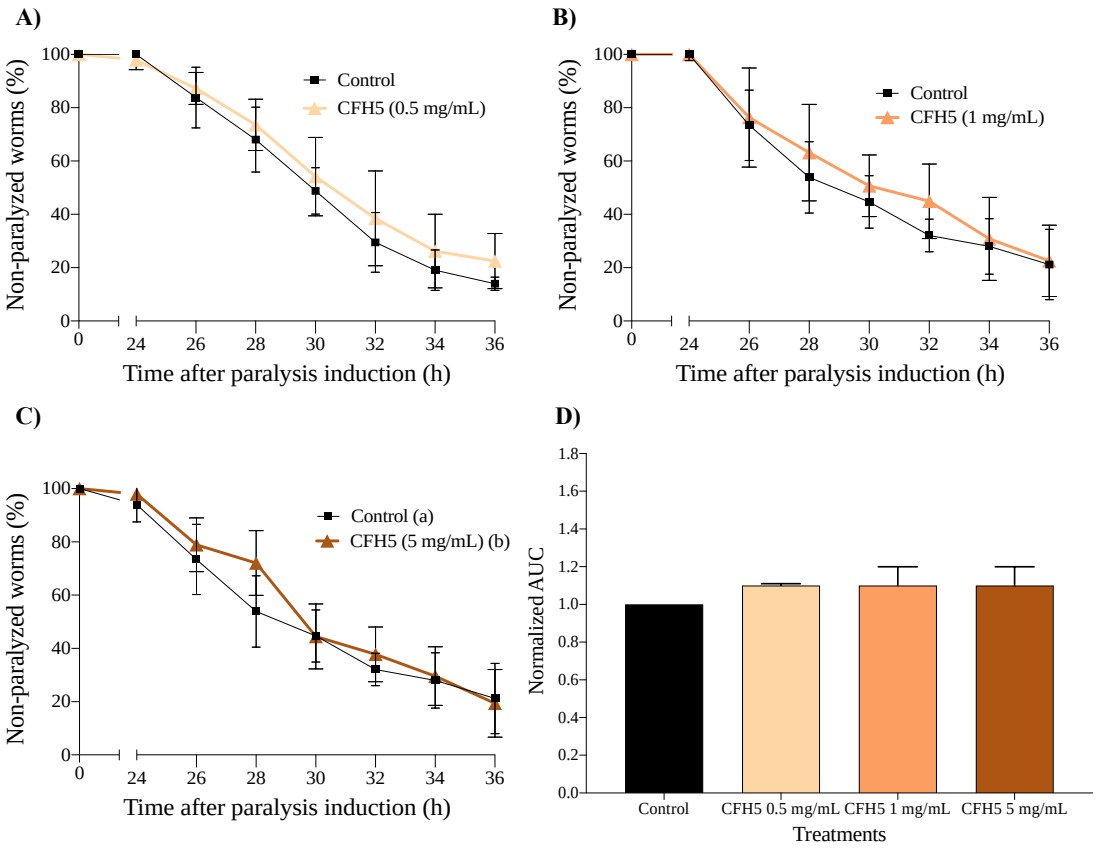

Figure 3. Effect of chicken foot hydrolysate CFH5 on β-amyloid (Aβ)-induced paralysis in the CL4176 *Caenorhabditis elegans* mutant strain. Egg-synchronized worms were grown on NGM plates in the absence (control) and presence of different doses of CFH5 (0.5, 1, and 5 mg protein/mL), and seeded with OP50 *E. coli* at 16 ºC for 48 h. Subsequently, worms were incubated at 25 ºC for 24 h to induce Aβ expression. Non-paralyzed worms were scored at 120-minute intervals for the next 12 h. **(A - C)** Obtained Aβ-induced paralysis curves in the absence (control) and presence of CFH5 at 0.5 mg/mL (A), 1 mg/mL (B), and 5 mg/mL (D). Different letters indicate significant differences between groups ($p < 0.05$; two-way ANOVA; post-hoc Bonferroni test). **(D)** The area under the curve (AUC) of *C. elegans* Aβ-induced paralysis curve, normalized by the control group AUC. No significant differences were found between groups ($p < 0.05$; one-way ANOVA; post-hoc Bonferroni test). Data are expressed as the mean of three independent experiments (n = 100 - 150 worms per experiment and group) ± standard deviation.

3.3. CFH3 hydrolysate characterization

The moisture and total protein contents of the freeze-dried hydrolysate CFH3 were 98.0% ± 0.0 and 78.7% ± 2.0, respectively. The collagen content, calculated using the hydroxyproline content, was 68.8%. The amino acid profile of CFH3 is shown in Table

3. Gly (15.5%) was the most abundant amino acid, comprising 15.5% of the total, followed by Glu (8.7%), Hyp (8.6%) and Pro (8.2%). In contrast, Trp, Cys, and Met were scarce, constituting only 0.1%, 0.3%, and 0.6% of the total, respectively.

Table 3. Amino acid profile and collagen content of the CFH3 hydrolysate.

Amino acids	Content (%)*
Aspartic acid	4.6 ± 0.5
Glutamic acid	8.7 ± 0.9
Alanine	6.8 ± 0.7
Arginine	5.7 ± 0.6
Cysteine	0.3 ± 0.1
Phenylalanine	2.0 ± 0.3
Glycine	15.5 ± 1.6
Histidine	0.7 ± 0.1
Isoleucine	1.3 ± 0.2
Leucine	2.7 ± 0.3
Lisine	3.0 ± 0.4
Methionine	0.6 ± 0.1
Proline	8.2 ± 0.8
Serine	2.1 ± 0.3
Tyrosine	0.8 ± 0.1
Threonine	1.7 ± 0.2
Tryptophan	0.1 ± 0.0
Valine	1.8 ± 0.2
Hydroxyproline	8.6 ± 0.4
Collagen	**68.8 ± 4.5**

* Results are expressed as a percentage (w/w) ± standard deviation.

3.4. Peptide identification in the hydrolysate CFH3

The <3 kDa fraction of CFH3 was separated into 16 fractions (F1 - F16) using semi-preparative RP-HPLC. Figure 4A shows the base peak chromatogram obtained for the fraction, revealing a complex mixture of peptides. The POPi activity of the 16 fractions ranged from 0 to 83% when measured at 0.01 mg of protein/mL (Figure 4B). Fraction F15 displayed the highest bioactivity, with a POP inhibition of 82.9% ± 4.2. This inhibitory activity was 47.6 and 49.2% higher than that of fractions F9 and F16,

respectively, which were the second most active fractions. The POPi IC_{50} for fraction F15 was determined as 1.7 ± 0.1 µg protein/mL.

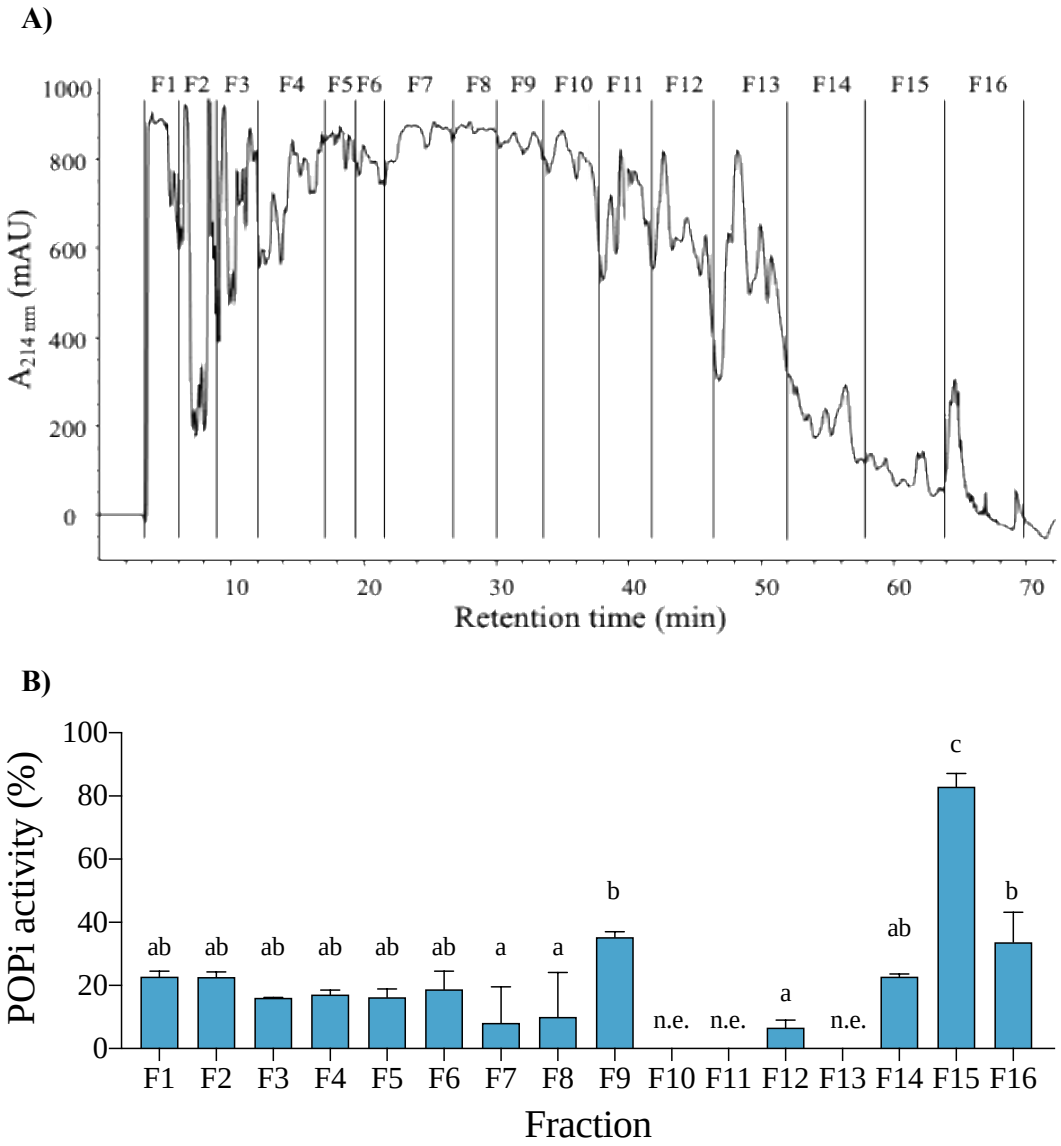

Figure 4. RP-HPLC chromatogram obtained for the <3 kDa fraction obtained from chicken foot hydrolysate CFH3 and peptide-collected fractions **(A)**. Prolyl oligopeptidase inhibitory (POPi) activity of the 16 RP-HPLC fractions obtained **(B)**. The results are expressed as the mean (n=3) ± standard deviation. Different letters indicate significant differences between the groups ($p < 0.05$; one-way ANOVA; post-hoc Bonferroni test). Abbreviations: n.e.: no effect.

Six hundred and sixty amino acid sequences were identified in the F15 fraction when analyzed by UHPLC-Orbitrap MS/MS. Twenty-three of them had a Mascot ion score ≥ 40 and relative ion abundance ≥ 10^8. The amino acid sequences, native protein, mass, charge, and *m/z* ratio of these selected 23 peptides are listed in Table 4.

Table 4. Peptides identified in the RP-HPLC fraction F15 with a Mascot ion score ≥ 40 and relative abundance ≥ 10^8.

Sequence[a]	Native protein	Mass (Da)	z	*m/z* (Da)	Relative abundance
PLRGPAL	TRPC4-AP	723.44	1	723.44	6.85E+09
VQHEELPLP	Ig-like domain-containing protein	1,061.56	1	1,061.56	2.57E+09
NLADDIMR	Vimentin	947.46	1	947.46	7.24E+08
LKGADPEDVIM	Myosin light chain, phosphorylable, fast skeletal muscle	1,188.61	2	594.30	4.93E+08
IITNWDDMEK	Actin, gamma-enteric smooth muscle	1,265.60	2	632.80	4.63E+08
LPGLGAL	M-phase inducer phosphatase	640.40	1	640.40	4.54E+08
DKNADGFIDIE	Troponin C, skeletal muscle	1,236.57	1	1236.57	3.57E+08
DNIKNTFSQL	Hemoglobin subunit epsilon 1	1,180.61	2	590.30	2.93E+08
PLGGLIA	CBY1 interacting BAR domain containing 1	640.40	1	640.40	2.90E+08
LPGALGL	Zinc finger protein 703	640.40	1	640.40	2.74E+08
FKFEEPNFEETPK	ATP synthase-coupling factor 6, mitochondrial	1,643.80	3	547.93	2.71E+08
VGDPNWFPK	Transgelin	1,060.53	2	530.27	2.42E+08
ITLEVEPSDTIENVKAK	Ubiquitin B	1,888.03	3	629.34	2.39E+08
IIDVVLK	Collagen type IX alpha 2 chain	799.53	1	799.53	2.14E+08
SADPEPGPGIDL	Cathelicidin-2	1,168.56	2	584.28	1.95E+08
PGENGLPGLPGARG EAGIPGPQG	Collagen, type X, alpha 1	2,099.06	2	1,049.53	1.94E+08
LGKFEDDMRT	Trafficking protein particle complex subunit 10	1,212.58	2	606.29	1.86E+08
FTTYPPTKTYFPH	Hemoglobin subunit alpha-A	1,601.80	3	533.93	1.55E+08
GERGFPGLPGPS	Collagen type I alpha 1 chain	1,170.59	1	1,170.59	1.29E+08
IDTWEKPF	ABI family member 3 binding protein	1,036.52	2	518.26	1.25E+08
AGNVLVI	Beta-1 adrenergic receptor	685.42	1	685.42	1.25E+08
IEAANHIDDIAGTL	Hemoglobin subunit alpha-A	1,453.74	2	726.87	1.12E+08
IDRQYDPDLEQL	Transgelin	1,505.73	2	752.87	1.07E+08

[a]Amino acids are designated using their one-letter codes.

The MS/MS spectra of 3 ions m/z (sequences VGDPNWFPK, SADPEPGPGIDL, and LPGALGL), are shown as an example in Figure 5. Those peptides were included in a wide variety of *Gallus gallus* proteins such as structural proteins (collagen), muscle proteins (actin, myosin, troponin C), and blood proteins (hemoglobin subunit A), among other cellular proteins. The length of these selected peptides ranged from 7 to 23 amino acid residues with a molecular weight between 640 and 2,099 Da (Table 4). The longest identified sequence was PGENGLPGLPGARGEAGIPGPQG. Thirteen of the identified peptides had the amino acid Pro in their structure.

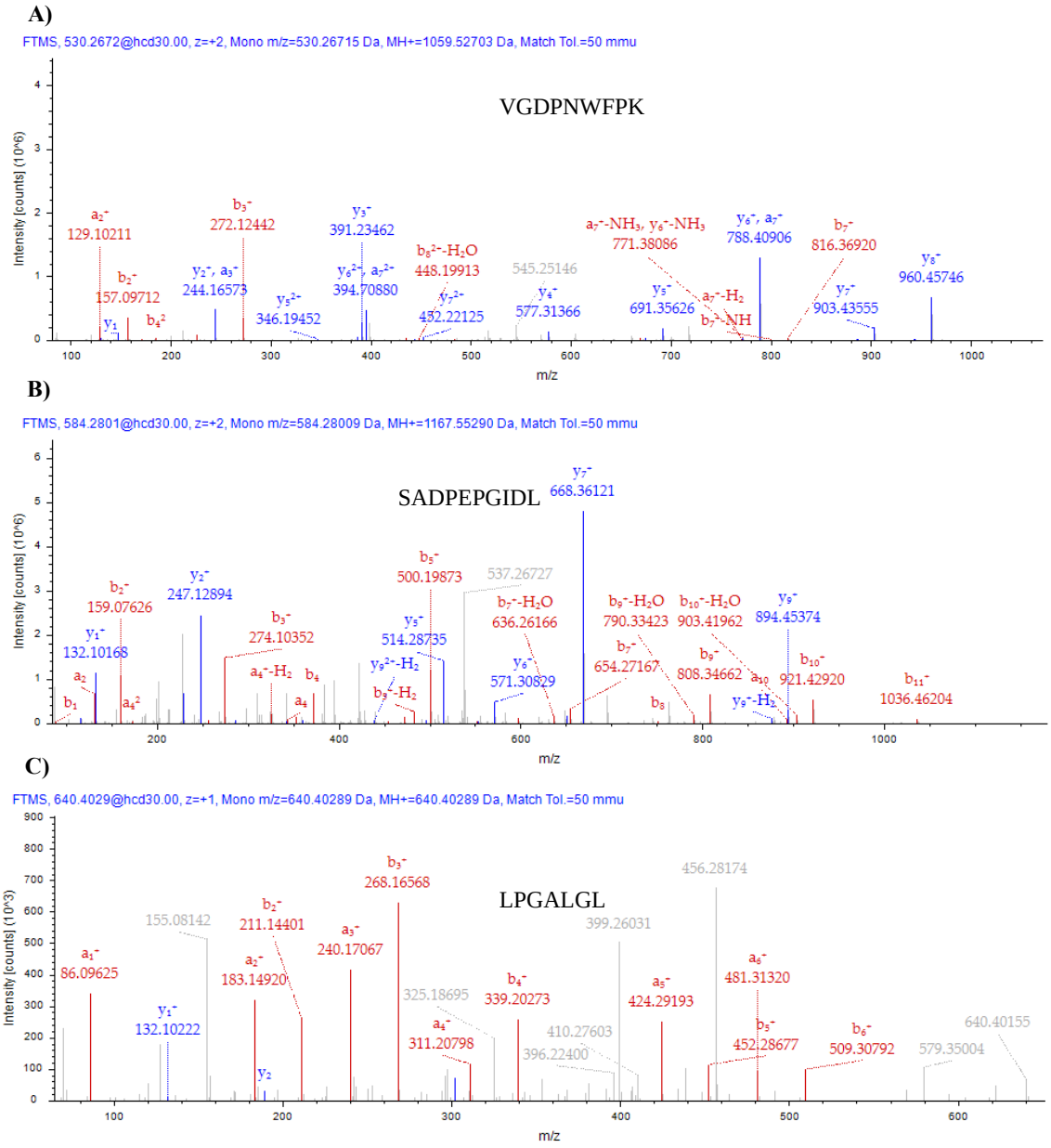

Figure 5. The MS/MS spectra of the peptides **(A)** VGDPNWFPK, **(B)** SADPEPGPGIDL, and **(C)** LPGALGL**.**

Table 5. PeptideRanker score of the identified peptides.

Sequence[a]	BIOPEP-UWM (POPi activity)	PeptideRanker score
PLRGPAL	GP	0.61
VQHEELPLP		0.28
NLADDIMR		0.50
LKGADPEDVIM		0.27
IITNWDDMEK		0.23
LPGLGAL	PG	0.59
DKNADGFIDIE		0.22
DNIKNTFSQL		0.23
PLGGLIA		0.43
LPGALGL	PG	0.63
FKFEEPNFEETPK		0.31
VGDPNWFPK		0.89
ITLEVEPSDTIENVKAK		0.06
IIDVVLK		0.13
SADPEPGPGIDL	PGP, PG, GP	0.64
PGENGLPGLPGARGEAGIPGPQG	PGP, PG, GP	0.61
LGKFEDDMRT		0.36
FTTYPPTKTYFPH		0.24
GERGFPGLPGPS	PGP, PG, GP	0.61
IDTWEKPF		0.60
AGNVLVI		0.22
IEAANHIDDIAGTL		0.22
IDRQYDPDLEQL		0.21

[a]Amino acids are designated using their one-letter codes.

3.5. Bioactivity prediction

None of the identified peptides were previously reported with neuroprotective or any other bioactivity in the BioPep database. However, SADPEPGPGIDL, LPGALGL, PLRGPAL, PGENGLPGLPGARGEAGIPGPQG, GERGFPGLPGPS, and LPGLGAL contained in their structures reported POPi peptides such as PGP, PG, and GP [34] (Table 5). The PeptideRanker score calculated for the identified peptides ranged between 0.89 and 0.06, with the peptide VGDPNWFPK being the one with the highest score and ITLEVEPSDTIENVKAK with the lowest one. Nine amino acid sequences showed a PeptideRanker score of 0.5 or higher (Table 5).

4. Discussion

Bioactive peptides obtained from different food and agri-food by-products have been reported to possess many functionalities, including neuroprotective, antihypertensive, anti-inflammatory, antidiabetic, or antioxidant effects [3,16,17]. Among others, these compounds could be useful to prevent or delay the development of neurodegenerative disorders, such as AD, through their consumption as functional foods and nutraceuticals. In this study, a hydrolysate from chicken feet, a collagen-rich by-product, was selected based on its capacity to inhibit POP enzyme and assessed for its ability to delay Aβ-induced paralysis in a *C. elegans* model of AD, and its constituent peptides were further identified. Hsieh et al. [36] reported a weak POPi activity of hydrolysates from different collagen-rich sources, such as porcine gelatin and fish skin. However, to the best of our knowledge, there was no evidence of the use of chicken feet as a source of POP inhibitory peptides up to the present study. Nonetheless, previous studies from our group demonstrated that bioactive peptides could be obtained from this material under specific enzymatic hydrolysis conditions. In this regard, a chicken foot hydrolysate prepared using Protamex (2 h, 50 °C) showed high ACE inhibitory activity *in vitro* and exerted antihypertensive effects after acute and long-term administration to hypertensive rats [37]. Furthermore, a hydrolysate obtained with Neutrase (24 h, 25 °C) was able to inhibit DPP-IV *in vitro* and exerted anti-hyperglycemic effects in two animal models of glucose intolerance [38]. Interestingly, other food-derived peptide/hydrolysates with POPi activity have also been reported to inhibit ACE or DPP-IV enzymes [20,39]. For instance, a protein hydrolysate (Papain, 4 h) obtained from the edible seeds of *Plukenetia volubilis* (commonly named *sacha inchi*) was found to possess ACE, DPP-IV, and POP inhibitory activities, with IC_{50} values of 1.17, 1.63, and 4.6 mg of protein/mL [39]. Also, 4 h Alcalase-digested hydrolysates from tuna heads, and tuna muscle and viscera were shown capable of inhibiting ACE (IC_{50} values of 0.27 and 4.59 mg dry weight/mL, respectively) and POP enzymes (0.24 and 3.30 mg dry weight/mL) [20].

In the present work, a total of 12 hydrolysates were prepared from chicken foot broth using three enzymes (E3, E4, and E5) at two different E/S ratios (C1 and C2), and two different hydrolysis times (t1 and t2). The hydrolysates tested at 0.2 mg product/mL, exerted a wide range of POPi activity (between 0 and 76%). Hydrolysates CFH3 (E1, C2, t2) and CFH5 (E4, C1, t1) exhibited the highest bioactivity (73.9 and 76.4%, respectively) with IC_{50} values of 57.9 and 40.1 µg protein/mL (73.7 µg

product/mL and 46.3 µg product/mL), respectively, revealing to be much more active than *sacha inchi* and tuna hydrolysates, which IC_{50} values between 3.30 and 4.60 mg/mL [20,39]. Similarly, POPi hydrolysates obtained from different Pro-rich sources, such as wheat gluten, soy, sodium caseinate, porcine gelatin, diverse fish skins, and tuna cooking juice, presented bioactivity ranging between 22 and 87% when tested at 0.6 mg/mL [36]. That concentration was 3-fold higher than the one of the products tested herein, which supports the POPi potential of the obtained chicken foot hydrolysates.

Hsieh et al. [36] observed that both the raw material and enzyme type affected the generation of POPi hydrolysates [36], with hydrolysates obtained from sodium caseinate, soy protein, and wheat gluten in most hydrolysis conditions showing greater bioactivity than those obtained from different collagen-rich sources. Additionally, the effect of the enzyme type was raw-material-dependent. Thus, Bromelain was the best enzyme for generating POPi hydrolysates from sodium caseinate, whereas Thermolysin was the best for wheat gluten [36]. This observation is in line with our results, where the POPi activity also varied according to the enzyme used, with E3 and E4 being the most effective in chicken foot proteins. Notwithstanding, the POPi activity of the hydrolysates also varied depending on the E/S ratio and hydrolysis time used. Higher E/S ratios for E3 and E5 (this last only at the highest hydrolysis time) led to increased POP inhibitory activity, whereas higher E/S ratios for E4 resulted in less bioactive hydrolysates. Moreover, increased hydrolysis time generally decreased POPi activity, except for E5 at the highest E/S ratio. Martorell et al. [22] also found that hydrolysates obtained from a high-protein cocoa by-product showed POP inhibition *in vitro* dependent on the type of enzyme, higher when the hydrolysis (1 h, 50 °C) was carried out with Alcalase (42.8%) than with Termamyl (22.8%) [22]. The hydrolysates also revealed in vivo neuroprotective effects in the CL4176 *C. elegans* strain [22] a mutant strain that expresses Aβ peptide in muscle cells when submitted to thermal stress [40] and is widely used as an in vivo screening model for AD drug discovery [41]. Taking this into account, the next step in the present study was to evaluate the effect of the two hydrolysates with the highest POPi activities (CFH3 and CFH5) in this *C. elegans* strain.

It was found that hydrolysate CFH3 delayed Aβ-induced paralysis in the CL4176 strain in a concentration-dependent manner (0.5 - 5 mg prot/mL), reducing the number of paralyzed worms by 20% at concentrations of 1 and 5 mg/mL in the cultivation medium, compared to non-treated worms at the end of the experiment (36 h after Aβ

induction). Conversely, hydrolysate CFH5 showed only a subtle effect at the highest concentration tested (5 mg protein/mL). Giraldo et al. [42] also tested in the CL4176 strain different concentrations of a whey protein hydrolysate (0.2 – 0.4 mg/mL). They found no mobile worms in the control group 30 h after the temperature upshifting, while the percentage of mobile worms in the hydrolysate-treated groups ranged between 20-30% [42]. In our assays, some worms in the controls were still non-paralyzed at the end of the assay (36 h). The differences in the paralysis progression among studies might be explained by the high sensitivity of the CL4176 strain to the temperature [43], so minor temperature variations in the incubators can lead to sensible changes in Aβ expression.

It is worth mentioning that unhydrolyzed chicken foot broth did not exhibit any effect on delaying worm paralysis at the highest dose tested (5 mg protein/mL). These results indicate that the beneficial effect observed for CFH3 was mediated by peptides released during the hydrolysis of chicken foot proteins under specific conditions. As above indicated, the lower rate of paralysis is associated with a reduction in Aβ accumulation in the peptide-treated worms compared to non-treated worms. Similarly, Martorell et al. [22] also observed that the hydrolysis of cocoa by-product proteins generated peptides, such as DNYDNSAGKWWVT, which delayed Aβ-induced paralysis in the CL4176 strain model. Although the mechanisms involved in the Aβ-induced paralysis-preventive effects of the POP-inhibitory CFH3 hydrolysate have not been the focus of the present study, there is evidence that POP inhibition enhances the clearance of protein aggregates in other neurodegenerative disorders and induces autophagy [8]. Therefore, this might be one of the potential mechanisms underlying the neuroprotective effects of CFH3 in the used AD model, although further research is needed to investigate it.

The next step was to characterize CFH3 and identify the peptides present. Chicken feet are a protein-rich source (approximately 75%) and are considered a good source of collagen (more than 50 g/100 g product) [27]. The composition of amino acids in collagen is unique among proteins, particularly in terms of its high concentration of Hyp. Moreover, it had prevalent amino acid sequence patterns, which are Gly-Pro-X and Gly-X-Hyp, with X representing any amino acid other than Gly, Pro, or Hyp [44]. As expected, the hydrolysate CFH3 showed a high protein content (78.7%), predominantly collagen (68.8%), with Gly, Glu, Hyp and Pro as the most abundant amino acids. Several studies have shown that POPi activity is associated with low molecular size peptides [20,39]. In this regard, Suwanangul et al. [39] observed that the

POP-inhibitory peptides of hydrolysate from *Plukenetia volubilis* were contained mainly in the < 1 kDa and 1 – 3 kDa fractions, which showed IC_{50} values of 0.67 and 2.01 mg protein/mL, compared to 3 - 5 kDa and 5 - 10 kDa fractions, with IC_{50} values of 13.41 and 20 mg prot/mL, respectively [39]. Thus, the < 3 kDa fraction of the hydrolysate CFH3 was obtained and fractionated by RP-HPLC into 16 peptide fractions. The analysis of POPi activity revealed that the bioactive peptides were located mainly in fraction F15 (82.9% POP inhibition), which was then analyzed by HPLC-Orbitrap MS/MS. The comparison with the *Gallus gallus* proteome gave 660 entries, providing an idea of the complexity of the sample. Among them, 23 amino acid sequences were selected based on ion intensity and a Mascot Ion Score ≥40, a widely recognized approach for assessing the validity of peptide and protein identification using MS/MS data [45]. This probability-based scoring system for an MS/MS match is defined as $-10 \times \log(p)$, where p indicates the likelihood that the observed match between the experimental data and the database sequence is a random occurrence [46]. Only 13% of the identified peptides were from collagen chains, despite CFH3 containing 68.8% collagen. This suggests that the hydrolysis conditions used were not sufficient to hydrolyze the complex collagen structure to obtain peptides with molecular weights lower than 3 kDa. Most peptides were derived from cellular and blood proteins such as actin, myosin, troponin C, several receptors, or hemoglobin subunit A. The amino acid lengths of these peptides ranged from 7 to 23, being worth mentioning that 65% of those peptides had the amino acid Pro in their structures.

POP is a serine endopeptidase that hydrolyzes the -Pro-X- bond in peptides up to 30 amino acids in length, where X is any amino acid different from Pro [47,48]. The presence of Pro in the amino acid sequence of POP inhibitory peptides, as well as in ACE and DPP-IVi peptides, has been associated with more active inhibitors [49–51]. Actually, all amino acid sequences included in the BIOPEP-UWM database (62 peptides) with POPi activity contained at least one Pro residue (accessed 20/03/2024) [34]. Hsieh et al. [36] also suggested that Pro presence could be more important than peptide length in the POPi bioactivity of peptides. None of the identified peptides was found in the BIOPEP-UWM database [34], denoting the novelty of these sequences. Only the peptides PGENGLPGLPGARGEAGIPGPQG, GERGFPGLPGPS, SADPEPGPGIDL, LPGALGL, PLRGPAL, and LPGLGAL, which contained in their structure reported POPi peptides, such as PGP, PG, and GP, are included in BIOPEP-UWM [34].

Moreover, the potential bioactivity of the identified peptides was predicted using the PeptideRanker prediction tool to obtain a PeptideRanker score [35], with values ranging from 0.0 to 1.0, where "0.0" represents improbable and "1.0" signifies high probability. Nine identified peptides had a PeptideRanker score ≥ 0.5, eight of which contained Pro in their structure. In addition, two of these were identified as collagen peptides (GERGFPGLPGPS and PGENGLPGLPGARGEAGIPGPQG). These sequences differed from the expected Gly-Pro-Hyp sequence, but modifications in these amino acids have been reported to be associated to different functionalities of the specific macromolecule [44,52]. Further research is required to evaluate the POPi activity of these nine selected peptides so as to ascertain if they are responsible of CFH3 bioactivity.

5. Conclusions

This study shows the potential of chicken feet as a source of peptides with potential neuroprotective effects when subjected to specific hydrolysis conditions. A hydrolysate from chicken feet, referred to as CFH3, showed good bioactivity, inhibiting POP enzyme *in vitro* and delaying the Aß-induced paralysis in a *C. elegans* model of Alzheimer's disease. Twenty-three peptides were identified in that hydrolysate, nine of which showed a good PeptideRanker score, indicating potential bioactivity. In addition, eight of them contained Pro in their amino acid sequences, which has been associated with increased POPi activity. In particular, the prepared hydrolysate might be useful as a functional ingredient in the prevention of cognitive decline. The valorization of chicken by-products as a source of bioactive peptides and hydrolysates would help promote the circular economy and environmental sustainability of the agri-food industry.

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8. Conflicts of interest

The authors declare no conflicts of interest.

9. Author contributions

Conceptualization, B.M, C.S-B, A.G-P, and F.I.B.; formal analysis, N.I-B; funding acquisition, B.M, and F.I.B.; methodology, N.I-B, L.G-G, B.A-D and F.I.B; supervision, C.S-B, A.G-P, and F.I.B.; writing—original draft, N.I-B.; writing—review and editing, N.I-B, F.I.B, and C.S-B All authors have read and agreed to the published version of the manuscript.

REFERENCES

1. Reitz, C.; Pericak-Vance, M.A.; Foroud, T.; Mayeux, R. A Global View of the Genetic Basis of Alzheimer Disease. *Nat Rev Neurol* **2023**, *19*, 261–277.
2. López, O.L.; DeKosky, S.T. Clinical Symptoms in Alzheimer's Disease. In *Handbook of Clinical Neurology*; Elservier, **2008**; Vol. 89, pp. 207–216.
3. Zhao, L.; Li, D.; Qi, X.; Guan, K.; Chen, H.; Wang, R.; Ma, Y. Potential of Food-Derived Bioactive Peptides in Alleviation and Prevention of Alzheimer's Disease. *Food Funct* **2022**, *13*, 10851–10869.
4. Lee, Y.-J.; Han, S.B.; Nam, S.-Y.; Oh, K.-W.; Hong, J.T. Inflammation and Alzheimer's Disease. *Arch Pharm Res* **2010**, *33*, 1539–1556.
5. Heneka, M.T.; Carson, M.J.; Khoury, J. El; Landreth, G.E.; Brosseron, F.; Feinstein, D.L.; Jacobs, A.H.; Wyss-Coray, T.; Vitorica, J.; Ransohoff, R.M.; et al. Neuroinflammation in Alzheimer's Disease. *Lancet Neurol* **2015**, *14*, 388–405.
6. Rea, I.M.; Gibson, D.S.; McGilligan, V.; McNerlan, S.E.; Denis Alexander, H.; Ross, O.A. Age and Age-Related Diseases: Role of Inflammation Triggers and Cytokines. *Front Immunol* **2018**, *9*.
7. Natunen, T.A.; Gynther, M.; Rostalski, H.; Jaako, K.; Jalkanen, A.J. Extracellular Prolyl Oligopeptidase Derived from Activated Microglia Is a Potential Neuroprotection Target. *Basic Clin Pharmacol Toxicol* **2019**, *124*, 40–49.
8. Svarcbahs, R.; Julku, U.; Kilpeläinen, T.; Kyyrö, M.; Jäntti, M.; Myöhänen, T.T. New Tricks of Prolyl Oligopeptidase Inhibitors – A Common Drug Therapy for Several Neurodegenerative Diseases. *Biochem Pharmacol* **2019**, *161*, 113–120.
9. Taraszkiewicz, A.; Sinkiewicz, I.; Sommer, A.; Staroszczyk, H. The Biological Role of Prolyl Oligopeptidase and the Procognitive Potential of Its Peptidic Inhibitors from Food Proteins. *Crit Rev Food Sci Nutr* **2023**, 1–14.
10. Serfozo, P.; Wysocki, J.; Gulua, G.; Schulze, A.; Ye, M.; Liu, P.; Jin, J.; Bader, M.; Myöhänen, T.; García-Horsman, J.A.; et al. Ang II (Angiotensin II) Conversion to Angiotensin-(1-7) in the Circulation Is POP (Prolyloligopeptidase)-Dependent and ACE2 (Angiotensin-Converting Enzyme 2)-Independent. *Hypertension* **2020**, *75*, 173–182.
11. Jiang, D.-X.; Zhang, J.-B.; Li, M.-T.; Lin, S.-Z.; Wang, Y.-Q.; Chen, Y.-W.; Fan, J.-G. Prolyl Endopeptidase Gene Disruption Attenuates High Fat Diet-Induced Nonalcoholic Fatty Liver Disease in Mice by Improving Hepatic Steatosis and Inflammation. *Ann Transl Med* **2020**, *8*, 218–218.
12. Höfling, C.; Kulesskaya, N.; Jaako, K.; Peltonen, I.; Männistö, P.T.; Nurmi, A.; Vartiainen, N.; Morawski, M.; Zharkovsky, A.; Võikar, V.; et al. Deficiency of Prolyl Oligopeptidase in Mice Disturbs Synaptic Plasticity and Reduces Anxiety-like Behaviour, Body Weight, and Brain Volume. *European Neuropsychopharmacology* **2016**, *26*, 1048–1061.
13. Hannula, M.J.; Myöhänen, T.T.; Tenorio-Laranga, J.; Männistö, P.T.; Garcia-Horsman, J.A. Prolyl Oligopeptidase Colocalizes with α-Synuclein, β-Amyloid, Tau Protein and Astroglia in the Post-Mortem Brain Samples with Parkinson's and Alzheimer's Diseases. *Neuroscience* **2013**, *242*, 140–150.
14. Crous-Bou, M.; Minguillón, C.; Gramunt, N.; Molinuevo, J.L. Alzheimer's Disease Prevention: From Risk Factors to Early Intervention. *Alzheimers Res Ther* **2017**, *9*, 1–9.
15. Scheltens, P.; De Strooper, B.; Kivipelto, M.; Holstege, H.; Chételat, G.; Teunissen, C.E.; Cummings, J.; van der Flier, W.M. Alzheimer's Disease. *Lancet* **2021**, *397*, 1577–1590.

16. Calfio, C.; Gonzalez, A.; Singh, S.K.; Rojo, L.E.; MacCioni, R.B. The Emerging Role of Nutraceuticals and Phytochemicals in the Prevention and Treatment of Alzheimer's Disease. *J Alzheimers Dis* **2020**, *77*, 33–51.
17. Arora, S.; Santiago, J.A.; Bernstein, M.; Potashkin, J.A. Diet and Lifestyle Impact the Development and Progression of Alzheimer's Dementia. *Front Nutr* **2023**, *10*.
18. Lin, L.; Li, C.; Li, T.; Zheng, J.; Shu, Y.; Zhang, J.; Shen, Y.; Ren, D. Plant-Derived Peptides for the Improvement of Alzheimer's Disease: Production, Functions, and Mechanisms. *Food Front* **2023**, *4*, 677–699.
19. Lajmi, K.; Gómez-Estaca, J.; Hammami, M.; Martínez-Alvarez, O. Upgrading Collagenous Smooth Hound By-Products: Effect of Hydrolysis Conditions, in Vitro Gastrointestinal Digestion and Encapsulation on Bioactive Properties. *Food Biosci* **2019**, *28*, 99–108.
20. Martínez-Alvarez, O.; Batista, I.; Ramos, C.; Montero, P. Enhancement of ACE and Prolyl Oligopeptidase Inhibitory Potency of Protein Hydrolysates from Sardine and Tuna By-Products by Simulated Gastrointestinal Digestion. *Food Funct* **2016**, *7*, 2066–2073.
21. Ribeiro, T.B.; Voss, G.B.; Coelho, M.C.; Pintado, M.E. Food Waste and By-Product Valorization as an Integrated Approach with Zero Waste: Future Challenges. *Future Foods: Global Trends, Opportunities, and Sustainability Challenges* **2022**, 569–596.
22. Martorell, P.; Bataller, E.; Llopis, S.; Gonzalez, N.; Alvarez, B.; Montón, F.; Ortiz, P.; Ramón, D.; Genovés, S. A Cocoa Peptide Protects Caenorhabditis Elegans from Oxidative Stress and β-Amyloid Peptide Toxicity. *PLoS One* **2013**, *8*, e63283.
23. Manzanares, P.; Martínez, R.; Garrigues, S.; Genovés, S.; Ramón, D.; Marcos, J.F.; Martorell, P. Tryptophan-Containing Dual Neuroprotective Peptides: Prolyl Endopeptidase Inhibition and Caenorhabditis Elegans Protection from β-Amyloid Peptide Toxicity. *International Journal of Molecular Sciences 2018, Vol. 19, Page 1491* **2018**, *19*, 1491.
24. Liu, Y.T.; Cheng, F.Y.; Takeda, S.; Lai, K.M.; Lin, L.C.; Sakata, R. Effects of Porcine Brain Hydrolysate on Impairment of Cognitive Learning Ability in Amyloid β(1-40) -Infused Rats. *Anim Sci J* **2019**, *90*, 271–279.
25. Alvarez, X.A.; Cacabelos, R.; Sampedro, C.; Aleixandre, M.; Linares, C.; Granizo, E.; Doppler, E.; Moessler, H. Efficacy and Safety of Cerebrolysin in Moderate to Moderately Severe Alzheimer's Disease: Results of a Randomized, Double-Blind, Controlled Trial Investigating Three Dosages of Cerebrolysin. *Eur J Neurol* **2011**, *18*, 59–68.
26. Lafarga, T.; Hayes, M. Effect of Pre-Treatment on the Generation of Dipeptidyl Peptidase-IV- and Prolyl Endopeptidase-Inhibitory Hydrolysates from Bovine Lung. *Irish Journal of Agricultural and Food Research* **2017**, *56*, 12–24.
27. Ibarz-Blanch, N.; Alcaide-Hidalgo, J.M.; Cortés-Espinar, A.J.; Albi-Puig, J.; Suárez, M.; Mulero, M.; Morales, D.; Bravo, F.I. Chicken Slaughterhouse By-Products: A Source of Protein Hydrolysates to Manage Non-Communicable Diseases. *Trends Food Sci Technol* **2023**, *139*, 104125.
28. Ayuda-Durán, B.; Garzón-García, L.; González-Manzano, S.; Santos-Buelga, C.; González-Paramás, A.M. Insights into the Neuroprotective Potential of Epicatechin: Effects against Aβ-Induced Toxicity in Caenorhabditis Elegans. *Antioxidants* **2024**, *13*, 79.
29. Chromý, V.; Vinklárková, B.; Šprongl, L.; Bittová, M. The Kjeldahl Method as a Primary Reference Procedure for Total Protein in Certified Reference Materials Used in Clinical Chemistry. I. A Review of Kjeldahl Methods Adopted by Laboratory Medicine. *Crit Rev Anal Chem* **2015**, *45*, 106–111.
30. Horwitz, W. Official Methods of Analysis of AOAC International. *Journal of AOAC International*. Vol 1, Agricultural Chemicals; Contaminants; Drugs. **2000**; ISBN 0935584676.

31. Government, M. of R. with the C., and the S. of the BOE-A-1991-18408. *BOE (Spanish)* **1991**, 23725–23730.
32. Safety, F.O. of C.P. and F. BVL - Official Collection of Methods of Analysis Available online: https://www.bvl.bund.de/EN/Tasks/09_Laboratories/01_Tasks/04_official_collection_methods_analysis/official_collection_methods_analysis_node.html (accessed on 20 March 2024).
33. Bravo, F.I.; Mas-Capdevila, A.; Margalef, M.; Arola-Arnal, A.; Muguerza, B. Novel Antihypertensive Peptides Derived from Chicken Foot Proteins. *Mol Nutr Food Res* **2019**, *63*.
34. Minkiewicz, P.; Iwaniak, A.; Darewicz, M. BIOPEP-UWM Database of Bioactive Peptides: Current Opportunities. *Int J Mol Sci* **2019**, *20*.
35. Mooney, C.; Haslam, N.J.; Pollastri, G.; Shields, D.C. Towards the Improved Discovery and Design of Functional Peptides: Common Features of Diverse Classes Permit Generalized Prediction of Bioactivity. *PLoS One* **2012**, *7*, e45012.
36. Hsieh, C.-H.; Wang, T.-Y.; Hung, C.-C.; Hsieh, Y.-L.; Hsu, K.-C. Isolation of Prolyl Endopeptidase Inhibitory Peptides from a Sodium Caseinate Hydrolysate. *Food Funct* **2016**, *7*.
37. Mas-Capdevila, A.; Iglesias-Carres, L.; Arola-Arnal, A.; Suarez, M.; Muguerza, B.; Bravo, F.I. Long-Term Administration of Protein Hydrolysate from Chicken Feet Induces Antihypertensive Effect and Confers Vasoprotective Pattern in Diet-Induced Hypertensive Rats. *J Funct Foods* **2019**, *55*, 28–35.
38. Casanova-Martí, À.; Bravo, F.I.; Serrano, J.; Ardévol, A.; Pinent, M.; Muguerza, B. Antihyperglycemic Effect of a Chicken Feet Hydrolysate *via* the Incretin System: DPP-IV-Inhibitory Activity and GLP-1 Release Stimulation. *Food Funct* **2019**, *10*, 4062–4070.
39. Suwanangul, S.; Aluko, R.E.; Sangsawad, P.; Kreungngernd, D.; Ruttarattanamongkol, K. Antioxidant and Enzyme Inhibitory Properties of Sacha Inchi (*Plukenetia Volubilis*) Protein Hydrolysate and Its Peptide Fractions. *J Food Biochem* **2022**, *46*, e14464.
40. Beeg, M.; Diomede, L.; Stravalaci, M.; Salmona, M.; Gobbi, M. Novel Approaches for Studying Amyloidogenic Peptides/Proteins. *Curr Opin Pharmacol* **2013**, *13*, 797–801.
41. Lublin, A.L.; Link, C.D. Alzheimer's Disease Drug Discovery: In Vivo Screening Using Caenorhabditis Elegans as a Model for β-Amyloid Peptide-Induced Toxicity. *Drug Discov Today Technol* **2013**, *10*, e115–e119.
42. Giraldo, N.D.; Sánchez, J.D.; López, A.; González, N.; Balaguer, F.; Redondo, J.; Llopis, S.; Barrena, M.; Klotz, B. Functional Assessment of a Whey Protein Hydrolysate: In Vivo Responses in Lipid Regulation, Oxidant Defense, and Neural Protection in Caenorhabditis Elegans. *Int Dairy J* **2024**, *154*, 105921.
43. Dostal, V.; Link, C.D. Assaying β-Amyloid Toxicity Using a Transgenic *C. Elegans* Model. *Journal of Visualized Experiments* **2010**, 2252.
44. Shoulders, M.D.; Raines, R.T. Collagen Structure and Stability. *Annu Rev Biochem* **2009**, *78*, 929–958.
45. Savitski, M.M.; Nielsen, M.L.; Zubarev, R.A. New Data Base-Independent, Sequence Tag-Based Scoring of Peptide MS/MS Data Validates Mowse Scores, Recovers below Threshold Data, Singles out Modified Peptides, and Assesses the Quality of MS/MS Techniques. *Molecular and Cellular Proteomics* **2005**, *4*, 1180–1188.
46. Bouyssié, D.; Gonzalez de Peredo, A.; Mouton, E.; Albigot, R.; Roussel, L.; Ortega, N.; Cayrol, C.; Burlet-Schiltz, O.; Girard, J.P.; Monsarrat, B. Mascot File Parsing and Quantification (MFPaQ), a New Software to Parse, Validate, and Quantify Proteomics Data Generated by ICAT and SILAC Mass Spectrometric Analyses: Application to the Proteomics Study of Membrane Proteins from Primary Human Endothelial Cells. *Molecular and Cellular Proteomics* **2007**, *6*, 1621–1637.

47. Cunningham, D.F.; O'Connor, B. A Study of Prolyl Endopeptidase in Bovine Serum and Its Relevance to the Tissue Enzyme. *Int J Biochem Cell Biol* **1998**, *30*, 99–114.
48. Myöhänen, T.T.; García-Horsman, J.A.; Tenorio-Laranga, J.; Männistö, P.T. Issues about the Physiological Functions of Prolyl Oligopeptidase Based on Its Discordant Spatial Association with Substrates and Inconsistencies among MRNA, Protein Levels, and Enzymatic Activity. *Journal of Histochemistry and Cytochemistry* **2009**, *57*, 831–848.
49. Lawandi, J.; Gerber-Lemaire, S.; Juillerat-Jeanneret, L.; Moitessier, N. Inhibitors of Prolyl Oligopeptidases for the Therapy of Human Diseases: Defining Diseases and Inhibitors. *J Med Chem* **2010**, *53*, 3423–3438.
50. Daskaya-Dikmen, C.; Yucetepe, A.; Karbancioglu-Guler, F.; Daskaya, H.; Ozcelik, B. Angiotensin-I-Converting Enzyme (ACE)-Inhibitory Peptides from Plants. *Nutrients* **2017**, *9*.
51. Li-Chan, E.C.Y.; Hunag, S.L.; Jao, C.L.; Ho, K.P.; Hsu, K.C. Peptides Derived from Atlantic Salmon Skin Gelatin as Dipeptidyl-Peptidase IV Inhibitors. *J Agric Food Chem* **2012**, *60*, 973–978.
52. Kirkness, M.W.; Lehmann, K.; Forde, N.R. Mechanics and Structural Stability of the Collagen Triple Helix. *Curr Opin Chem Biol* **2019**, 98-105.

UNIVERSITAT ROVIRA I VIRGILI
PROTEIN HYDROLYSATES AND PEPTIDES FROM ANIMAL BY-PRODUCTS AS A PREVENTIVE STRATEGY FOR LOCAL INFLAMMATION AND NEUROINFLAMMATION
Néstor Ibarz Blanch

GENERAL DISCUSSION

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Inflammation is a natural response of an organism to harmful stimuli such as infection or trauma [1]. The inflammatory response is widely observed in numerous diseases and pathologies such as inflammatory bowel disease, metabolic syndrome-associated diseases, or arthritis [2,3]. It is tightly regulated by several mechanisms, pathways, and key molecules, including prostaglandins, NO, and other inflammatory mediators [4]. One of the most important enzymes involved in this process is COX, mainly COX isoform 2 (COX-2). COX enzymes catalyze the conversion of arachidonic acid into PGH_2 through the bis-oxygenation of arachidonic acid. PGH_2 serves as a precursor for various prostaglandins, thromboxanes, and prostacyclins [5]. COX-2 is induced in response to inflammatory stimuli and is expressed in fibroblasts, activated macrophages, and other cells [6]. In contrast, COX-1 is constitutively expressed in many tissues and is mainly involved in the regulation of homeostatic functions through the production of thromboxane A_2 and several PGs [5]. Inhibition of COX enzymes is the target of NSAIDs, such as ibuprofen, diclofenac, or naproxen [7]. However, most of them inhibit both COX-2 and COX-1. COX-1 inhibition is one of the causes of undesirable side effects associated with prolonged NSAID use [8]. Thus, there is a trend toward the development or discovery of new anti-inflammatory compounds that selectively target COX-2 [7,9].

Inflammation also plays an important role in neurodegenerative disorders. Neuroinflammation exerts a substantial influence on disease progression and severity of the diseases [10]. Microglial activation (immune cells in the central nervous system), leads to the secretion of different cytokines and enzymes to the extracellular space such as the serine protease POP [11,12]. Many functionalities have been attributed to POP, and it has been associated with amnesia and other neurodegenerative disorders, such as AD and Parkinson's disease [13,14]. Moreover, the POP enzyme has been found alongside Aβ aggregates in the brains of deceased patients with AD, indicating its involvement in generating neurotoxic peptides [15]. POP inhibition has become the target for the development of new neuroprotective drugs [16].

Bioactive peptides are promising candidates for natural health-promoting functional foods. They have shown a wide range of biological activities, such as antihypertensive, antioxidant, antidiabetic, anti-inflammatory, or neuroprotective activities [17]. These bioactive peptides have been obtained from various protein sources, including animals, vegetables, and fungi [18–20]. The most commonly used sources have been eggs [21], milk [22], and soybean proteins [23], although other less

conventional products, such as agri-food by-products, have recently been used [24,25]. Notably, the development of high-value products from these by-products could be beneficial in terms of valorizing them and supporting the circular economy within the sector [26]. Many by-products of animal origin are rich in proteins such as collagen [24]. Collagen, known for its high Pro and Hyp content, yields peptides with significant bioactivity [17]. Peptides that include hydrophobic amino acids such as Pro have been linked to an increased ability of a peptide to inhibit different biological enzymes such as angiotensin-converting enzyme, dipeptidyl peptidase-IV, and POP [27–29]. They also showed COX-2 inhibitory and anti-inflammatory effects [30–32]. The anti-inflammatory effects of collagen-derived hydrolysates have been reported, although only a few studies have evaluated their effects *in vivo* and only after oral consumption [33]. Hydrolyzed collagen, such as that obtained from pigskin, is commonly commercialized for joint and skin health and some are included in cosmetic creams [34]. However, there is no evidence that hydrolyzed collagen or any food-derived protein hydrolysate can exert anti-inflammatory effects after topical application. Therefore, collagen-rich by-products could be good sources of bioactive peptides to prevent inflammation and inflammation-related pathologies, such as muscle inflammation, arthritis, or AD. Thus, the objective of this doctoral thesis was to obtain protein hydrolysates from several collagen-rich sources with acute anti-inflammatory properties after topical application, or neuroprotective effects *in vivo,* and to identify their bioactive peptides.

Firstly, the study was focused on the development of a hydrolysate from pigskin collagen able to prevent focal acute inflammation after topical application and to identify the underlying mechanisms and bioactive peptides **(*Chapter 1*).** For that, it was studied the optimal hydrolysis conditions to obtain the pigskin-collagen hydrolysate with selective COX-2 inhibitory activity and also the ability to prevent or ameliorate carrageenan-induced acute inflammation and response to acute painful stimuli **[Manuscript 1]**.

Pigskin collagen was selected for this finality as it is commonly commercialized and many commercial hydrolyzed collagen is provided from this source. Twelve hydrolysates were obtained using different hydrolysis conditions, including the factors: enzyme type (E1, E2, or E3), enzyme/substrate (E/S) ratio (C1 < C2), and hydrolysis time (t1 < t2). The hydrolysates exhibited variable COX-2 inhibition (0 - 51 % at 1 mg/mL), indicating that hydrolysis conditions are crucial for generating COX-2

peptides from pigskin collagen. In addition, it was observed that the three tested hydrolysis factors affected the release of these bioactive collagen-derived peptides, being necessary to use enzymes E1 or E3, the longest hydrolysis time (t2), and the highest E/S ratio (at least with E1). Considering the obtained results, the best hydrolysis conditions to generate pigskin-collagen hydrolysates were enzyme E1, E/S C2, and hydrolysis time t2, which resulted in hydrolysate H8. There is no previous evidence regarding the influence of hydrolysis factors on the production of COX-2 inhibitory peptides from collagen. Moreover, it is worth to mentioning that the bioactivity of H8 was in the range of COX-2 inhibitory activity shown by hydrolysates obtained from lupine and several edible insects [35,36]. Considering this fact, hydrolysate H8 was further produced on a pilot scale (H8p), and its inhibitory activity was increased. Additionally, the <10 kDa fraction of H8p was obtained because different studies have shown that low-molecular-weight peptides can exert greater anti-inflammatory bioactivity than whole hydrolysate [37,38]. However, no differences in COX-2 inhibitory activity were observed between the peptide fraction and whole hydrolysate in the present study. Interestingly, neither of the samples inhibited COX-1 activity, indicating that both samples selectively inhibited COX-2 activity. As mentioned, this is desirable because COX-1 inhibition has been associated with side effects in the intestinal tract, cardiovascular events, and renal dysfunction due to the long-term consumption of NSAIDs [39].

Subsequently, the *in vivo* anti-inflammatory effects of H8p and its <10 kDa fraction were evaluated in a well-known model of acute inflammation (carrageenan-induced paw edema) after topical application. This method of application was chosen because there is no evidence that hydrolyzed collagen or any food-derived protein hydrolysates can exert anti-inflammatory effects after topical application. In contrast to the *in vitro* results, the whole hydrolysate did not present any anti-inflammatory effect, whereas its peptide fraction (only 15 mg) reduced edema volume by 54 % in the first hour after carrageenan injection, which gradually attenuated over time until 3 h. It is known that peptides up to 1,500 kDa can cross the outer skin layer [40]. Thus, the higher proportion of small peptides in the <10 kDa fraction than in the whole hydrolysate could explain these differences in bioactivity. It is worth mentioning that the anti-inflammatory effect of the <10 kDa fraction was faster than that of the commercial cream containing sodium diclofenac but was less maintained over time (24 h). Given that at 24 h (animal sacrifice time), the anti-inflammatory effect of the <10 kDa fraction no longer remained, LPS-

stimulated macrophages were used to elucidate the mechanisms underlying the anti-inflammatory effect of this peptide fraction **[Manuscript 1]**. This model is widely used as a screening tool for anti-inflammatory drugs, especially natural compounds [41–43]. The results showed that this fraction exerted its anti-inflammatory effects reducing NO production, iNOS and COX-2 protein expression, and COX-2 activity. COX-2, as mentioned above, is involved in the pro-inflammatory PGE_2 synthesis [6], while iNOS is the enzyme responsible for NO production, a regulator of inflammatory processes [44]. However, the potential effects of peptides contained in the <10 kDa fraction on other inflammatory-related and stress-oxidative molecules such as bradykinin, histamine, or serotonin should be not discarded although further research is needed to study it.

Considering that some natural anti-inflammatory compounds [45–47] and selective COX-2 drugs have antinociceptive effects [48], this activity was also evaluated in the H8p <10 kDa fraction using the tail-withdrawal test **[Manuscript 1]**. This is a well-known test used to determine the nociceptive response to painful stimuli, where the rat tail is submerged in 40 - 55 °C warm water, and the latency to purposely withdraw the tail from the water is measured [49]. The <10 kDa fraction retarded by 1.4-fold the time required to flick the tail compared to non-treated rats in the 55 ºC-water tail-withdrawal test. Interestingly, this antinociceptive effect was similar to that observed with diclofenac. Although some protein hydrolysates have shown antinociceptive effects [50,51], to the best of our knowledge, this is the first time to demonstrate antinociceptive effects of a dietary-derived hydrolysate following topical administration.

Finally, the peptides contained in hydrolysate H8p were identified **[Manuscript 2]**. The <3 kDa fraction of the hydrolysate was used to isolate small peptides (up to 3 kDa), which are usually responsible for the bioactivity of protein hydrolysates, including anti-inflammatory activity [52]. They can interact with enzymes, be absorbed, and penetrate the skin better than longer peptides [53]. This fraction was separated into 22 fractions by RP-HPLC. The COX-2 inhibitory activity of the 22 fractions showed that the most active peptides were present in fractions F3 and F20, which were further analyzed by UHPLC-Orbitrap MS/MS to identify their peptides. A total of nine sequences were identified (AGERGEQ, GPAGSVG, and GSKGRPG in fraction F3, and AGPAGKP, AGPPGAK, GPAGPAG, GPAGPAGPRG, PGPAGPV, and TGPIGPPGPA in the fraction F20. It is worth mentioning that any bioactivity has not been previously

reported for these amino acid sequences, according to the BIOPEP-UWM database [54]. Moreover, all identified peptides satisfied the suggested characteristics of potentially anti-inflammatory peptides (2 – 20 amino acids, less than 1 kDa, and the presence of hydrophobic or positively charged amino acids in their structure) [18,37]. Additional *in vitro* results showed that AGERGEQ, GSKGRPG, AGPAGKP, and PGPAGPV selectively inhibited COX-2 by up to 41 % at a concentration of 9 µg/mL. Interestingly, this bioactivity was higher than that exerted by several COX-2 inhibitory peptides obtained from edible insects or red microalga [32,55]. Moreover, the anti-inflammatory effects of these four identified peptides were evaluated in LPS-stimulated macrophages **[Manuscript 2]**. These peptides reduced NO production in LPS-stimulated macrophages pre-incubated with the peptides. Peptides from many different sources, including collagen-rich sources, have been reported to reduce NO production in this cell inflammatory model [37,56]. The results indicated that our identified peptides, mainly the most active ones (AGERGEQ and GSKGRPG), might exert higher anti-inflammatory effects than those reported for other anti-inflammatory peptides such as peptides SNPSVAGVR or PAY [30,31]. Moreover, peptides AGERGEQ and GSKGRPG significantly reduced the protein expression of iNOS and COX-2 in the LPS-stimulated macrophages. These peptides could be responsible for the anti-inflammatory effects of the <10 kDa fraction **[Manuscript 1]**, although it should be noted that other peptides could be involved, considering that the <10 kDa fraction is a complex mixture of different peptide sequences.

Therefore, these studies showed that pigskin collagen is a good source for the production of COX-2 inhibitory, anti-inflammatory, and antinociceptive peptides **(*Chapter 1*)**. Moreover, a peptide fraction (<10 kDa) was obtained with selective COX-2 inhibition, which also exerted acute anti-inflammatory and antinociceptive effects *in vivo* after topical application. The peptides contained in the H8p <10 kDa could exert their effects by targeting multiple components of the inflammatory response. To the best of our knowledge, this is the first time that dietary-derived hydrolysates have shown these effects after topical application, which opens doors to the use of this peptide fraction to prevent or ameliorate a focal inflammatory response, such as those observed in muscle inflammation, swelling or arthritis. Moreover, the anti-inflammatory peptides AGERGEQ and GSKGRPG, identified in H8p, may be also useful for the management of inflammatory diseases in the pharmacological sector. The collagen-derived

hydrolysate, the methodology to obtain it, and the identified peptides have already been protected by an international patent.

Inflammation is also involved in the development and progression of neurodegenerative diseases [57]. For example, in AD, an inflammatory response occurs when there is a failure in the clearance of Aβ aggregates, promoting the progression of this neurodegenerative disease [10]. AD is the most prevalent form of dementia, accounting for approximately 40 million people [58]. Unfortunately, there are currently no effective treatments for AD, and new treatments are being developed. In this context, one target of these new compounds is POP inhibition [16]. Several studies have shown that this enzyme can be inhibited by food-derived peptides or protein hydrolysates [13]. Some were obtained from agri-food by-products of animal origin containing collagen [59,60]. Moreover, food-derived peptides with POPi and neuroprotective properties typically feature at least one Pro residue in their sequence [61]. As mentioned, collagen has a high Pro content. Considering this evidence, the next objective of this doctoral thesis was to develop a hydrolysate from a chicken by-product rich in collagen that can prevent the development of AD and identify the peptides that it contains **(*Chapter 2)***. In this case, pigskin was substituted by a chicken by-product rich in collagen because the use of pigskin collagen may cause rejection in some cultures and religions as well as organoleptic consequences that might be unpleasant [62]. Chicken dominates global meat consumption and has few religious or cultural restrictions. Moreover, the chicken meat industry generates over 24 million metric tons of by-products yearly [63], and they are less valorized than pork by-products. The use of a collagen-rich by-product to obtain of bioactive peptides would reduce the impact of waste management, add value to the by-product, and contribute to the sustainability and circular economy of this sector. To achieve the proposed objective, the first step was to review the potential of chicken by-products as sources of bioactive peptides and select the best candidate for generating POPi peptides [**Manuscript 3**]. The search revealed that chicken feet are one of the chicken by-products with the highest collagen content and is rich in proteins [64]. Moreover, the potential of chicken feet for the production of POPi peptides or neuroprotective peptides has not been explored. Additionally, our research group has previously demonstrated that chicken feet, enzymatically hydrolyzed under specific conditions, are a good source of ACE inhibitory peptides with *in vivo* antihypertensive effects [65] and DPP-IV inhibitory peptides with *in vivo* anti-hyperglycemic effects [66]. Interestingly, some reported food-derived peptides and hydrolysates with POPi

activity also showed the ability to inhibit ACE or DPP-IV enzymes [60,67]. The presence of Pro in the amino acid sequence of POPi peptides as well as ACE and DPP-IVi peptides has been associated with peptide more active [27–29]. Moreover, we also observed that chicken foot hydrolysates could exert anti-inflammatory effects in LPS-stimulated RAW 264.7 cells (data not published). Therefore, chicken feet were selected to obtain protein hydrolysates with neuroprotective effects **[Manuscript 4]**. To achieve this objective, chicken feet were minced and boiled to solubilize proteins including collagen. Then, the protein broth was hydrolyzed with three different proteases (E3, E4, and E5), two different E/S ratios (C1 < C2), and two hydrolysis times (t1 < t3), resulting in twelve chicken foot hydrolysates. The hydrolysates exerted a wide range of POPi activity (0 - 76 %). Similar to the developed pigskin-collagen hydrolysates with COX-2 inhibitory activity [**Manuscript 1**], hydrolysis conditions were also crucial for the release of POPi peptides. However, in contrast to COX-2 inhibition, POP inhibition was higher with short hydrolysis times (t1), whereas the effects of E/S ratio were enzyme-dependent. Chicken-foot hydrolysates CFH3 and CFH5 exhibited the highest POP inhibition (IC_{50} = 57.9 and 40.1 µg protein/mL, respectively). This effect was stronger than that of hydrolysates obtained from wheat gluten, soy, sodium caseinate, porcine gelatin, diverse fish skins, and tuna cooking juice [61].

Considering the potential of CFH3 and CFH5, and that reported POPi hydrolysates have shown neuroprotective effects in the CL4176 *C. elegans* strain, an AD model [68], the effect of CFH3 and CFH5 hydrolysates on Aβ-induced paralysis in the *Caenorhabditis elegans* CL4176 mutant strain were studied [**Manuscript 4**]. This mutant model is characterized by inducible expression of Aβ peptides when exposed to 25 ºC. The accumulation of Aß peptides in muscle cells hinders the mobility of the worm, triggering paralysis [69]. Interestingly, hydrolysate CFH3 delayed Aβ-induced paralysis in this AD animal model in a concentration-dependent manner, reducing the content of paralyzed worms by 20 % (at 1 and 5 mg/mL) compared with non-treated worms at the end of the experiment. In contrast, CFH5 and unhydrolyzed chicken broth did not exert this effect, indicating that the bioactivity of CFH3 was due to the specific peptides generated during the hydrolysis process.

Finally, the identification of the peptides responsible for the neuroprotective effects of CFH3 was performed. The peptides contained in CFH3 were fractionated using ultrafiltration and RP-HPLC and 16 fractions were collected during the last separation. Fraction F15 had the greatest POPi activity, and its peptides were isolated and identified

using UHPLC-Orbitrap MS/MS. In total, 660 amino acid sequences were identified. 23 amino acid sequences were selected based on ion intensity and a Mascot Ion Score ≥40. This probability-based scoring system for an MS/MS match is defined as $-10 \times \log(p)$, where p indicates the likelihood that the observed match between the experimental data and database sequence is a random occurrence [70]. Only 13 % of the identified peptides were from collagen chains, despite CFH3 containing 68.8 % collagen. This suggests that the hydrolysis conditions used might be insufficient to hydrolyze the complex collagen structure to obtain peptides with molecular weights lower than 3 kDa. This is in agreement with the results observed for the COX-2 inhibitory hydrolysate (H8p), as collagen hydrolysis required prolonged hydrolysis times to generate bioactive peptides [**Manuscript 1**]. CFH3 was obtained at a lower hydrolysis time (more than 15 h less than H8p). Although the bioactivity of these peptides has not yet been analyzed, there is evidence that some of them might exert bioactivity. In this regard, 65 % of the identified peptides contained the amino acid Pro in their structures. POP is a serine endopeptidase that hydrolyzes the -Pro-X- bond in peptides up to 30 amino acids in length, where X is an amino acid different from Pro [71,72]. In addition, the PeptideRanker score, which calculates the probability of a peptide exerting any bioactivity, showed that nine peptides (PGENGLPGLPGARGEAGIPGPQG, PLRGPAL, LPGLGAL, LPGALGL, VGDPNWFPK, SADPEPGPGIDL, GERGFPGLPGPS, IDTWEKPF, and NLADDIMR) could have bioactivity. None of the identified sequences have been previously reported in the BIOPEP-UWM database [54] (consulted 26/03/2024). However, further research is needed to evaluate whether these identified peptides are responsible for the POPi and neuroprotective effects of CFH3.

Therefore, the studies collected in ***Chapter 2*** showed that chicken feet subjected to specific hydrolysis conditions are a good source for obtaining POPi hydrolysates. Moreover, CFH3 showed neuroprotective effects by delaying the Aβ-induced paralysis of the *Caenorhabditis elegans* CL4176 mutant strain, indicating its potential to be used as a functional ingredient in the management of AD. Moreover, nine peptides identified in the hydrolysate might be responsible for the bioactivity of this hydrolysate, although further research is needed to confirm this. Nowadays, the potential of protecting this hydrolysate under patent is being evaluated.

Considering the results obtained in this doctoral thesis, it was demonstrated that collagen-rich by-products such as pigskin and chicken feet subjected to specific hydrolysis conditions can release bioactive peptides for the prevention of inflammation

and inflammation-related diseases (AD). The results presented in this thesis endorse the potential use of pigskin-collagen hydrolysate H8 and chicken foot hydrolysate CFH3 as novel preventive tools for localized inflammation and neuroprotection, respectively. Moreover, the use of agri-food by-products for this finality helps to reduce waste and contributes to the sustainability and circular economy.

REFERENCES

1. Medzhitov, R. Origin and Physiological Roles of Inflammation. *Nature* **2008**, *454*, 428–435.
2. Lee, J.; Abbas, A.A.; Cheah, T.; Simanjuntak, R.N.; Sockalingam, S.; Roohi, S. Topical Nonsteroidal Anti-inflammatory Drugs for Management of Osteoarthritis Pain: A Consensus Recommendation. *Journal of Orthopaedic Research* **2023**, *41*, 1916–1924.
3. Beaugerie, L.; Itzkowitz, S.H. Cancers Complicating Inflammatory Bowel Disease. *New England Journal of Medicine* **2015**, *372*, 1441–1452.
4. Sun, S.-C. The Non-Canonical NF-KB Pathway in Immunity and Inflammation. *Nat Rev Immunol* **2017**, *17*, 545–558.
5. Lee, Y.; Rodriguez, C.; Dionne, R. The Role of COX-2 in Acute Pain and the Use of Selective COX-2 Inhibitors for Acute Pain Relief. *Curr Pharm Des* **2005**, *11*, 1737–1755.
6. Williams, J.A.; Shacter, E. Regulation of Macrophage Cytokine Production by Prostaglandin E2. *Journal of Biological Chemistry* **1997**, *272*, 25693–25699.
7. Everts, B.; Währborg, P.; Hedner, T. COX-2-Specific Inhibitors--the Emergence of a New Class of Analgesic and Anti-Inflammatory Drugs. *Clin Rheumatol* **2000**, *19*, 331–343.
8. Süleyman, H.; Demircan, B.; Karagöz, Y. Anti-Inflammatory and Side Effects of Cyclooxygenase Inhibitors. *Pharmacological Reports* **2007**, *59*, 247–258.
9. Ju, Z.; Li, M.; Xu, J.; Howell, D.C.; Li, Z.; Chen, F.E. Recent Development on COX-2 Inhibitors as Promising Anti-Inflammatory Agents: The Past 10 Years. *Acta Pharm Sin B* **2022**, *12*, 2790–2807.
10. Heneka, M.T.; Carson, M.J.; Khoury, J. El; Landreth, G.E.; Brosseron, F.; Feinstein, D.L.; Jacobs, A.H.; Wyss-Coray, T.; Vitorica, J.; Ransohoff, R.M.; et al. Neuroinflammation in Alzheimer's Disease. *Lancet Neurol* **2015**, *14*, 388–405.
11. Zhang, W.; Xiao, D.; Mao, Q.; Xia, H. Role of Neuroinflammation in Neurodegeneration Development. *Signal Transduction and Targeted Therapy 2023 8:1* **2023**, *8*, 1–32.
12. Natunen, T.A.; Gynther, M.; Rostalski, H.; Jaako, K.; Jalkanen, A.J. Extracellular Prolyl Oligopeptidase Derived from Activated Microglia Is a Potential Neuroprotection Target. *Basic Clin Pharmacol Toxicol* **2019**, *124*, 40–49.
13. Taraszkiewicz, A.; Sinkiewicz, I.; Sommer, A.; Staroszczyk, H. The Biological Role of Prolyl Oligopeptidase and the Procognitive Potential of Its Peptidic Inhibitors from Food Proteins. *Crit Rev Food Sci Nutr* **2023**, 1–14.
14. García-Horsman, J.A. The Role of Prolyl Oligopeptidase, Understanding the Puzzle. *Ann Transl Med* **2020**, *8*, 983–983.
15. Hannula, M.J.; Myöhänen, T.T.; Tenorio-Laranga, J.; Männistö, P.T.; Garcia-Horsman, J.A. Prolyl Oligopeptidase Colocalizes with α-Synuclein, β-Amyloid, Tau Protein and Astroglia in the Post-Mortem Brain Samples with Parkinson's and Alzheimer's Diseases. *Neuroscience* **2013**, *242*, 140–150.
16. Svarcbahs, R.; Julku, U.; Kilpeläinen, T.; Kyyrö, M.; Jäntti, M.; Myöhänen, T.T. New Tricks of Prolyl Oligopeptidase Inhibitors – A Common Drug Therapy for Several Neurodegenerative Diseases. *Biochem Pharmacol* **2019**, *161*, 113–120.
17. Ibarz-Blanch, N.; Alcaide-Hidalgo, J.M.; Cortés-Espinar, A.J.; Albi-Puig, J.; Suárez, M.; Mulero, M.; Morales, D.; Bravo, F.I. Chicken Slaughterhouse By-Products: A Source of Protein Hydrolysates to Manage Non-Communicable Diseases. *Trends Food Sci Technol* **2023**, *139*, 104125.

18. Tawalbeh, D.; Al-U'datt, M.H.; Wan Ahmad, W.A.N.; Ahmad, F.; Sarbon, N.M. Recent Advances in In Vitro and In Vivo Studies of Antioxidant, ACE-Inhibitory and Anti-Inflammatory Peptides from Legume Protein Hydrolysates. *Molecules* **2023**, *28*, 2423.
19. Liu, H.; Yang, Y.; Liu, Y.; Cui, L.; Fu, L.; Li, B. Various Bioactive Peptides in Collagen Hydrolysate from Salmo Salar Skin and the Combined Inhibitory Effects on Atherosclerosis in Vitro and in Vivo. *Food Research International* **2022**, *157*, 111281.
20. Li, M.; Ge, Q.; Du, H.; Lin, S. Tricholoma Matsutake-Derived Peptides Ameliorate Inflammation and Mitochondrial Dysfunction in RAW264.7 Macrophages by Modulating the NF-KB/COX-2 Pathway. *Foods* **2021**, *10*.
21. Liu, Y.-F.; Oey, I.; Bremer, P.; Carne, A.; Silcock, P. Bioactive Peptides Derived from Egg Proteins: A Review. *Crit Rev Food Sci Nutr* **2018**, *58*, 2508–2530.
22. Punia, H.; Tokas, J.; Malik, A.; Sangwan, S.; Baloda, S.; Singh, N.; Singh, S.; Bhuker, A.; Singh, P.; Yashveer, S.; et al. Identification and Detection of Bioactive Peptides in Milk and Dairy Products: Remarks about Agro-Foods. *Molecules* **2020**, *25*, 3328.
23. Chatterjee, C.; Gleddie, S.; Xiao, C.-W. Soybean Bioactive Peptides and Their Functional Properties. *Nutrients* **2018**, *10*, 1211.
24. Mora, L.; Reig, M.; Toldrá, F. Bioactive Peptides Generated from Meat Industry By-Products. *Food Research International* **2014**, *65*, 344–349.
25. Ramakrishnan, S.R.; Jeong, C.-R.; Park, J.-W.; Cho, S.-S.; Kim, S.-J. A Review on the Processing of Functional Proteins or Peptides Derived from Fish By-Products and Their Industrial Applications. *Heliyon* **2023**, *9*, e14188.
26. Ribeiro, T.B.; Voss, G.B.; Coelho, M.C.; Pintado, M.E. Food Waste and By-Product Valorization as an Integrated Approach with Zero Waste: Future Challenges. *Future Foods: Global Trends, Opportunities, and Sustainability Challenges* **2022**, 569–596.
27. Lawandi, J.; Gerber-Lemaire, S.; Juillerat-Jeanneret, L.; Moitessier, N. Inhibitors of Prolyl Oligopeptidases for the Therapy of Human Diseases: Defining Diseases and Inhibitors. *J Med Chem* **2010**, *53*, 3423–3438.
28. Daskaya-Dikmen, C.; Yucetepe, A.; Karbancioglu-Guler, F.; Daskaya, H.; Ozcelik, B. Angiotensin-I-Converting Enzyme (ACE)-Inhibitory Peptides from Plants. *Nutrients* **2017**, *9*.
29. Li-Chan, E.C.Y.; Hunag, S.L.; Jao, C.L.; Ho, K.P.; Hsu, K.C. Peptides Derived from Atlantic Salmon Skin Gelatin as Dipeptidyl-Peptidase IV Inhibitors. *J Agric Food Chem* **2012**, *60*, 973–978.
30. Inkanuwat, A.; Sukaboon, R.; Reamtong, O.; Asawanonda, P.; Pattaratanakun, A.; Saisavoey, T.; Sangtanoo, P.; Karnchanatat, A. Nitric Oxide Synthesis Inhibition and Anti-Inflammatory Effect of Polypeptide Isolated from Chicken Feather Meal in Lipopolysaccharide-Stimulated RAW 264.7 Macrophages. *Food Technol Biotechnol* **2019**, *57*, 200–212.
31. Ahn, C.-B.; Cho, Y.-S.; Je, J.-Y. Purification and Anti-Inflammatory Action of Tripeptide from Salmon Pectoral Fin Byproduct Protein Hydrolysate. *Food Chem* **2015**, *168*, 151–156.
32. Hayes, M.; Aluko, R.E.; Aurino, E.; Mora, L. Generation of Bioactive Peptides from Porphyridium Sp. and Assessment of Their Potential for Use in the Prevention of Hypertension, Inflammation and Pain. *Mar Drugs* **2023**, *21*, 422.
33. Galland, F.; de Espindola, J.S.; Lopes, D.S.; Taccola, M.F.; Pacheco, M.T.B. Food-Derived Bioactive Peptides: Mechanisms of Action Underlying Inflammation and Oxidative Stress in the Central Nervous System. *Food Chemistry Advances* **2022**, *1*, 100087.

34. Dias Campos, L.; De Almeida, V.; Junior, S.; Demuner Pimentel, J.; Lusi, G.; Carregã, F.; Baú, C.; Cazarin, B. Collagen Supplementation in Skin and Orthopedic Diseases: A Review of the Literature. *Heliyon* **2023**, *9*, 2405–8440.
35. Zielińska, E.; Baraniak, B.; Karaś, M. Antioxidant and Anti-Inflammatory Activities of Hydrolysates and Peptide Fractions Obtained by Enzymatic Hydrolysis of Selected Heat-Treated Edible Insects. *Nutrients* **2017**, *9*, 970.
36. Millán-Linares, M.D.C.; Yust, M.D.M.; Alcaide-Hidalgo, J.M.; Millán, F.; Pedroche, J. Lupine Protein Hydrolysates Inhibit Enzymes Involved in the Inflammatory Pathway. *Food Chem* **2014**, *151*, 141–147.
37. Rivera-Jiménez, J.; Berraquero-García, C.; Pérez-Gálvez, R.; García-Moreno, P.J.; Espejo-Carpio, F.J.; Guadix, A.; Guadix, E.M. Peptides and Protein Hydrolysates Exhibiting Anti-Inflammatory Activity: Sources, Structural Features and Modulation Mechanisms. *Food Funct* **2022**, *13*, 12510–12540.
38. Hong, H.; Fan, H.; Chalamaiah, M.; Wu, J. Preparation of Low-Molecular-Weight, Collagen Hydrolysates (Peptides): Current Progress, Challenges, and Future Perspectives. *Food Chem* **2019**, *301*, 125222.
39. Rocha-González, H.I.; Sánchez-Mendoza, M.E.; Cruz-Antonio, L.; Flores-Murrieta, F.J.; Cornelio-Huerta, X.I.; Arrieta, J. Antinociceptive Interaction and Pharmacokinetics of the Combination Treatments of Methyleugenol Plus Diclofenac or Ketorolac. *Molecules* **2020**, *25*, 5106.
40. Kumar, S.; Zakrewsky, M.; Chen, M.; Menegatti, S.; Muraski, J.A.; Mitragotri, S. Peptides as Skin Penetration Enhancers: Mechanisms of Action. *Journal of Controlled Release* **2015**, *199*, 168–178.
41. Facchin, B.M.; dos Reis, G.O.; Vieira, G.N.; Mohr, E.T.B.; da Rosa, J.S.; Kretzer, I.F.; Demarchi, I.G.; Dalmarco, E.M. Inflammatory Biomarkers on an LPS-Induced RAW 264.7 Cell Model: A Systematic Review and Meta-Analysis. *Inflammation Research* **2022**, *71*, 741–758.
42. Kim, H.S.; Lee, J.H.; Moon, S.H.; Ahn, D.U.; Paik, H.-D. Ovalbumin Hydrolysates Inhibit Nitric Oxide Production in LPS-Induced RAW 264.7 Macrophages. *Food Sci Anim Resour* **2020**, *40*, 274–285.
43. Guevara, M.; Tejera, E.; Iturralde, G.A.; Jaramillo-Vivanco, T.; Granda-Albuja, M.G.; Granja-Albuja, S.; Santos-Buelga, C.; González-Paramás, A.M.; Álvarez-Suarez, J.M.; Américas, L.; et al. Anti-Inflammatory Effect of the Medicinal Herbal Mixture Infusion, Horchata, from Southern Ecuador against LPS-Induced Cytotoxic Damage in RAW 264.7 Macrophages. *Food Chem Toxicol* **2019**, *131*, 110594.
44. Spiller, F.; Oliveira Formiga, R.; Fernandes da Silva Coimbra, J.; Alves-Filho, J.C.; Cunha, T.M.; Cunha, F.Q. Targeting Nitric Oxide as a Key Modulator of Sepsis, Arthritis and Pain. *Nitric Oxide* **2019**, *89*, 32–40.
45. Sofidiya, M.O.; Imeh, E.; Ezeani, C.; Aigbe, F.R.; Akindele, A.J. Antinociceptive and Anti-Inflammatory Activities of Ethanolic Extract of Alafia Barteri. *Revista Brasileira de Farmacognosia* **2014**, *24*, 348–354.
46. Islam, S.; Shajib, Md.S.; Rashid, R. Bin; Khan, M.F.; Al-Mansur, Md.A.; Datta, B.K.; Rashid, M.A. Antinociceptive Activities of Artocarpus Lacucha Buch-Ham (Moraceae) and Its Isolated Phenolic Compound, Catechin, in Mice. *BMC Complement Altern Med* **2019**, *19*, 214.
47. Galehdar, N.; Rezaeifar, M.; Rezaeifar, M.; Rezaeifar, M. Antinociceptive and Anti-Inflammatory Effects of Amygdalus Eburnea Shell Root Extract in Mice. *Biomedical Research and Therapy* **2018**, *5*, 2746–2751.

48. European Medicines Agency. New Safety Advice for Diclofenac-CMDh Endorses PRAC Recommendation. *Lancet Publishing Group* **2013**,Vol. 382.
49. Langford, D.J.; Mogil, J.S. Pain Testing in the Laboratory Mouse. In *Anesthesia and Analgesia in Laboratory Animals*; Fish, Richard.E., Brown, M.J., Danneman, P.J., Karas, A.Z., Eds.; *Elsevier*, **2008**; pp. 549–560.
50. Narayana Raju, K.V.S.; Ashok Kumar, D.; Arutselvan, N.; Thejomoorthy, P.; Puvanakrishnan, R. Antinociceptive and Antipyretic Effects of a Derivatized Tetrapeptide from Lactoferrin in Rats. *Peptides (N.Y.)* **2005**, *26*, 615–619.
51. Tavares, T.G.; Spindola, H.; Longato, G.; Pintado, M.E.; Carvalho, J.E.; Malcata, F.X. Antinociceptive and Anti-Inflammatory Effects of Novel Dietary Protein Hydrolysate Produced from Whey by Proteases of Cynara Cardunculus. *Int Dairy J* **2013**, *32*, 156–162.
52. Gao, R.; Shen, Y.; Shu, W.; Bai, F.; Jin, W.; Wang, J.; Yuan, L. Optimization of Enzymatic Conditions of Sturgeon Muscles and Their Anti-Inflammatory Potential. **2020**.
53. Dini, I.; Mancusi, A. Food Peptides for the Nutricosmetic Industry. *Antioxidants* **2023**, *12*, 788.
54. Minkiewicz, P.; Iwaniak, A.; Darewicz, M. BIOPEP-UWM Database of Bioactive Peptides: Current Opportunities. *Int J Mol Sci* **2019**, *20*.
55. Zielińska, E.; Baraniak, B.; Karaś, M. Identification of Antioxidant and Anti-inflammatory Peptides Obtained by Simulated Gastrointestinal Digestion of Three Edible Insects Species (*Gryllodes Sigillatus*, *Tenebrio Molitor*, *Schistocerca Gragaria*). *Int J Food Sci Technol* **2018**, *53*, 2542–2551.
56. Abril, A.G.; Pazos, M.; Villa, T.G.; Calo-Mata, P.; Barros-Velázquez, J.; Carrera, M. Proteomics Characterization of Food-Derived Bioactive Peptides with Anti-Allergic and Anti-Inflammatory Properties. *Nutrients* **2022**, *14*.
57. Lee, Y.-J.; Han, S.B.; Nam, S.-Y.; Oh, K.-W.; Hong, J.T. Inflammation and Alzheimer's Disease. *Arch Pharm Res* **2010**, *33*, 1539–1556.
58. Reitz, C.; Pericak-Vance, M.A.; Foroud, T.; Mayeux, R. A Global View of the Genetic Basis of Alzheimer Disease. *Nat Rev Neurol* **2023**, *19*, 261–277.
59. Lajmi, K.; Gómez-Estaca, J.; Hammami, M.; Martínez-Alvarez, O. Upgrading Collagenous Smooth Hound By-Products: Effect of Hydrolysis Conditions, in Vitro Gastrointestinal Digestion and Encapsulation on Bioactive Properties. *Food Biosci* **2019**, *28*, 99–108.
60. Martínez-Alvarez, O.; Batista, I.; Ramos, C.; Montero, P. Enhancement of ACE and Prolyl Oligopeptidase Inhibitory Potency of Protein Hydrolysates from Sardine and Tuna By-Products by Simulated Gastrointestinal Digestion. *Food Funct* **2016**, *7*, 2066–2073.
61. Hsieh, C.-H.; Wang, T.-Y.; Hung, C.-C.; Hsieh, Y.-L.; Hsu, K.-C. Isolation of Prolyl Endopeptidase Inhibitory Peptides from a Sodium Caseinate Hydrolysate. *Food Funct* **2016**, *7*, 565–573.
62. Gómez-Guillén, M.C.; Giménez, B.; López-Caballero, M.E.; Montero, M.P. Functional and Bioactive Properties of Collagen and Gelatin from Alternative Sources: A Review. *Food Hydrocoll* **2011**, *25*, 1813–1827.
63. Food and Agriculture Organization of the United Nations, (FAO) POULTRY DEVELOPMENT REVIEW. **2013**.
64. Bravo, F.I.; Calvo, E.; López-Villalba, R.A.; Torres-Fuentes, C.; Muguerza, B.; García-Ruiz, A.; Morales, D. Valorization of Chicken Slaughterhouse Byproducts to Obtain Antihypertensive Peptides. *Nutrients* **2023**, *15*.

65. Bravo, F.I.; Mas-Capdevila, A.; Margalef, M.; Arola-Arnal, A.; Muguerza, B. Novel Antihypertensive Peptides Derived from Chicken Foot Proteins. *Mol Nutr Food Res* **2019**, *63*, 1801176.
66. Casanova-Martí, À.; Bravo, F.I.; Serrano, J.; Ardévol, A.; Pinent, M.; Muguerza, B. Antihyperglycemic Effect of a Chicken Feet Hydrolysate *via* the Incretin System: DPP-IV-Inhibitory Activity and GLP-1 Release Stimulation. *Food Funct* **2019**, *10*, 4062–4070.
67. Suwanangul, S.; Aluko, R.E.; Sangsawad, P.; Kreungngernd, D.; Ruttarattanamongkol, K. Antioxidant and Enzyme Inhibitory Properties of Sacha Inchi (*Plukenetia Volubilis*) Protein Hydrolysate and Its Peptide Fractions. *J Food Biochem* **2022**, *46*, e14464.
68. Martorell, P.; Bataller, E.; Llopis, S.; Gonzalez, N.; Alvarez, B.; Montón, F.; Ortiz, P.; Ramón, D.; Genovés, S. A Cocoa Peptide Protects Caenorhabditis Elegans from Oxidative Stress and β-Amyloid Peptide Toxicity. *PLoS One* **2013**, *8*, e63283.
69. Beeg, M.; Diomede, L.; Stravalaci, M.; Salmona, M.; Gobbi, M. Novel Approaches for Studying Amyloidogenic Peptides/Proteins. *Curr Opin Pharmacol* **2013**, *13*, 797–801.
70. Bouyssié, D.; Gonzalez de Peredo, A.; Mouton, E.; Albigot, R.; Roussel, L.; Ortega, N.; Cayrol, C.; Burlet-Schiltz, O.; Girard, J.P.; Monsarrat, B. Mascot File Parsing and Quantification (MFPaQ), a New Software to Parse, Validate, and Quantify Proteomics Data Generated by ICAT and SILAC Mass Spectrometric Analyses: Application to the Proteomics Study of Membrane Proteins from Primary Human Endothelial Cells. *Molecular and Cellular Proteomics* **2007**, *6*, 1621–1637.
71. Cunningham, D.F.; O'Connor, B. A Study of Prolyl Endopeptidase in Bovine Serum and Its Relevance to the Tissue Enzyme. *Int J Biochem Cell Biol* **1998**, *30*, 99–114.
72. Myöhänen, T.T.; García-Horsman, J.A.; Tenorio-Laranga, J.; Männistö, P.T. Issues about the Physiological Functions of Prolyl Oligopeptidase Based on Its Discordant Spatial Association with Substrates and Inconsistencies among MRNA, Protein Levels, and Enzymatic Activity. *Journal of Histochemistry and Cytochemistry* **2009**, *57*, 831–848.

CONCLUSIONS

UNIVERSITAT ROVIRA I VIRGILI
PROTEIN HYDROLYSATES AND PEPTIDES FROM ANIMAL BY-PRODUCTS AS A PREVENTIVE STRATEGY FOR LOCAL INFLAMMATION AND NEUROINFLAMMATION
Néstor Ibarz Blanch

1. The hydrolysis of pigskin collagen under optimized conditions (enzyme E1, time t2, and E/S C2) resulted in the obtainment of the hydrolysate H8, which showed a high COX-2 inhibitory activity.
2. The COX-2 bioactivity of the hydrolysate (H8) was maintained when it was elaborated at the pilot scale and its inhibitory activity was selective for COX-2.
3. The <10 kDa fraction of pigskin-collagen H8 exerted acute anti-inflammatory effects in a carrageenan-induced paw edema model and antinociceptive effects in a tail-withdrawal model after topical application and at a low dose.
4. The reduction in NO production, and iNOS and COX-2 protein expression as well as inhibition of COX-2 activity were anti-inflammatory mechanisms underlying the effect of the H8 <10 kDa fraction in LPS-stimulated RAW 264.7 cells.
5. Peptides AGERGEQ and GSKGRPG could be involved in the anti-inflammatory effect of the < 10 kDa fraction of H8 in LPS-stimulated RAW 264.7 cells.
6. Chicken-foot hydrolysates showed POPi activity under specific hydrolysis conditions.
7. Chicken-foot hydrolysates CFH3 and CFH5, produced by different enzymes, showed the highest POPi activity *in vitro*.
8. Chicken-foot hydrolysate CFH3 delayed Aß-induced paralysis in the CL4176 *C. elegans* mutant strain in a concentration-dependent manner, showing its neuroprotective effects.
9. Nine peptides were identified in the chicken-foot hydrolysate CFH3, which could be responsible for CFH3 bioactivity.

Collagen-rich by-products, in particular pigskin and chicken feet, subjected to specific hydrolysis conditions can release bioactive peptides for the prevention of inflammatory and inflammation-related diseases (such as AD). The results presented in this thesis endorse the potential use of pigskin-collagen hydrolysate H8 and chicken-foot hydrolysate CFH3 as novel preventive tools for localized inflammation and neuroprotection, respectively. The H8 is already patented and the CFH3 is in the process of being protected too. Moreover, the use of agri-food by-products for this finality helps to reduce waste and contributes to their sustainability and circular economy.

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LIST OF PUBLICATIONS

A) Published Papers

Ibarz-Blanch, N.; Morales, D.; Calvo, E.; Ros-Medina, L.; Muguerza, B.; Bravo, F.I.; Suárez, M. Role of Chrononutrition in the Antihypertensive Effects of Natural Bioactive Compounds. *Nutrients* **2022**, *14*.Impact Factor (2022) 5.9. SI Journal Citation Reports © Ranking 17/88 (Q1) Nutrition & Dietetics.

Ibarz-Blanch, N.; Alcaide-Hidalgo, J.M.; Cortés-Espinar, A.J.; Albi-Puig, J.; Suárez, M.; Mulero, M.; Morales, D.; Bravo, F.I. Chicken Slaughterhouse By-Products: A Source of Protein Hydrolysates to Manage Non-Communicable Diseases. *Trends Food Sci Technol* **2023**, *139*, 104125. Impact Factor (2022): 15.3. SI Journal Citation Reports © Ranking 2/142 (Decile 1) in Food Science & Technology.

Cortés-Espinar, A.J.; **Ibarz-Blanch, N**.; Soliz-Rueda, J.R.; Calvo, E.; Bravo, F.I.; Mulero, M.; Ávila-Román, J. Abrupt Photoperiod Changes Differentially Modulate Hepatic Antioxidant Response in Healthy and Obese Rats: Effects of Grape Seed Proanthocyanidin Extract (GSPE). *Int J Mol Sci* **2023**, *24*, 17057. Impact Factor (2022): 5.6. SI SI Journal Citation Reports © Ranking 66/285 (Q1) in Biochemistry & Molecular Biology.

Cortés-Espinar, A.J.; **Ibarz-Blanch, N**.; Soliz-Rueda, J.R.; Bonafos, B.; Feillet-Coudray, C.; Casas, F.; Bravo, F.I.; Calvo, E.; Ávila-Román, J.; Mulero, M. Rhythm and ROS: Hepatic Chronotherapeutic Features of Grape Seed Proanthocyanidin Extract Treatment in Cafeteria Diet-Fed Rats. Antioxidants (Basel) 2023, 12(8) 1606. Impact Factor (2022): 7.0. SI Journal Citation Reports © Ranking 13/142 (Decile 1) in Food Science & Technology.

B) In preparation Papers

Ibarz-Blanch, N.; Escoté, X.; Calvo, E, Cortés-Espinar, A.J.; del Bas, J.M.,; Mulero, M.; Muguerza, B.; Bravo,F.I. Anti-inflammatory and anti-nociceptive effects of a selective COX-2 inhibitory collagen hydrolysate fraction.

Ibarz-Blanch, N.; Alcaide-Hidalgo, J.M.; Calvo, E, Cortés-Espinar, A.J.; Mulero, M.; Muguerza, B.; Bravo,F.I. Pigskin collagen peptides alleviate LPS-induced inflammatory response in RAW 264.7 macrophages.

Ibarz-Blanch, N.; Garzón-García, L.; Muguerza, B.; Santos-Buelga**, C. ;** González-Paramás, A.M.; Bravo,F.I A POPi chicken-foot hydrolysate delayed amyloid-β-induced paralysis in *Caenorhabditis elegans* and identification of its peptides.

C) Patents

F. I. Bravo Vázquez, **N. Ibarz Blanch**, C. Urueña Díaz, N. Hospital Ramón, M. Mulero Abellán, X. Escoté Miró, J.M Alcaide-Hidalgo, J.M del Bas Prior, R. M. Raventós Colomer, B. Muguerza. Hydrolyzed collagen, process for its preparation and uses thereof. Application number: P220144/IT/MBE. Priority date: 30/10/2022.

R. Subirós, A. Soley Astals, N. Alminana Domènech, C. Bonell, C. Garcia Hernández, C. Youssif, S. Méndez, **N. Ibarz Blanch**, B. Muguerza, F. I. Bravo Vázquez. Protein hydrolysates of azuki beans and cosmetic uses thereof Application number: EP22382248.7. Priority date: 16/03/2022. PCT/IB2023/052524 Title-holder entity: Lubrizol. Advanced Materials, Inc.

N. Ibarz Blanch, L. Garzón García, B. Muguerza, C. Santos Buelga, A.M. González Paramás, F.I. Bravo. Method for obtaining a chicken foot hydrolysate with POP inhibitory activity and neuroprotective effects, the hydrolysate that is obtained, and the peptides it contains. Patent in progress: currently performing the patentability report.

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