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THIAGO SANTOS ARAÚJO

POTENCIAL BIOATIVO DE HIDROLISADOS PROTEICOS E PEPTÍDEOS DE ORIGEM AVÍCOLA

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Tese apresentada ao Programa de Pós-Graduação em Biologia Aplicada à Saúde do Centro de Biociências da Universidade Federal de Pernambuco como parte dos requisitos exigidos para obtenção do título de Doutor.

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Orientador: Profª Drª Ana Lúcia Figueiredo Porto

Coorientador: Profª Drª Maria Taciana Cavalcanti Vieira Soares

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RESUMO

A carne de aves, além de apresentar elevada qualidade nutricional, é uma excelente fonte de hidrolisados proteicos e peptídeos bioativos. O objetivo do presente trabalho foi estudar as propriedades bioativas de hidrolisados proteicos e peptídeos de origem avícola. Inicialmente, por meio de uma revisão sistemática, verificou-se a eficácia de hidrolisados e peptídeos de proteínas de frango em reduzir a pressão arterial em ratos hipertensos. Foram realizadas buscas em quatro banco de dados internacionais (Scopus, ScienceDirect, ISI Web of Science e PubMed) por artigos que avaliaram o efeito da administração de hidrolisados e peptídeos bioativos obtidos por hidrólise das proteínas de frango neste modelo animal. Foi possível verificar que os hidrolisados e peptídeos avaliados apresentaram efeitos anti-hipertensivos positivos independente das condições de hidrólise empregada no processo de obtenção. Além disso, confirmou-se, a partir dos artigos selecionados, que os hidrolisados de frango podem ser uma alternativa eficaz e mais barata para potenciais aplicações biotecnológicas se comparados aos peptídeos purificados. Uma vez que a obtenção de compostos bioativos (hidrolisados proteicos e peptídeos bioativos) a partir de outras fontes avícolas, tais como codornas ainda carece de estudos que possam permitir novos usos para estes produtos para além do seu valor alimentício, procurou-se com este trabalho realizar o primeiro estudo das propriedades antioxidantes e inibitória da enzima conversora de angiotensina de hidrolisados proteicos de *Coturnix japonica* (CJPH), além de determinar os prováveis peptídeos bioativos que podem ser formados após a sua digestão gastrointestinal *in vitro*. CJPH mostrou ser atóxico, apresentou além de uma elevada atividade antioxidante de eliminação do radical 2,2-Difenil-1-picril hidrazilo (55,07-69,28%) e uma forte inibição da enzima conversora de angiotensina (ECA) (IC_{50} = 0,10 $mg.mL^{-1}$). Embora tenha sido verificada uma baixa capacidade de eliminação do radical ácido 2,2-azinobis (3-etilbenzotiazolina-ácidosulfônico) (19,97%), após a digestão gastrointestinal *in vitro*, houve uma melhora nesta atividade em torno de 3,33 vezes. O tamanho dos peptídeos foi crucial para as atividades biológicas, os estudos cinéticos revelaram que o padrão de inibição da ECA pelo CJPH e sua fração gastrointestinal (GiPH(3)) era do tipo incompetitivo e vários potenciais peptídeos podem ser encontrados no GiPH(3). As proteínas de origem avícola são uma alternativa promissora para a produção de ingredientes funcionais.

Palavras-chave: Peptídeos bioativos. Hidrolisados proteicos. Frango. Codorna japonesa. Enzima Conversora de Angiotensina. Atividade antioxidante.

ABSTRACT

The poultry meat, besides presenting high nutritional quality, is an excellent source of protein hydrolysates and bioactive peptides. The objective of the present work was to study the functional properties of protein hydrolysates and peptides of poultry origin. Initially, through a systematic review, it was verified the efficacy of hydrolysates and peptides of chicken proteins in reducing blood pressure in hypertensive rats. Searches were performed in four international databases (Scopus, ScienceDirect, ISI Web of Science and PubMed) for articles that evaluated the effect of the administration of protein hydrolysates and bioactive peptides obtained from chicken proteins hydrolysis in rats. It was possible to verify that the hydrolysates and peptides evaluated showed positive antihypertensive effects independent of the hydrolysis conditions employed in the preparation process. In addition, it has been confirmed from the selected articles that chicken hydrolysates may be an effective and cheaper alternative for potential biotechnological applications as compared to purified peptides. Since obtaining bioactive compounds (protein hydrolysates and bioactive peptides) from other poultry sources, such as quails, still lacks studies that may allow new uses for these products beyond their food value, was carried out the first study of the antioxidant and angiotensin-converting enzyme inhibitory properties of the *Coturnix japonica* protein hydrolysates (CJPH). Besides this, the potential bioactive peptides that could be formed after *in vitro* gastrointestinal digestion was also determined. CJPH showed to be nontoxic and presented a high 2,2-Diphenyl-1-Picrylhydrazyl radical-scavenging activity (55.07-69.28%) besides a strong ACE inhibition (IC_{50} = 0.10 $mg.mL^{-1}$). Although a low capacity to scavenge the 2,2-azino-bis- (3-ethylbenzothiazoline) -6-sulphonic acid radical (19.97%) has been found, an improvement around 3.33 folds in this activity was verified after *in vitro* gastrointestinal digestion. The peptide size was crucial for the biological activities, the kinetics studies revealed that ACE inhibition pattern of CJPH and its gastrointestinal fraction (GiPH (3)) was uncompetitive and several potential peptides were found in the GiPH(3). Poultry proteins are a promising alternative for the production of functional ingredients.

Keywords: Bioactive peptides. Protein hydrolysates. Chicken. Japanese quail. Angiotensin Converting Enzyme. Antioxidant activity.

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LISTA DE ABREVIATURAS E SIGAS

ABTS	2,2-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid
ACE	Angiotensin-Converting Enzyme
ACEi	Angiotensin-Converting Enzyme inhibition
Ang I	Angiotensin I
Ang II	Angiotensin II
BW	Body weight
CA	Chromosomal aberrations
CBSH	Chicken breast skin protein hydrolysate
CJPH	*Coturnix japonica* protein hydrolysate
CTSH	Chicken thigh skin protein hydrolysate
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ECA	Enzima Conversora de Angiotensina
FA	Fatty acids
FAPGG	N-(3-[2-furyl] acryloyl)-phenylalanylglycylglycine
GiPH(3)	Gastrointestinal protein hydrolysate with 3 kDa MWCO
GiPH(3-10)	Gastrointestinal protein hydrolysate with 3-10 kDa MWCO
GPH(3)	Gastric protein hydrolysate with 3 kDa MWCO
GPH(3-10)	Gastric protein hydrolysate with 3-10 kDa MWCO
HA	Hippuric acid
HHL	Hippuryl-L-histidyl-L-leucine
HPLC	High-Performance Liquid Chromatography
MI	Mitotic index
MRG	Mean root growth

MW	Molecular weight
MWCO	Molecular weight cut-off
NC	Negative control
PA	Pressão arterial
PC	Positive control
RAS	Renin-angiotensin system
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive rat
SRA	Sistema renina-angiotensina
Trolox	6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid
UF	Ultrafiltrated
WKY	Wistar-Kyoto rat

SUMÁRIO

1 INTRODUÇÃO

A crescente a demanda por alimentos que possam influenciar de maneira positiva a nossa saúde e ajudar a prevenir doenças, tem levado a um aumento na busca por novas fontes proteicas que possam atuar como ingredientes funcionais. Neste contexto, os hidrolisados proteicos e peptídeos bioativos se caracterizam como uma alternativa promissora. Obtidos a partir da quebra de diversas proteínas alimentares, esses compostos são conhecidos por apresentar uma ampla gama de aplicações como: produção de ingredientes alimentícios de valor agregado, modulação de funções fisiológicas, servem como fonte de aminoácidos essenciais e contribuem para o metabolismo energético.

Sabe-se que os peptídeos bioativos são sequências curtas de 2-20 aminoácidos, que, embora permaneçam inativos dentro de suas proteínas "precursoras", tornam-se ativos após a sua liberação durante digestão gastrointestinal, por hidrólise enzimática, hidrólise química *in vitro* e por fermentação microbiana. Uma vez absorvidos pelo epitélio intestinal, estes fragmentos proteicos podem apresentar diversas atividades biológicas, tais como anti-hipertensiva, antioxidante, imunomoduladora, hipocolesterolemiante, anticâncer, entre outras.

Os peptídeos bioativos são vulneráveis química e fisicamente às condições de produção e ao processo de digestão, por este motivo a sua utilização em escala industrial ainda se constitui como um grande desafio tecnológico. Alguns fatores que influenciam diretamente em seus valores nutricionais e respostas biológicas incluem: as condições experimentais e o método empregado para sua obtenção. Isso porque a depender do método utilizado serão formados hidrolisados contendo peptídeos com distintos comprimentos da cadeia e composições de aminoácidos, os quais, ao serem administrados num estado livre, podem não resistir à ação das enzimas do trato gastrointestinal (pepsina e tripsina por exemplo), sofrendo degradação com consequente perda de sua atividade.

Dentre as fontes alimentares existentes, a carne de aves de produção tem-se destacado devido ao seu elevado valor nutricional e também por serem uma importante fonte de compostos bioativos (hidrolisados proteicos e peptídeos bioativos) com excelentes propriedades anti-hipertensivas *in vivo* e *in vitro* e capacidade antioxidante. Dentre os tipos de aves de produção utilizadas neste campo da ciência, os frangos comerciais sempre foram os mais utilizados, sendo assim, a obtenção de compostos bioativos a partir de outras fontes avícolas, tais como codornas e avestruzes ainda carece de estudos que possam permitir novos usos para estes produtos para além do seu valor alimentício.

1.1 OBJETIVOS

1.1.1 Objetivo geral

Estudar as propriedades bioativas de hidrolisados proteicos e peptídeos de origem avícola.

1.1.2 Objetivos específicos

a) Estudar, por meio de uma revisão sistemática, a possível eficácia relatada pelos artigos selecionados de hidrolisados e peptídeos obtidos por meio da hidrólise de proteínas de frango na redução e/ou manutenção da pressão arterial em ratos;
b) Obter hidrolisados proteicos por meio da hidrólise ácida das proteínas musculares do peito de codorna (*Coturnix japonica*);
c) Verificar a resistência dos hidrolisados de codornas japonesas frente a ação de proteases gastrointestinais;
d) Analisar as propriedades antioxidante e inibitória da Enzima Conversora da Angiotensina (ECA) dos hidrolisados do músculo do peito de codornas japonesas;
e) Avaliar os possíveis efeitos tóxicos, citotóxicos, genotóxicos e mutagênicos dos hidrolisados de codorna;
f) Correlacionar a variável peso molecular com as atividades biológicas testadas;
g) Compreender o mecanismo de inibição da Enzima Conversora de Angiotensina pelos hidrolisados proteicos de músculo do peito de codornas japonesas;
h) Determinar o perfil de peptídeos presentes nos hidrolisados de codorna japonesa após a digestão gastrointestinal *in vitro*;

2 REFERENCIAL TEÓRICO

2.1 INGREDIENTES FUNCIONAIS

O conceito de alimento funcional surgiu no Japão, no início dos anos 80, após o governo daquele país ter promovido maciços investimentos em pesquisas voltadas à prevenção de doenças ligadas ao envelhecimento da população, ao antever a elevação de casos diversas doenças crônicas (diabetes, hipertensão, câncer, doenças cardiovasculares e osteoporose). No entanto foi só no ano de 1993 que este termo foi utilizado pela primeira vez em uma publicação científica. Na época, o conceito que prevalecia era o de que para os alimentos receberem um selo de aprovação do Ministério de Saúde e Bem-estar japonês por conter ingredientes funcionais era necessário que melhorassem as condições relacionadas à saúde; apresentassem efeitos benéficos nas funções fisiológicas e promovessem a saúde (SANCHO; PASTORE, 2016).

Anos mais tarde, em 1999, o Brasil veio a se tornar o primeiro país da América Latina a possuir uma legislação sobre os alimentos funcionais (SANCHO; PASTORE, 2016). Conforme resolução nº 18, de 30 de Abril de 1999, da Agência Nacional de Vigilância Sanitária, para um alimento ou ingrediente ser considerado funcional, além das suas funções nutricionais básicas, quando consumido como parte da dieta usual, deve ser capaz de produzir efeitos metabólicos, fisiológicos ou benéficos à saúde, sendo seguro para o seu consumo sem a supervisão médica (ANVISA, 99). Sendo assim, a eficácia e segurança destes alimentos precisam necessariamente ser comprovadas (VITAL et al., 2018).

Vários trabalhos científicos têm demonstrado que uma grande variedade de constituintes dos alimentos (lipídeos, vitaminas, antioxidantes, peptídeos, entre outros) estão associados à prevenção e tratamento de doenças como câncer, hipertensão, osteoporose, diabetes, entre outras. Desta maneira, são considerados ingredientes de grande interesse para a saúde dos indivíduos (LEÓN-ESPINOSA; JIMÉNEZ-MARTÍNEZ; DÁVILA-ORTIZ, 2015; VITAL et al., 2018). Contudo vale ressaltar que a resposta do organismo à ingestão de um alimento funcional depende de fatores genéticos, do estado fisiológico e da composição da dieta (LEÓN-ESPINOSA; JIMÉNEZ-MARTÍNEZ; DÁVILA-ORTIZ, 2015).

Considerados um dos fatores responsáveis pelas grandes transformações que os conceitos relacionados à alimentação e aos alimentos passaram nas últimas décadas (SANCHO; PASTORE, 2016), estes estudos, juntamente com outros fatores-chave, tais como o aumento da expectativa de vida com o envelhecimento da população, elevação nos custos com cuidados

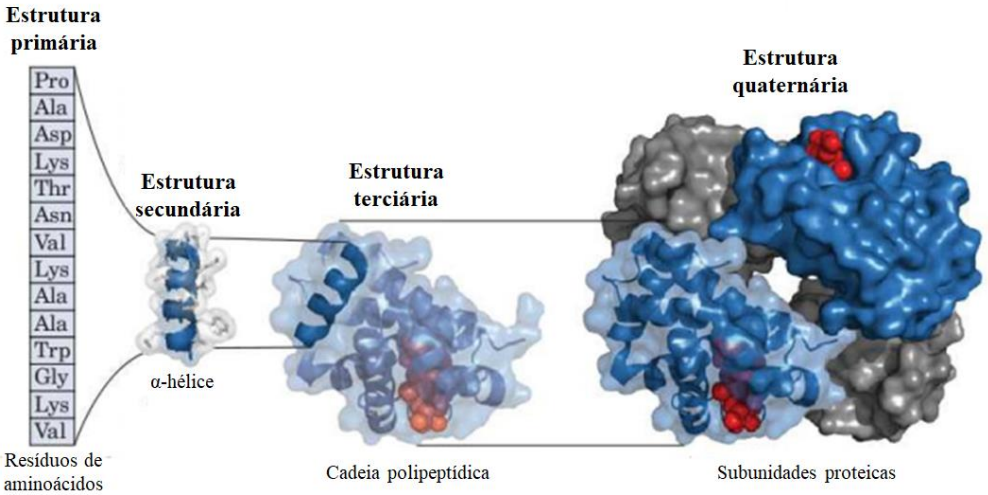
da saúde e mudanças na regulação dos alimentos, têm gerado destaque à importância do consumo de alimentos e ingredientes chamados funcionais para a saúde humana (LEÓN-ESPINOSA; JIMÉNEZ-MARTÍNEZ; DÁVILA-ORTIZ, 2015; SANCHO; PASTORE, 2016). Segundo Sancho e Pastore (2016), é interessante notar que hoje, para uma grande parte dos consumidores, alguns alimentos, além de atrativos e seguros, já são correlacionados a prevenção de doenças crônicas, além de ser capazes de contribuir para o bem-estar físico e mental.

Sabendo que é grande a demanda por estes alimentos que podem influenciar de maneira positiva a nossa saúde e ajudar a prevenir doenças, é crescente o interesse científico em encontrar cada vez novos componentes que possam atuar como ingredientes funcionais (MENDOZA-JIMÉNEZ et al., 2018; PANGESTUTI; ARIFIN, 2018).

2.2 AS PROTEÍNAS DE ORIGEM ANIMAL

As proteínas são macromoléculas fundamentais dos alimentos que sempre foram apreciadas por suas propriedades nutricionais e funcionais (FERNÁNDEZ, 2012; VALDERRAMA, 2016). Quimicamente, são polímeros (Fig. 1), cujas unidades básicas são os aminoácidos, moléculas orgânicas que contêm um grupo funcional ácido carboxílico (-COOH), um grupo amino ($-NH_2$) e um hidrogênio (-H), unidos entre si por ligações peptídicas (CENTENARO, 2011; RODRÍGUEZ, 2018).

Figura 1 – Estrutura das proteínas



A estrutura primária é definida pela sequência linear de resíduos de aminoácidos. A estrutura secundária descreve as conformações locais, por ex. α-hélices ou β-pregueada. O arranjo tridimensional da cadeia polipeptídica é a sua estrutura terciária e o arranjo tridimensional de diferentes cadeias polipeptídicas é a sua estrutura terciária. Fonte: Adaptado de Rashid, Khatib e Sattar (2015).

A carne, primeira escolha de proteína animal para muitos consumidores, é um produto pecuário valioso, pois apresenta aminoácidos essenciais em alta disponibilidade que não são normalmente encontrados em proteínas vegetais (FERNÁNDEZ, 2012; LAFARGA; HAYES, 2014; LIU et al., 2016). Essa fonte proteica pode ser dividida nas seguintes categorias: carne crua; subprodutos da carne, produzidos durante o processamento industrial (tais como ossos, pele, penas, tecidos conectivos, entre outros) e normalmente são utilizados como ração animal, fertilizantes ou alimentos para animais de estimação (FU et al., 2018) e produtos do processamento da carne (hambúrgueres, presuntos, salsichas, por exemplo) (BOURSCHEID, 2015; LIU et al., 2016; TOLDRÁ et al., 2018).

Em relação a carne crua, sabe-se que podem ser classificadas amplamente como proteínas sarcoplasmáticas, proteínas miofibrilares e proteínas estromáticas (insolúveis em sal) (LIU et al., 2016). As proteínas miofibrilares correspondem à miosina, actina, proteína C, proteína M, tropomiosina, α-actina, β-actina e γ-actina. Representam 52 a 56% das proteínas musculares e formam os miofilamentos grossos e finos que constituem a miofibrila, organela que desempenha a função de contração muscular (ALVES; GOES; MANCIO, 2005; COSTA, 2009). As proteínas sarcoplasmáticas constituem 30 a 35% das proteínas musculares e correspondem à mioglobina, todas as enzimas da glicólise e a maior parte das enzimas da síntese de carboidratos e de proteínas (ALVES; GOES; MANCIO, 2005; COSTA, 2009). Apresentam como função principal proporcionar energia imediata para a contração muscular e podem atuar anaerobicamente (COSTA, 2009). As proteínas estromáticas ou proteínas do tecido conjuntivo representam de 10 a 15% de toda a proteína dos músculos esqueléticos e são menos solúveis, quando comparadas às proteínas sarcoplasmáticas. Colágeno, proteína estrutural do tecido conjuntivo, e elastina são as duas proteínas que representam a maior parte da fração proteica estromática (ALVES; GOES; MANCIO, 2005; COSTA, 2009).

Muito embora o consumo de carne possa estar tradicionalmente associado ao aumento dos ricos de câncer, obesidade e outras doenças (SÁNCHEZ; VÁZQUEZ, 2017), algumas proteínas presentes nesta fonte alimentar possuem importantes atividades fisiológicas. O colágeno, por exemplo, influencia positivamente a liberação e bioatividade da proteína morfogenética óssea-2 e a formação ectópica de tecidos ósseos, melhorando a cicatrização deste tecido (LAFARGA; HAYES, 2014). A carnosina e a anserina, peptídeos antioxidantes, existem endogenamente no tecido muscular e atuam como sequestradores de radicais livres e quelantes de íons metálicos (LIU et al., 2016).

2.2.1 As proteínas da carne de origem avícola

A carne de aves se destaca por ter uma aceitação quase universal entre as diversas culturas e tradições culinárias (CENTENARO, 2011) e a evolução no seu consumo, ao longo dos anos, está principalmente vinculada ao crescente interesse do consumidor na relação entre saúde, prevenção de doenças e bem-estar (KALAFOVA et al., 2018; KIM; DO; CHUNG, 2017; TAVANIELLO et al., 2017).

Segundo maior produtor mundial, ficando atrás apenas dos Estados Unidos, o Brasil, em 2017, produziu cerca de 13,05 milhões de toneladas de carne de frango, de acordo com os dados divulgados pela Associação Brasileira de Proteína Animal – ABPA (ABPA, 2018; FRANCO, 2017). Desse total, cerca de 66,9% permaneceram no mercado interno e 33,1% foram destinados à exportação, o que levou ao país a posição de maior exportador mundial, chegando a marca de 4,32 milhões de toneladas de carne de frango exportadas (ABPA, 2018). Dentre os Estados brasileiros, o Paraná é o maior produtor e exportador de frango do país, tendo comercializado, no ano de 2016, U$ 2,1 bilhões, o que equivale a 1,4 milhões de toneladas de carne de frango *in natura* (FRANCO, 2017).

No tocante a produção de codornas no país (coturnicultura), esta atividade, por exigir um baixo investimento, com rápido retorno econômico, se comparada à avicultura de frangos de corte, tem apresentado, ao longo dos anos, um desenvolvimento bastante acentuado (SILVA et al., 2018; SILVEIRA, 2018). Ao adequar-se as novas tecnologias de produção, a coturnicultura no Brasil passou de um atividade tida como de subsistência, para uma atividade altamente tecnificada com resultados promissores aos investidores (PASTORE; OLIVEIRA; MUNIZ, 2012). De acordo com Pastore, Oliveira e Muniz (2012), apesar do baixo consumo de carne de codorna per capta ano, o Brasil produz uma quantidade significativa de carne dessa ave. Segundo Silveira (2018) e com base nos dados publicados pelo Instituto Brasileiro de Geografia e Estatística (IBGE), o efetivo de codornas no país, em 2015, foi de 21,99 milhões de aves, estando o país entre os 10 maiores produtores de carne de codorna no cenário mundial. Os maiores centros coturnicultores brasileiros estão nos estados de Minas Gerais e São Paulo, porém há presença da criação de codornas em outras regiões do Brasil, porém há presença da criação de codornas em outras regiões do Brasil (SILVA et al., 2018).

Em geral, a composição da carne de aves (valores nutricionais) depende de seu sexo, idade, dieta e principalmente de sua espécie (CENTENARO, 2011). Os quadros 1 e 2 mostram, respectivamente, as diferenças nutricionais e o conteúdo de aminoácidos e proteínas entre a

carne crua do peito de frango e de codorna. Em resumo, ambas as carnes são consideradas de boa qualidade nutricional, tanto pelos seus elevados teores proteicos, quanto aos seus baixos níveis de colesterol e teores de ácidos graxos saturados, quando comparada a outros produtos pecuários. Além disso, são ricas em ácidos graxos insaturados e são excelente fontes de minerais (sódio, potássio, ferro, selênio, entre outros) e aminoácidos essenciais (CENTENARO, 2011; KALAFOVA et al., 2018; KIM; DO; CHUNG, 2017; TAVANIELLO et al., 2017).

Quadro 1 – Valores nutricionais da carne crua do peito de frango e de codorna (100g)

	Carne de frango	Carne de codorna
Calorias totais	263,1 kcal	134 kcal
Carboidratos	58,1 kcal	0 kcal
Gordura	142,1 kcal	40,9 kcal
Proteína	62,9 kcal	92,9 kcal
% Valores diários*		
Gorduras totais	16,42 g (24%)	4,53 g (7%)
Gordura saturada	3,26 g (16%)	1,32 (7%)
Gordura monoinsaturada	6,48 g	1,28 g
Gordura poliinsaturada	3,34 g	1,17 g
Ácidos graxos ômega-3	0,143 g	0,03 g
Ácidos graxos ômega-6	3,197 g	1,05 g
Colesterol	41 mg (14%)	70 mg (23%)
Carboidratos Totais	15,01 g (5%)	0 g
Fibras dietéticas	1,1 g (4%)	0 g
Açucares	0 g	0 g
Vitaminas		
Vitamina A	0 IU	57 IU (1%)
Vitamina B6	0,041 mg (2%)	0,53 mg (26%)
Vitamina B12	0,27 mcg (5%)	0,47 mcg (8%)
Niancina	5,71 mg (29%)	8,2 mg (41%)
Riboflavina	0,091 mg (5%)	0,285 mg (17%)
Minerais		
Cálcio	19 mg (2%)	13 mg (1%)
Fósforo	211 mg (21%)	307 mg (31%)
Selênio	24,7 mcg (35%)	17,4 mcg (25%)
Sódio	451 mg (20%)	51 mg (2%)
Ferro	1,11 mg (6%)	4,51 mg (25%)

*Porcentagem de valores diários, baseados em uma dieta de 2.000 calorias. Valores diários podem ser maiores ou menores dependendo da necessidade energética individual. Fonte: https://skipthepie.org (2019) (Acessado em 05/03/2019)

Quadro 2 – Conteúdo de proteínas e aminoácidos presentes na carne crua do peito de frango e de codorna (100g)

	Carne de frango	Carne de codorna
% Ingestão diária recomendada*		
Proteína	14,730 g (29%)	24,760 g (44%)
Aminoácidos essenciais		
Histidina	0,435 g (62%)	0,825 g (118%)
Isoleucina	0,603 g (43%)	1,187 (85%)
Leucina	1,019 g (37%)	1,866 (68%)
Lisina	0,988 g (47 %)	1,905 (91%)
Metionina	0,300 g	0,689 g
Fenilalanina	0,525 g	0,944 g
Treonina	0,557 g (53 %)	1,090 g (104%)
Valina	0,659 g (36%)	1,180 g (65%)
Aminoácidos não-essenciais		
Arginina¥	0,804 g	1,379 g
Alanina	0,722 g	1,341 g
Aspartato	1,189 g	1,814 g
Cisteína¥	0,167 g	0,380 g
Glutamato	2,242 g	2,820 g
Glicina	0,632 g	1,442 g
Hidroxiprolina	0,075 g	~
Prolina	0,675 g	0,797 g
Serina	0,538 g	1,062 g
Tirosina¥	0,331 g	1,010 g
*Metionina + Cisteína*ǂ	0,467 g	1,069 g
Fenilalanina + Tirosina	0,856 g	1,954 g

*Baseado na ingestão diária recomendada pela Organização Mundial de Saúde para um adulto pesando 70kg; ¥São requeridos por bebês e crianças em crescimento, portanto podem ser incluídos na lista de aminoácidos essenciais; ǂA Organização Mundial de Saúde recomenda a ingestão de uma única dose diária; (~): Dados não disponíveis. Fonte: https://skipthepie.org (2019) (Acessado em 05/03/2019)

2.3 AS PROTEÍNAS DE ORIGEM ANIMAL COMO FONTE DE HIDROLISADOS PROTEICOS E PEPTÍDEOS BIOATIVOS

Sabendo que muitas proteínas de origem animal são capazes de apresentar efeitos fisiológicos benéficos ao organismo humano e que aquelas de origem avícola são extremamente ricas em aminoácidos essenciais, tem crescido, nos últimos anos, a busca pela a obtenção de hidrolisados proteicos e peptídeos bioativos a partir destes produtos com o intuito de melhor compreender não apenas as suas propriedades nutricionais e funcionais, como também a possibilidade de sua utilização como alimentos funcionais (FERNÁNDEZ, 2012; VALDERRAMA, 2016).

Os hidrolisados proteicos consistem de uma mistura de cadeias peptídicas de tamanhos

variados e com distintos aminoácidos em sua composição e aminoácidos livres que podem ser obtidos por diferentes métodos: proteólise enzimática, hidrólise química "*in vitro*", por fermentação microbiana e por digestão gastrointestinal (LEÓN-ESPINOSA; JIMÉNEZ-MARTÍNEZ; DÁVILA-ORTIZ, 2015; MENDOZA-JIMÉNEZ et al., 2018; SILVA, R. R., 2018). Estes compostos apresentam uma ampla gama de aplicações, tais como, produção de ingredientes alimentícios de valor agregado (ingredientes funcionais) (CHALAMAIAH; YU; WU, 2018); preparação de dietas especiais para alimentação enteral de bebês e para manutenção do estado nutricional de pacientes impossibilitados de digerir proteínas (PACHECO et al., 2005); fórmulas hipoalergênicas (LEÓN-ESPINOSA; JIMÉNEZ-MARTÍNEZ; DÁVILA-ORTIZ, 2015); cosméticos (COQUET et al., 2019; VERMELHO et al., 2010) e na área biomédica, como suplementos dietéticos (MARTÍNEZ-ALVAREZ; CHAMORRO; BRENES, 2015; ZAMORA-SILLERO et al., 2019) e na produção biomoléculas (peptídeos bioativos) visando a melhora de importantes atividades biológicas (HAYES, 2018; LORENZO et al., 2018; MARTÍNEZ-ALVAREZ; CHAMORRO; BRENES, 2015).

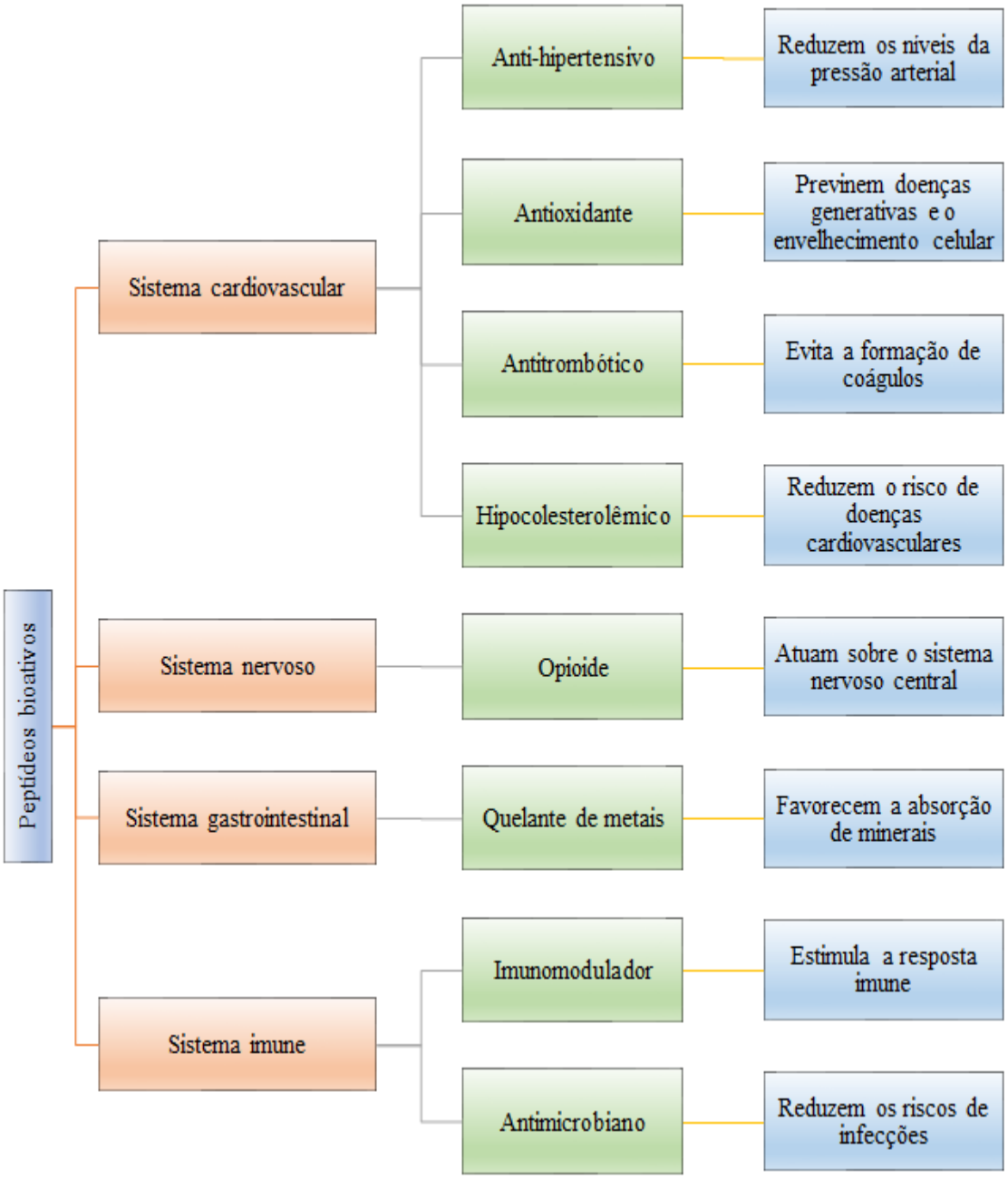
Para que sejam considerados bioativos, um componente da dieta deve conferir um efeito biológico mensurável a nível fisiológico. Além disso, essa bioatividade deve apresentar o potencial de afetar a saúde humana de maneira benéfica, excluindo, portanto, seus efeitos potencialmente tóxicos e mutagênicos (PACHAS, 2018). Neste contexto, o termo peptídeos bioativos refere-se a sequências curtas de 2-20 aminoácidos que podem modular funções fisiológicas específicas, além de fornecer aminoácidos essenciais e contribuir para o metabolismo energético. No entanto, fragmentos proteicos contendo uma quantidade superior a 20 resíduos também já foram relatados (HOU et al., 2017; RYAN et al., 2011).

Os peptídeos bioativos encontram-se criptografados dentro da estrutura primária de diversas proteínas alimentares e apenas tornam-se ativos após sua liberação da "proteína fonte" por proteólise enzimática "*in vivo*" ou "*in vitro*", durante a digestão gastrointestinal ou o processamento do alimento e posterior absorção pelos enterócitos (LEÓN-ESPINOSA; JIMÉNEZ-MARTÍNEZ; DÁVILA-ORTIZ, 2015; MENDOZA-JIMÉNEZ et al., 2018; SÁNCHEZ; VÁZQUEZ, 2017).

Diversos estudos relatam que hidrolisados proteicos e peptídeos bioativos derivados de diferentes proteínas de origem animal apresentam uma ampla gama de atividades, incluindo efeitos anti-hipertensivos, antioxidantes, anticâncer e imunomoduladores, além de variadas propriedades físico-químicas (Fig. 2) (CHALAMAIAH; YU; WU, 2018). Ressalta-se que grande parte dessas características são determinados pelo o número de ligações peptídicas

quebradas (grau de hidrólise), bem como a sequência e sua composição de aminoácidos (HOU et al., 2017; LEÓN-ESPINOSA; JIMÉNEZ-MARTÍNEZ; DÁVILA-ORTIZ, 2015).

Figura 2 – Efeitos dos peptídeos bioativos no organismo humano



Fonte: Adaptado de Pachas (2018)

Em relação aos hidrolisados e peptídeos bioativos derivados da carne, de seus subprodutos e do seu processamento, já foram descritos hidrolisados e peptídeos biologicamente ativos de distintas fontes apresentando diversas aplicações potenciais (Tabela 1). Adicionalmente, vários peptídeos bioativos gerados durante a proteólise *post-mortem* também já foram descritos e estão

relacionados ao desenvolvimento das características organolépticas e maciez do produto (FERNÁNDEZ, 2012).

Tabela 1 - Hidrolisados proteicos e peptídeos biologicamente ativos derivados da proteína animal e sua aplicação potencial

Fonte proteica	Aplicações	Referências
Frango	Antioxidante	Fukada et al. (2016)
Peru	Antioxidante	Wang e Shahidi (2018)
Pato	Antioxidante	Wang et al. (2015)
Peixe	Anticâncer	Hsu, Li-Chan e Jao (2011)
Vísceras de aves	Antioxidante e anti-hipertensivo	Jamdar, Rajalakshmi e Sharma (2012)
Vísceras de aves	Anti-hipertensivo	Mane e Jamdar (2017)
Penas	Antioxidante e anti-hipertensivo	Callegaro, Welter e Daroit (2018)
Ossos de frango	Antioxidante	Nie et al. (2017)
Colágeno	Antioxidante	Liu et al. (2016) e Fu et al. (2018)
Presunto chinês	Antioxidante	Xing et al. (2016)
Presunto chinês	Antioxidante e anti-hipertensivo	Wang et al. (2018)
Presunto espanhol	Antioxidante e anti-hipertensivo	Escudero et al. (2012)
Presunto espanhol	Anti-hipertensivo	Escudero et al. (2013)
Sangue de frango	Antioxidante	Zheng et al. (2018)
Sangue de boi	Antimicrobiano	Nedjar-Arroume et al. (2008)
Sangue de boi	Opioide	Piot et al. (1992)
Porco	Anti-hipertensivo	Margalef et al. (2017)
Porco	Anti-trombótico	Shimizu et al. (2008)
Carne bovina	Antimicrobiano	Jang et al. (2008)
Pele de frango	Antioxidante	Sarbon, Badii e Howell (2018)

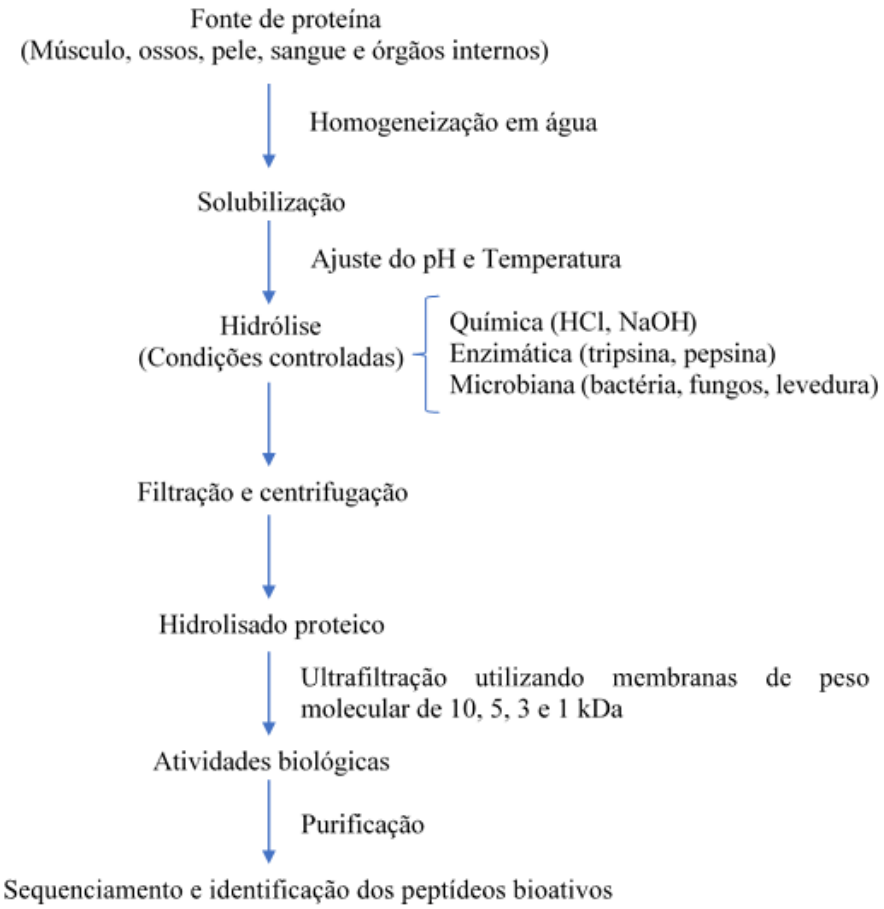
Uma vez que qualquer proteína animal, independentemente de suas funções e qualidade nutricional, pode ser empregada como fonte de compostos bioativos (hidrolisados proteicos e peptídeos bioativos), pode-se dizer que a geração destas biomoléculas é um critério para se estabelecer o valor de uma proteína (LEÓN-ESPINOSA; JIMÉNEZ-MARTÍNEZ; DÁVILA-ORTIZ, 2015).

2.4 OBTENÇÃO DE HIDROLISADOS PROTEICOS E PEPTÍDEOS BIOATIVOS A PARTIR DA PROTEÍNA ANIMAL

Conforme já explicado na secção anterior, os hidrolisados proteicos e peptídeos bioativos podem ser obtidos a partir de distintos métodos, tais como fermentação, hidrólise enzimática ou química, por processos de digestão gastrointestinal *in vivo* ou *in vitro* ou ainda por extração direta (ALUKO, 2015; MENDOZA-JIMÉNEZ et al., 2018; SÁNCHEZ; VÁZQUEZ, 2017). Entretanto, verificou-se que a combinação de mais um processo pode ser crucial durante a obtenção de peptídeos funcionais de tamanho pequeno (PEREIRA, 2016).

Os procedimentos gerais empregados na produção de hidrolisados proteicos e peptídeos bioativos de proteínas de origem animal encontram-se sumarizados na Figura 3. No entanto, a depender da fonte de proteína utilizada, esses procedimentos precisam ser modificados.

Figura 3 - Procedimentos gerais para a produção de hidrolisados proteicos e peptídeos bioativos de proteínas animais



Os compostos bioativos podem ser produzidos por meio de hidrólise química, enzimática, por extração direta, fermentação microbiana, e/ou por processos de digestão gastrointestinal *in vivo* ou *in vitro*. Fonte: Adaptado de Hou et al. (2017)

2.4.1 Extração direta

A extração é considerada uma etapa essencial nas análises de hidrolisados proteicos e peptídeos bioativos originados da carne, pois sabe-se que a partir da extração direta, já é possível obter hidrolisados proteicos tanto da carne crua quanto de produtos obtidos pelo processamento da carne com capacidade antioxidante inibitória da enzima conversora de angiotensina (LIU et al., 2016; WANG et al., 2018). Nesta etapa, o tecido muscular, livre de gordura e tecido conectivo, deve ser cortado em tamanhos menores e homogeneizado com um dos seguintes solventes de extração: água destilada (FOLCH; LEES; SLOANE STANLEY, 1957); soluções aquosas ácidas (ESCUDERO et al., 2012, 2013; WANG, L. et al., 2018); soluções salinas diluídas (FERNÁNDEZ, 2012) ou tampão fosfato neutro (XING et al., 2016).

Após extração, é indicado que as amostras passem por uma etapa de desproteinização. Segundo Martínez-Maqueda et al. (2013), este é o mais importante procedimento preliminar de limpeza da amostra em análises com peptídeos, podendo também atuar, segundo os autores, e com base nos achados de Cheng, Chen e Xiong (2010) e Cheng, Xiong e Chen (2010), como um procedimento de fracionamento, uma vez que a solubilidade dos peptídeos obtidos depende do agente precipitante, bem como de sua proporção. Os solventes mais empregados para a desproteinização da amostra incluem a acetronila, o metanol ou o etanol e ao final da etapa de desproteinização, a amostra irá conter um extrato de peptídeos solúveis e aminoácidos livres (hidrolisado proteico) (FERNÁNDEZ, 2012).

2.4.2 Hidrólise enzimática

A hidrolise enzimática é método mais empregado na obtenção de hidrolisados e peptídeos com atividade biológica, uma vez que a hidrólise pode modificar e melhorar as propriedades funcionais das proteínas, diminuindo a viscosidade, aumentando a solubilidade e a capacidade espumante sem afetar o seu valor nutritivo, ao mesmo tempo que as converte em peptídeos de tamanho desejado (MENDOZA-JIMÉNEZ et al., 2018; PEREIRA, 2016). Entretanto, este processo apresenta algumas desvantagens, como a necessidade de utilizar processos químicos ou térmicos para interromper a reação de proteólise, o que pode afetar as características finais das proteínas hidrolisadas, além de que o tratamento pode induzir alterações na degradação das proteínas em matrizes alimentares complexas (LEÓN-ESPINOSA; JIMÉNEZ-MARTÍNEZ; DÁVILA-ORTIZ, 2015).

Existe uma vasta gama de enzimas que são consideradas naturais e seguras para serem

utilizadas na obtenção de compostos bioativos. Muito embora as enzimas de origem animal, tais como a tripsina, pepsina e a quimotripsina, sejam as mais utilizadas neste processo; alguns estudos relatam a utilização de enzimas derivadas de plantas (papaína e bromelina) e de proteases bacterianas, sobretudo as provenientes de *Bacillus licheniformis* e *Bacillus subtilis*, sozinhas ou em combinação (PEREIRA, 2016; VERMA et al., 2018; XUE et al., 2018). Outras enzimas e/ou proteases já utilizadas incluem, alcalase (ONUH et al., 2013), Flavourzyme® (NIKOLAEV et al., 2016), Protamex® (MIRDHAYATI et al., 2016), pronase E (SARBON; BADII; HOWELL, 2018), protease de *Aspergillus* (O'KEEFFE et al., 2017), entre outras.

A maior parte das proteases utilizadas no processo de hidrólise enzimática são específicas (pepsina, tripsina, quimotripsina), ou seja, não hidrolisam a molécula de proteína em qualquer ligação peptídica, mas entre aminoácidos específicos. Porém, também existem as proteases não específicas (carboxipeptidase A), que podem hidrolisar a proteína em vários fragmentos da molécula (EVANGELHO, 2014; LIU et al., 2016). Desta maneira, o tipo da protease utilizada irá influenciar diretamente no tamanho, quantidade, composição e sequência dos aminoácidos dos peptídeos bioativos formados, afetando assim em seus valores nutricionais e respostas biológicas (LIU et al., 2016; PEREIRA, 2016). Outros parâmetros de hidrólise que também são conhecidos por afetarem a bioatividade dos hidrolisados e peptídeos incluem concentração do substrato, razão enzima/substrato, tempo de incubação, condições físico-químicas, como pH e temperatura (LEÓN-ESPINOSA; JIMÉNEZ-MARTÍNEZ; DÁVILA-ORTIZ, 2015; MENDOZA-JIMÉNEZ et al., 2018; SILVA, 2018; WANG; SHAHIDI, 2018).

2.4.3 Digestão gastrointestinal

Hidrolisados proteicos e peptídeos bioativos podem ser gerados a partir da proteína da carne durante o processo de digestão gastrointestinal. Nele, as proteínas são quebradas por ação das enzimas que atuam no trato digestório, como a pepsina, a tripsina e a quimotripsina, gerando hidrolisados contendo peptídeos bioativos de baixo peso molecular, os quais, uma vez absorvidos pelos enterócitos, podem apresentar atividade inibitória da enzima conversora de angiotensina e antioxidante (FERNÁNDEZ, 2012; LEÓN-ESPINOSA; JIMÉNEZ-MARTÍNEZ; DÁVILA-ORTIZ, 2015). Além disso, foi relatado que muitos dos peptídeos gerados durante a digestão gastrointestinal podem ser resultantes da quebra de proteínas musculares por ação da protease pancreática (FERNÁNDEZ, 2012).

Levando-se em consideração que a resistência frente as proteases gastrointestinais é um dos pré-requisitos para que compostos bioativos possam apresentar suas ações *in vivo* (ZHENG et

al., 2018), diversos autores têm procurado verificar se os hidrolisados proteicos e/ou peptídeos bioativos avaliados em seus trabalhos são capazes de sobreviver a digestão gastrointestinal por meio de estudos *in vitro* (KETNAWA; WICKRAMATHILAKA; LICEAGA, 2018; PHONGTHAI et al., 2018; SANGSAWAD; ROYTRAKUL; YONGSAWATDIGUL, 2017; WANG, L. et al., 2018). Wang et al. (2018), por exemplo, verificaram que as amostras do presunto Xuanwei após cozimento e digestão apresentaram redução na atividade antioxidante sequestradora do radical DPPH (2,2-difenil-1-picril hidrazilo), ao mesmo tempo em que houve um aumento na atividade antioxidante sequestradora do radical ABTS (2,2-azinobis (3-etilbenzotiazolina-acidosulfónico)). Adicionalmente, Sangsawad, Roytrakul e Yongsawatdigul (2017) analisando a atividade inibitória da enzima conversora de angiotensina (ECA) de peptídeos de origem do musculo de frango, verificaram que a digestão gastrointestinal *in vitro* destas amostras gerou um grande número de potentes peptídeos inibidores da ECA.

2.5 HIPERTENSÃO ARTERIAL

As VI Diretrizes Brasileiras de Hipertensão (2010) definem a hipertensão arterial sistêmica (HAS) como uma condição clínica multifatorial caracterizada por níveis elevados e sustentados de pressão arterial. Frequentemente associada a alterações funcionais e/ou estruturais de órgãos-alvo além de alterações metabólicas no organismo, a HAS é um dos principais fatores de risco modificáveis e um dos mais importantes problemas de saúde pública (CORREA et al., 2005; NOBRE et al., 2013). A hipertensão arterial sistêmica apresenta alta prevalência, baixas taxas de controle, elevado custo econômico-social e grande impacto na morbimortalidade mundial. A sua prevalência mundial é estimada na ordem de 1 bilhão de indivíduos hipertensos e aproximadamente 7,1 milhões de óbitos por ano podem ser atribuídos ao aumento da pressão arterial (PA) (CORREA et al., 2005; NEVES; CAMPOS; MARQUEZ, 2006)

Particularmente no Brasil, de acordo com as informações divulgadas pelo Ministério da Saúde, as doenças do aparelho circulatório ainda constituem a causa determinante de morte mais frequente na população. Estudos no país por meio de inquéritos populacionais em cidades brasileiras nos últimos 20 anos revelaram uma prevalência de HAS acima de 30% (média de 32,5%), chegando a mais de 50% entre 60 e 69 anos e 75% acima de 70 anos. Em relação aos gêneros pesquisados, foi constatado uma prevalência de 35,8% nos homens e de 30% em mulheres (NOBRE et al., 2013).

Evidências científicas mostram que além da terapia farmacológica, os fatores nutricionais desempenham um papel significativo na prevenção e tratamento da hipertensão, sendo assim,

são crescentes os esforços da ciência para a produção de alimentos funcionais com atividade anti-hipertensiva que, associados a mudanças na dieta e estilo de vida, afetam positivamente a pressão arterial do indivíduo (JÄKÄLÄ; VAPAATALO, 2010)

2.6 HIDROLISADOS E PEPTÍDEOS ANTI-HIPERTENSIVOS

Dentre os peptídeos bioativos derivados de proteínas de origem animal, os peptídeos hipertensivos tem sido extensivamente estudados, uma vez que sua grande maioria atua inibindo o funcionamento da ECA, enzima que está relacionada com a regulação da pressão arterial por meio da modulação do sistema renina-angiotensina (PEREIRA, 2016; VALDERRAMA, 2016).

Após a descoberta do primeiro inibidor da ECA no veneno de serpentes, vários inibidores sintéticos desta enzima foram desenvolvidos ao longo dos anos, sendo o captopril o primeiro e mais conhecido (FERNÁNDEZ, 2012). No entanto, este e outros inibidores sintéticos da ECA comumente utilizados no tratamento da hipertensão arterial (como enalapril), são conhecidos por apresentarem vários efeitos colaterais indesejáveis, como tosse seca, náuseas, erupções cutâneas e insuficiência renal aguda (DENG et al., 2018; HUANG et al., 2016). Estes efeitos colaterais, juntamente com o fato anteriormente relatado de que a hipertensão arterial é um dos mais importantes problemas de saúde pública, com grande impacto na morbimortalidade mundial, têm contribuído para o aumento na busca por peptídeos bioativos com atividade inibidora da ECA derivados das proteínas alimentares que possam ser utilizados como agentes anti-hipertensivos em alimentos funcionais ou nutracêuticos (DENG et al., 2018; FERNÁNDEZ, 2012)

Vários autores demostraram que a partir do tratamento de proteínas de origem animal com enzimas e/ou proteases é possível obter peptídeos inibidores da ECA significativamente eficazes em suprimir ou reduzir o desenvolvimento da hipertensão arterial em ratos espontaneamente hipertensos (SHR) com efeitos colaterais reduzidos ou nulos (SAIGA et al., 2003). Arihara et al. (2001) identificaram dois peptídeos inibidores da ECA a partir da digestão da miosina do porco com a enzima termolisina. A administração destes peptídeos em ratos hipertensos resultou em um decréscimo na pressão sanguínea sistólica destes animais de aproximadamente 10,26 % após 6 horas da administração.

Saiga et al. (2003) e Terashima et al. (2010) também obtiveram resultados hipotensores significativos utilizando peptídeos inibidores da ECA extraídos de proteínas do músculo ou osso de frangos em seus experimentos. A força desses peptídeos foi avaliada pelo valor de IC_{50}

e verificou-se que, enquanto o captopril apresenta valor IC_{50} na ordem de 0,022 μM, os peptídeos derivados de proteínas alimentares mostram valor de IC_{50} variando entre 0,1 μM a algumas centenas de micromolares dependendo de suas sequências de aminoácidos (TERASHIMA et al., 2010).

2.6.1 Mecanismo de inibição da enzima conversora de angiotensina

A ECA, identificada e isolada primeiramente em 1954 por Skeggs a partir do plasma de cavalo, é uma dipeptidil-carboxipeptidase responsável pela vasoconstrição, possuindo um papel importante na regulação da pressão arterial periférica (FERNÁNDEZ, 2012; PEREIRA, 2016). Esta enzima encontra-se associada tanto ao sistema renina-angiotensina (SRA), uma via importante envolvida no controle homeostático da PA, quanto ao sistema calicreína-cinina. No SRA, a cascata começa com o angiotensinogênio, substrato da renina (PACHAS, 2018). Sob a ação de alterações na pressão da arteríola aferente renal e concentração de sódio na mácula densa, a renina irá interagir com seu substrato plasmático o angiotensinogênio, convertendo-o em angiotensina I, um decapeptídeo inativo (FERNÁNDEZ, 2012; NOBRE et al., 2013; PEREIRA, 2016). Uma vez formada, a angiotensina I será convertida, através da ação da ECA, em angiotensina II, um octapeptídeo com potente ação hipertensiva (FERNÁNDEZ, 2012; PACHAS, 2018; PEREIRA, 2016). Adicionalmente, a ECA atua degradando a bradicinina, um peptídeo vasodilatador, estimula a liberação de aldosterona pelo córtex da adrenal e de hormônio antidiurético, que levam a uma diminuição na excreção de sódio e água, levando a um aumento gradual no volume do líquido extracelular e, consequentemente, elevando a pressão arterial (FERNÁNDEZ, 2012; FUJITA; YOKOYAMA; YOSHIKAWA, 2000).

Estudos prévios demonstraram que a ECA parece possuir uma preferência por peptídeos inibidores do tipo competitivos, conhecidos por competirem com o substrato pelo sítio ativo da enzima, e que contenham resíduos de aminoácidos hidrofóbicos nas três posições mais próximas a sua região C-terminal (FERNÁNDEZ, 2012; LAU; ABDULLAH; SHUIB, 2013; MANE; JAMDAR, 2017; MAS-CAPDEVILA et al., 2018). Contudo, diversos autores relatam que peptídeos inibidores da ECA do tipo incompetitivo (GIRGIH et al., 2016; JAKUBCZYK; BARANIAK, 2014), não competitivos (DENG et al., 2018) e do tipo misto (ONUH et al., 2015; TU et al., 2018), também podem ser encontrados.

2.7 HIDROLISADOS E PEPTÍDEOS ANTIOXIDANTES

Protegendo o organismo contra a ação de radicais livres, os antioxidantes são valiosamente

benéficos à saúde humana. Os radicais livres são uma espécie química (orgânica ou inorgânica), geralmente extremamente instável e, portanto, com grande poder reativo. São fisiologicamente produzidos e exercem funções de sinalização celular e de defesa do organismo contra infecções. Contudo, estes radicais também podem causar efeitos negativos à saúde ao longo da vida, devido a sua capacidade de oxidar o DNA, lipídeos, carboidratos e proteínas, aumentando o risco para diversas doenças crônicas (câncer, diabetes, arteriosclerose) (EVANGELHO, 2014; FERNÁNDEZ, 2012).

Os métodos mais comumente utilizados para avaliar a atividade antioxidante *in vitro* são os ensaios que se baseiam na transferência de elétrons de um composto antioxidante para um oxidante: ABTS●+ (2,2-azinobis (3-etilbenzotiazolina-acidosulfónico)), DPPH (2,2-difenil-1-picril hidrazilo) e o poder redutor. O método do ABTS●+ consiste em monitorar o decaimento do cátion-radical ABTS●+ produzido a partir de sua oxidação na presença de uma agente antioxidante (KETNAWA; WICKRAMATHILAKA; LICEAGA, 2018; PHONGTHAI et al., 2018). Por outro lado, o método DPPH está baseado na capacidade do radical livre DPPH reagir com doadores de hidrogênio (EVANGELHO, 2014). Nas avaliações do poder redutor, a atividade antioxidante é analisada pela redução do Fe3+, a Fe2+ (EVANGELHO, 2014).

A hidrólise enzimática tem sido amplamente utilizada na produção de hidrolisados e peptídeos antioxidantes a partir de proteínas alimentares. Contudo em produtos alimentícios, os peptídeos antioxidantes também podem ser produzidos pela ação de micro-organismos ou enzimas proteolíticas endógenas (LEÓN-ESPINOSA; JIMÉNEZ-MARTÍNEZ; DÁVILA-ORTIZ, 2015).

Em geral, a maioria dos peptídeos antioxidantes derivados de fontes alimentares possuem pesos moleculares variando de 500 a 3000 Da (SUN et al., 2016). Além disso, apresentam resíduos hidrofóbicos, como Val e Leu, na região N terminal dos peptídeos e Pro, His, Tyr, Trp, Met, Cys em suas sequências (LEÓN-ESPINOSA; JIMÉNEZ-MARTÍNEZ; DÁVILA-ORTIZ, 2015).

3 MÉTODOS

3.1 EFEITO ANTI-HIPERTENSIVO DE HIDROLISADOS E PEPTÍDEOS OBTIDOS PELA HIDRÓLISE DAS PROTEÍNAS DE FRANGO

3.1.1 Busca de artigos e critérios científicos adotados para a sua seleção

Para esta revisão sistemática, por quatro pesquisadores diferentes realizaram pesquisas eletrônicas, nos meses de fevereiro e outubro de 2018, em quatro banco de dados internacionais: Scopus (http://www.scopus.com/), ScienceDirect (http://www.sciencedirect.com/), ISI Web of Science (http://apps.isiknowledge.com) e PubMed (http://www.ncbi.nlm.nih.gov/pubmed), utilizando as seguintes palavras-chave: efeito anti-hipertensivo, peptídeos bioativos, proteína de frango, ratos, com conector "e". Não foram feitas restrições quanto à linhagem de ratos utilizadas nos artigos, método empregado na obtenção dos peptídeos e/ou hidrolisados avaliados, tampouco dosagem, forma e seu período de administração. Contudo, apenas trabalhos de pesquisa originais, publicados em inglês, em periódicos com revisão realizada por pares e nos últimos quinze anos foram selecionados.

Os critérios científicos para a seleção dos artigos foram definidos para avaliar os prováveis efeitos inibitórios e protetores dos peptídeos/hidrolisados sobre a ECA e a pressão arterial de ratos, respectivamente, bem como a qualidade metodológica dos artigos. A qualidade metodológica dos trabalhos publicados foi avaliada segundo Greenhalgh (1997) e outras revisões sistemáticas (ANDRADE et al., 2015; FERREIRA et al., 2013; PEREIRA et al., 2011; SILVA et al., 2012). Os estudos foram qualitativamente categorizados em adequado (escore: 2) e não claro/parcialmente adequado (escore: 1), sendo classificados pelos seguintes critérios: grupo controle, tipo de grupo controle, dosagem, avaliações cegas, estudos randomizados, tamanho da amostra e tipo de análise efetuada. A pontuação máxima que poderia ser recebida por cada artigo foi de 14 pontos.

3.2 POTENCIAL BIOATIVO DE HIDROLISADOS PROTEICOS DO MÚSCULO DO PEITO DE CODORNAS JAPONESAS

3.2.1 Preparo dos hidrolisados proteicos de *Coturnix japonica* (CJPH) e suas frações gastrointestinais

A metodologia utilizada para a extração e desproteinização do músculo do peito de codornas japonesas foi a proposta por Escudero et al. (2013), com modificações. 20 carcaças de codornas, adquiridas em um estabelecimento comercial e pesando aproximadamente 100g, foram

incialmente desossadas e, posteriormente, o músculo do peito (50 g) foi cortado e homogeneizado (Scilogex D160 Homogenizer, 30000 rpm, 10 min) com HCL (200 mL; 0,01 N). Após sucessivas etapas de filtração (PTFE; 25 mm x 0,22 µm) e centrifugação (12000 g; 20 min; 4ºC), foram adicionados 3 volumes de etanol (20 min; 4°C), para desproteinizar as amostras. O etanol foi evaporado e os extratos secos foram dissolvidos em água Milli-Q (30 mL) e armazenados (-20ºC; CJPH) até sua posterior utilização.

A digestão gastrointestinal *in vitro* foi realizada de acordo com o método descrito por Versantvoort et al., (2005). A digestão *in vitro* foi iniciada adicionando amostras de CJPH (3.63 mL; 7000 µg.mL^{-1}) a uma solução de saliva (3 mL; pH 6.8), ficando sob agitação por 5 min. Após este período, o pH da solução foi ajustado para 2 e foi adicionado a enzima pepsina (1%) à mistura. Após 120 min, a reação foi neutralizada (pH 8) e então foi adicionada pancreatina (1%). Ao final de 1 hora, o pH da solução foi novamente ajustado para 8,2 e a solução de bile foi adicionada à reação, permanecendo em incubação por 60 min. Todas as incubações foram realizadas a 37°C e sob agitação (100 rpm). As amostras foram coletadas em diferentes tempos (120 min e 240 min), representando as digestões gástrica e gastrointestinal, respectivamente e, posteriormente, foram fracionadas utilizando membranas de ultrafiltração de pesos moleculares de 10 e 3 kDa (Millipore Co., Bedford, MA). Os permeados foram coletados, liofilizados, reconstituídos em água Milli-Q e armazenados (-20ºC) até posterior análises.

As amostras utilizadas nas análises subsequentes foram assim definidas: CJPH – hidrolisado proteico de *Coturnix japonica* (não digerido e desproteinizado); GPH(3) e GPH(3-10) – hidrolisados proteicos obtidos após digestão gástrica do CJPH e com pesos moleculares <3 e 3–10 kDa, respectivamente; GiPH(3) e GiPH(3-10) – hidrolisados proteicos obtidos após a digestão gastrointestinal do CJPH e com pesos moleculares <3 e 3–10 kDa, respectivamente.

3.2.2 Atividades antioxidantes de eliminação dos radicais 2,2-Difenil-1-picril hidrazilo (DPPH) e 2,2-azinobis (3-etilbenzotiazolina-ácidosulfônico) (ABTS)

A capacidade do CJPH em eliminar o radical 2,2-Difenil-1-picril hidrazilo (DPPH) foi mensurada pelo método descrito por Brand-Williams, Cuvelier e Berset (1995), modificado por Fukumoto e Mazza (2000). CJPH (22 µL) em três concentrações (2,5; 5,0 e 10,0 mg.mL^{-1}) foi adcionado a uma solução de 2,2-Difenil-1-picril hidrazilo (DPPH) preparada previamente. A mistura foi incubada por 120 min, a temperatura ambiente e ao abrigo da luz, e a absorbância resultante da eliminação dos radicais DPPH foi mensurada a 595 nm. O branco de cada concentração foi preparado substituindo CJPH por água destilada.

Para análise da capacidade do CJPH em eliminar o radical 2,2-azinobis (3-etilbenzotiazolina-acidosulfónico) foi utilizado o método descrito por Re et al. (1999), com modificações. Primeiramente, uma solução estoque de ABTS radical (7 mM) contendo persulfato de amônio (140 mM) foi preparada e incubada a temperatura ambiente e ao abrigo da luz por 16 horas antes de sua utilização. A solução de trabalho foi obtida a partir de uma alíquota da solução estoque de ABTS que foi diluída em etanol e apresentou uma absorbância de 0,7 (± 0,02; 734 nm). CJPH (30 μL) a diferentes concentrações (2,5; 5,0 e 10,0 $mg.mL^{-1}$) foram adicionados a 3 mL da solução de trabalho e a absorbância foi mensurada a 734 nm após 06 min de incubação. Trolox foi utilizado para construção da curva de calibração.

A porcentagem de eliminação dos radicais DPPH e ABTS foram calculadas utilizando a seguinte formula:

$$\text{Atividade antioxidante (\%)} = (A - B)/A \times 100$$

onde A é a absorbância do branco e B a absorbância da amostra. Quando possível, o valor de IC_{50} foi calculado pela regressão linear da curva mostrando a atividade de eliminação do radical DPPH ou ABTS (%) *versus* concentração do hidrolisado de codorna ($mg.mL^{-1}$). Todos os testes foram realizados em triplicata.

3.2.3 Atividade inibitória da enzima conversora de angiotensina e mecanismo de inibição da ECA

A inibição da ECA *in vitro* foi analisada de acordo com o método proposto por Cushman and Cheung (1971), adaptado por Meira et al. (2012). Foram incubados os seguintes componentes (37 ºC; 30 min): tampão contendo HEPES-HCL (50 mM; pH 8,3), substrato hipuril-histidil-leucina (HHL) (5 mM), NaCl (300 mM), ECA purificada de pulmão de coelho (40 μL; 0,1 $U.mL^{-1}$) e CJPH (20 μL) em três concentrações (2,5; 5,0 e 10,0 $mg.mL^{-1}$). Afim de parar a reação, HCl (150 μL; 1 M) foi adicionado à mistura. A taxa de hidrólise de HHL foi determinada pela medida da absorbância da liberação do ácido hipúrico (HA) (228 nm) após sua extração com acetato de etilo, evaporação da fase orgânica e a dissolução do resíduo em água. Captopril (1 mM) foi utilizado como controle positivo. A inibição da ECA (ACEi) (%) foi calculada utilizando a seguinte formula:

$$\text{ACEi (\%)} = (A - B)/(A - C) \times 100$$

onde A é o conteúdo de HA do controle sem amostra, B é o conteúdo de HA do controle sem a ECA, C é o conteúdo de HA da reação com a amostra e com a ECA. A concentração do inibidor da ECA necessária para inibir 50% da atividade da enzima foi expressa como IC_{50}.

A cinética de inibição da ECA pelas amostras CPJH e GiPH(3) foi estudada para três concentrações distintas (0; 5,0 e 10,0 mg.mL^{-1}) e utilizando o substrato hipuril-histidil-leucina nas seguintes concentrações: 6; 3,5, 2, 1 e 0,5 mM. A equação de Michaelis-Menten foi usada para estimar os parâmetros cinéticos K_m and V_{max}, utilizando o programa GraphPad Prism versão 7.04 (GraphPad Software, Inc., San Diego, USA). Gráficos de Lineweaver—Burk foram construídos e aplicados para confirmar o modo de inibição da ECA (competitivo, não competitivo, incompetitivo ou misto). Todos os experimentos foram realizados em triplicata.

3.2.4 Perfil de peptídeos

A fim de verificar o perfil dos potenciais peptídeos formados após o processo de digestão gastrointestinal *in vitro*, as amostras de CJPH e GiPH(3) foram analisadas por espectrometria de massa (MALDI-TOF-MS/MS) utilizando o espectrofotômetro Autoflex II (Bruker Daltonics, Bremen, Germany). Inicialmente, as amostras foram misturadas à matriz contendo ácido alfa-ciano-4-hidroxicinâmico (10 mg.mL^{-1}) em acetonitrila (50%) com ácido trifluoroacético (0,3%) (proporção 1:1). Posteriormente, 1 µL desta mistura foi aplicado a placa MALDI (MTP 384 ground steel, Bruker Daltonics, Bremen, Germany) e deixado em temperatura ambiente até secar. O modo refletor de íons positivos foi utilizado para análise de cada fração cromatográfica utilizando uma voltagem de aceleração de 19 kV e frequência do laser de 100 Hz. O procedimento realizado no modo refletor utilizou a solução Peptide Mix (Brucker Daltonics) para a calibração e faixa de detecção de íons foi de 0,4 – 5 kDa.

A partir dos espectros de MS, foram escolhidos os picos de interesse a serem fragmentados utilizado o método LIFT DEFAULT a uma voltagem de aceleração de 6 kV. Todos os dados foram obtidos usando o software Flex Control, e os espectros foram processados usando o software Flex Analysis One (Versão 3.0, Bruker Daltonics). O processamento automático dos spectra foi realizado utilizando o software SearchGui v3.3.13 (CompOmics, Ghent, Belgium) (http://compomics.github.io/projects/searchgui.html) (BARSNES; VAUDEL, 2018) e como mecanismo de busca foi escolhido o MS Amanda (DORFER et al., 2014). Os dados obtidos a partir das análises das amostras no MALDI-TOF-MS/MS foram pesquisados contra os subdiretórios de *Coturnix* ou *Gallus* do banco de dados de proteínas SwissProt (UniProt, EBI, UK). O software PeptideShaker v1.16.38 (CompOmics, Ghent, Bélgica) (http://compomics.github.io/projects/peptide-shaker.html) foi utilizado para o pós-processamento das identificações (VAUDEL et al., 2015).

3.2.5 Potencial tóxico dos hidrolisados proteicos de *Coturnix japonica* pelo teste *Allium cepa*

O teste *Allium cepa* foi realizado de acordo com o proposto por Fiskejö (1985), com modificações. Bulbos não germinados e saudáveis de cebola comum (n = 55; Ø 20-30 cm; 30–40 g) foram adquiridos em um estabelecimento comercial em Recife-Pernambuco e, antes de iniciar os experimentos, foram colocados em um refrigerador (4 °C) por 24 horas, para reduzir possíveis efeitos dos inibidores de brotamento. Após este período, os bulbos foram posicionados randomicamente em tubos de polipropileno esterilizados (15 mL) para germinação, durante cinco dias a temperatura ambiente, com a parte inferior imersa nas soluções contendo: água desclorada (controle negativo), sulfato de cobre (0,6 $\mu g.mL^{-1}$; controle positivo) e CJPH em três concentrações (2,5; 5,0 e 10,0 $mg.mL^{-1}$). A média do crescimento das raízes foi usada para determinar o potencial tóxico do CJPH, a capacidade antiproliferativa (citotoxicidade) dos tratamentos foi verificada pelo índice mitótico e as análises de genotoxicidade e mutagenicidade foram avaliados, respectivamente, pela contagem das anomalias cromossômicas (quebra cromossômica, células binucleadas, pontes cromossômicas) e micronúcleos nas células do meristema da raiz de *Allium cepa*.

4 HYDROLYSATES AND BIOACTIVE PEPTIDES GENERATED FROM CHICKEN PROTEIN HYDROLYSIS: A SYSTEMATIC REVIEW OF ANTIHYPERTENSIVE EFFECTS IN RATS

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Conflict of interest statement

The authors declare no conflict of interest.

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Abstract

Background: In recent years, obtaining protein hydrolysates and peptides from food proteins has been extensively studied in order to better understand not only their nutritional and functional properties, but also the possibility of their use as ingredients in functional foods. It is known that many dietary proteins have, within their primary structure, peptide sequences which upon release can be absorbed by enterocytes and modulate specific physiological functions, as well as provide essential amino acids and contribute to energy metabolism

Scope and approach: To obtain a better insight into chicken protein hydrolysates and bioactive peptides with antihypertensive activity, a systematic review was conducted to evaluate the efficacy of these bioactive compounds in controlling the blood pressure in rats. In addition, the methodological quality of the published articles was evaluated.

Key findings and conclusions: Searches in Scopus, ScienceDirect, ISI Web of Science and PubMed databases were focused on studies about the use of hydrolysates and peptides obtained from the enzymatic hydrolysis of various chicken tissues in rats. It was possible to verify that the positive antihypertensive effects were mainly dependent on the enzyme and enzymatic hydrolysis conditions and the monitoring of vital organ functions is essential to the regulation of blood pressure. Due to their efficacy and low cost, chicken protein hydrolysates can be an interesting alternative for biotechnological applications. Administration of chicken protein-derived hydrolysates and peptides to spontaneously hypertensive rats (SHR) exhibits a potential antihypertensive activity with likely cardiac protective effects against end-organ damage.

Keywords: protein hydrolysates, bioactive peptides, chicken protein, antihypertensive activity, enzymatic hydrolysis, spontaneously hypertensive rats

1. INTRODUCTION

Systemic hypertension is a multifactorial clinical condition characterized by high and sustained levels of arterial blood pressure with consequent metabolic alterations in organs such as heart, brain, kidneys and blood vessels (Nobre, Coelho, Lopes, & Geleilete, 2013). Hypertension is known as a risk factor for cardiovascular diseases, including coronary artery disease, peripheral arterial disease, acute myocardial infarction, heart failure, and stroke; which makes it one of the most important causes of morbidity and mortality worldwide (Jäkälä & Vapaatalo, 2010; Onuh, Girgih, Malomo, Aluko, & Aliani, 2015).

The renin-angiotensin system (RAS) plays an important role in the blood pressure control and sodium homeostasis through integrated processes in the kidney, cardiovascular system, and central nervous system (Sparks, Crowley, Gurley, Mirotsou, & Coffman, 2014). In response to a drop in blood pressure, renin cleaves the N-terminus of angiotensinogen, releasing the angiotensin I (Ang I), a 10 amino acid peptide. After the removal of two amino acids from its C-terminus by the angiotensin-converting enzyme (ACE), the Ang I is converted to a potent vasoconstrictor peptide, the angiotensin II (Ang II), which induces several biological responses by binding to specific membrane receptors (Sahay & Sahay, 2012; Sparks et al., 2014). Since RAS plays a critical role in the circulatory homeostasis, the abnormal activation of this system can contribute to the development of cardiovascular disorders like hypertension, heart failure, and hypertrophy (Sparks et al., 2014).

In addition to the pharmacologic therapy, nutritional factors are essential in the prevention and treatment of hypertension, a fact which motivates the search for functional food with antihypertensive properties (Jäkälä & Vapaatalo, 2010). It is known that many food proteins have in their primary structure some peptide sequences easily released after *in vivo* or *in vitro* enzymatic proteolysis during gastrointestinal digestion or food processing, that are able to

modulate specific physiological functions (Sharma, Singh, & Rana, 2011). The angiotensin-converting enzyme (ACE) inhibitory peptides (particularly those derived from animal protein) have been extensively studied and the findings have indicated that they are significantly effective in eliminating or reducing the development of hypertension in the spontaneously hypertensive rat (SHR) and human patients (Iwai et al., 2009; Lau, Abdullah, & Shuib, 2013; Yu, Yin, Zhao, Chen, & Liu, 2014; Zhuang, Sun, Zhang, & Liu, 2012).

Due to a high nutritional quality of poultry tissues, obtaining new hydrolysates and peptides from enzymatic hydrolysis of their proteins is of considerable interest for the development of new products, such as nutraceutical compounds. Once ingested they are able to perform basic nutritional functions, as well as trigger beneficial metabolic effects to the human health (Iwaniak, Minkiewicz, & Darewicz, 2014; Jamdar, Rajalakshmi, & Sharma, 2012; Jao, Huang, & Hsu, 2012; Kurozawa, Park, & Hubinger, 2009).

Different animal models are often used in biomedical research to simulate *in vivo* physiological and pathological conditions in order to improve current practices and technologies. However, it is important that the results obtained with experimental species should be extrapolated to other species that cannot be directly investigated, due to ethical, financial and/or facilities management issues (Fagundes & Taha, 2004; Neves, Campos, & Marquez, 2006; V. D. O. Silva et al., 2012). This systematic review was conducted aiming to determine the effectiveness of hydrolysates and peptides derived from chicken protein hydrolysis in reducing and/or maintaining blood pressure in rats.

2. MATERIALS AND METHODS

a. Database search and aim

Electronic searches were carried out on Scopus (http://www.scopus.com/), ScienceDirect (http://www.sciencedirect.com/), ISI Web of Science (http://apps.isiknowledge.com) and

PubMed (http://www.ncbi.nlm.nih.gov/pubmed) databases, using the following keywords: antihypertensive effect, bioactive peptides, chicken protein, rats with connector "and". The searches were conducted between February and October 2018 by four different researchers in order to obtain an independent analysis of the selected papers. This present review aimed to provide an overview of the *in vivo* effects of peptides and/or hydrolysates derived from chicken protein enzymatic hydrolysis on rats.

b. Selection criteria

No restrictions were made regarding the rat lineage, method for obtaining peptides and/or hydrolysates, dosage, administration form and period. However, only original research papers published in English in peer-reviewed journals and in the last fifteen years were selected.

The scientific selection criteria were defined to evaluate the inhibitory and protective effects of peptides/hydrolysates on the ACE and the blood pressure, respectively, as well as the methodological quality of the articles. The methodological quality of the published works was assessed according to Greenhalgh (1997) and other systematic reviews (Andrade et al., 2015; Ferreira et al., 2013; Pereira, Oliveira, Mesquita, Costa, & Pereira, 2011; V. D. O. Silva et al., 2012). The studies were qualitatively categorized on a scale of adequate (score: 2) and unclear/partially adequate (score: 1) and classified by the following criteria: control group, type of control group, dosage, blind assessments, randomized studies, sample size, type of analysis (Table 1).

The maximum score was 14 points (Table 2). Other parameters such as type of bioactive compound administered (protein hydrolysate or bioactive peptide), chicken protein source, dose, administration route, among others, did not score but were taken into consideration to subsequent discussions. Table 3 shows a summary of the selected articles.

3. RESULTS

The overview of the database search and the reasons for the exclusion of some retrieved studies are shown in Figure 1. A search in Scopus found 134 studies, from which 13 were disregarded because they were literature reviews; 7 did not address the use of hydrolysates or bioactive peptides; 92 of them evaluated hydrolysates or peptides derived from sources outside the scope of this study; 13 articles performed only *in vitro* tests, evaluated different biological activities or were not animal studies and 1 article was not written in English. Finally, a total of 8 papers were identified to fit the scope of this review.

A search in ScienceDirect resulted in 30 studies, 18 of which were previously found in Scopus Database. The remaining studies (12) were not considered because 2 were literature reviews, 9 referred to hydrolysates or peptides derived from a different source (outside the scope of this paper) and 1 paper did not regard hydrolysates or bioactive peptides. The literature survey in the ISI Web of Science database retrieved 5 papers, four of which were duplicates of articles found in Scopus and/or ScienceDirect and 1 was a literature review. In this way, the ISI Web of Science database search did not identify additional papers. The literature search in the PubMed database retrieved 4 papers, three of which were duplicates of articles found in Scopus, ScienceDirect and/or ISI Web of Science databases. Thus, 1 paper was selected for this study. Table 3 shows the experimental design of the 9 selected articles.

Out of the selected articles, 6 (66.70%) reported the use of spontaneous hypertensive rat (SHR) as the animal model and 3 (33.30%) used this lineage in combination with the normotensive Wistar-Kyoto (WKY) rat. The number of animals per group varied in the studies. The smallest sample size comprised 4 animals/group (Nakade et al., 2008; Onuh et al., 2015; Udenigwe et al., 2017), whilst the largest one used 8 animals/group (Cheng et al., 2008) (Table 3).

Although all articles have reported the presence of control groups in the experiments, 44.44% of the studies mentioned the use of positive and negative controls. In these articles,

captopril (Mas-Capdevila, Pons, Aleixandre, Bravo, & Muguerza, 2018; Onuh et al., 2015; Udenigwe et al., 2017) and Ile-Pro-Pro (Nakade et al., 2008), a tripeptide with antihypertensive activity, were used as positive control, while phosphate-buffered saline (PBS) (Onuh et al., 2015), distilled water (Nakade et al., 2008), tap water (Mas-Capdevila et al., 2018) and saline (Udenigwe et al., 2017) were used as negative controls. Of the remaining studies, only 1 (11.12%) reported the use of a single type of control group, with the captopril as the positive one (Cheng et al., 2008). Although 4 (44.44%) papers have not informed the type of control group used, casein (Onuh et al., 2016) and saline (Saiga et al., 2003, 2006, 2008) were administered to the animals in the control group (Table 3).

The majority of the retrieved articles (77.8%) evaluated hydrolysates prepared from proteins of various chicken parts such as skins from thigh and breast muscle, collagen, bone legs, breast muscle and chicken feet. The other papers (22.2%) studied peptides obtained from hydrolysis of chicken bone (Tyr-Tyr-Arg-Ala) (Nakade et al., 2008) or synthesized an ACE inhibitory peptide (Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-Phe) found in the extract digested by gastric enzyme in a previous work (Saiga et al., 2006) (Tables 3 and 4).

The following techniques for the hydrolysate fractionation and peptide purification were used in the papers included in this systematic review: High-Performance Liquid Chromatography (HPLC) (Nakade et al., 2008), ultrafiltration membrane (Onuh et al., 2015) and a 0.45 μm membrane (Mas-Capdevila et al., 2018). A combination of two distinct methods was also used (Saiga et al., 2003, 2008) and in 4 articles no fractionation techniques were applied (Cheng et al., 2008; Onuh et al., 2016; Saiga et al., 2006; Udenigwe et al., 2017) (Table 3).

Of the 9 selected papers, only 2 (22.22%) were randomized experiments (Cheng et al., 2008; Onuh et al., 2016) and none of the studies reported having conduced a blind evaluation (Table 2). There was no consensus about the hydrolysates and/or peptides administration

conditions. The lowest dose found was 10 mg/kg bw (body weight), administered once by intubation (Nakade et al., 2008), while the highest one was 3,000 mg/kg bw, single and long-term (four weeks) orally administered (Saiga et al., 2008) (Tables 3 and 4)

All selected articles used the tail-cuff method to measure the systolic blood pressure (SBP) of the rats and made *in vitro* analysis of the ACE inhibitory activity. Other mechanisms involved in the antihypertensive effect of the hydrolysate tested, such plasmatic ACE activity, vascular effects or metabolomics studies were also investigated (Mas-Capdevila et al., 2018; Onuh et al., 2016) (Table 3). Additional parameters analysed in the selected papers included: *in vitro* renin-inhibition analysis (Onuh et al., 2015; Udenigwe et al., 2017), kinetics of enzyme inhibition studies (Mas-Capdevila et al., 2018; Onuh et al., 2015; Saiga et al., 2006), *in vitro* antioxidant capacity (Onuh et al., 2016; Udenigwe et al., 2017), peptide sequence identification (Onuh et al., 2016; Saiga et al., 2006, 2008; Udenigwe et al., 2017), weight gain; heart weight and heart/body weight ratio (Cheng et al., 2008), analysis of the blood components (Saiga et al., 2008), amino acid composition and molecular weight/size determination (Onuh et al., 2015; Udenigwe et al., 2017).

4. DISCUSSION

Reviews provide significant insights into a particular research field as well as suggest the best protocols to assist future studies (Sampaio & Mancini, 2007; V. D. O. Silva et al., 2012). This present study provides an overview of the efficacy of hydrolysates and/or peptides obtained from chicken protein hydrolysis on reducing and/or maintaining blood pressure in rats, focused on targeting which protocols were associated with the most promising results in this species and could be used as a guide to future studies.

According to Huang et al. (2016), hypertension is a threat to human health due to the concomitant risks of cardiovascular and renal diseases. The common drugs prescribed to treat hypertension are mostly “Prils”, such as enalapril, fosinopril and captopril (Deng et al., 2018;

Huang et al., 2016). Acting as ACE inhibitors, these synthetic medicines, once ingested, can produce a strong and rapid hypotensive effect (Deng et al., 2018). Cheng et al. (2008), Onuh et al. (2015), Mas-Capdevila et al. (2018) and Udenigwe et al. (2017) reported this powerful antihypertensive effect: the captopril-treated animals showed a rapid significant SBP reduction observed 2 hours after administration (around -30, -25, -15 and -10 mmHg, respectively) and this effect could still be observed during 8 weeks of chronic drug administration (Cheng et al., 2008). Despite its beneficial effects in the blood pressure regulation, the "Prils" drugs can also cause various undesirable side-effects (e.g. dry cough, nausea, rashes and acute renal failure) (Deng et al., 2018; Huang et al., 2016). For this reason, it is growing the scientific efforts to obtain less harmful alternatives (Deng et al., 2018).

It has been reported that through enzymatic proteolysis, *in vitro* chemical hydrolysis, microbial fermentation and gastrointestinal digestion of plants and animals proteins, e.g. winged bean seeds (Chay, Tan, & Saari, 2015), egg (Jahandideh et al., 2014), pork (Inoue et al., 2013) and feather (Fontoura et al., 2014), it is possible to obtain hydrolysates and bioactive peptides which can act like drugs with anti-hypertensive activity by regulating ACE, renin and angiotensin II receptor activities, without or with reduced side-effects to human health (Aluko, 2015; Deng et al., 2018; Jahandideh et al., 2014; Jamdar et al., 2012).

In relation to the proteins of poultry origin, it is known that they are also an excellent source of protein hydrolysates and bioactive peptides. Several of these bioactive compounds have already been reported and, independent of the animal part used, like meat (Fukada et al., 2016; D. Wang & Shahidi, 2018; L.-S. Wang, Huang, Chen, Huang, & Zhou, 2015); viscera (Jamdar et al., 2012; Mane & Jamdar, 2017); feather (Callegaro, Welter, & Daroit, 2018; Fontoura et al., 2014, 2018), skin (S. J. Lee et al., 2010, 2012; Sarbon, Badii, & Howell, 2018) and bones (Nie, Xu, Zhao, & Meng, 2017), in all works, the evaluated bioactive compound presented a great antioxidant and/or angiotensin-converting enzyme (ACE) inhibitory capacities.

Although the protein hydrolysates and bioactive peptides with poultry origin could exert their biological activities after direct extraction, a fast method that employ distinct solvents extraction (distilled water, phosphate buffer and aqueous acidic solution), the enzymatic hydrolysis is the method widely-used (Liu, Xing, Fu, Zhou, & Zhang, 2016; L. Wang et al., 2018). This method involves the use of one or more enzymes, usually at the optimum temperature and pH conditions for each protease, that can modify and improve the functional properties of the proteins, enhancing its solubility, without affecting the nutritional value, while converts them into various peptides (Aluko, 2015; Mendoza-Jiménez et al., 2018).

The antihypertensive activity reported to the protein hydrolysates and bioactive peptides studied in the papers retrieved to this systematic review were based on the use of various enzymes and/or proteases, alone (e.g. *Bacillus licheniformis* proteinase; Pepsin), or in combination, (e.g. Alcalase and Pepsin + Pancreatin; Pepsin + Pancreatin; *Bacillus licheniformis* alcalase and porcine Pepsin with pancreatin) (Table 4).

The majority of these proteases are specific, cleaving peptide bonds between specific amino acids (Liu et al., 2016). In this way, the type of protease used in this process, as well the hydrolysis conditions affect the acquisition of antihypertensive hydrolysates and peptides (Bhat, Mason, Morton, Bekhit, & Bhat, 2018; S. Y. Lee & Hur, 2017; Sánchez & Vázquez, 2017). The hydrolysates should contain peptides with a wide range of chain lengths and composed by distinct amino acids that could influence their nutritional values and biological effectiveness (Aluko, 2015; Iwaniak et al., 2014; Liu et al., 2016). Other hydrolysis parameters which are also known to affect the bioactivity of the hydrolysates and peptides include substrate concentration, enzyme/substrate ratio, incubation time and physicochemical conditions, such as pH and temperature (Mendoza-Jiménez et al., 2018; R. R. Silva, 2018; D. Wang & Shahidi, 2018).

Regarding to the ACE inhibition property, researchers observed that most of the antihypertensive peptides are generally short chains of two to 12 amino acids. Besides that, hydrolysates and peptides with most effective antihypertensive activities likely contain, at the N-terminal position, basic amino acids (such as Lys) or aliphatic amino acids (such as Val and Leu) and, in the C-terminal, hydrophobic amino acids residues (such as Pro, Leu, Phe and Trp) (Huang et al., 2016; Lemes et al., 2016; Sangsawad, Roytrakul, & Yongsawatdigul, 2017). For this purpose, the separation of hydrolysates and peptides into groups based on their hydrophobicity, net charge and chain length, and peptide purification by membrane ultrafiltration and chromatography techniques are often applied (Aluko, 2015; Iwaniak et al., 2014).

Although purified peptides and protein hydrolysates with low molecular weight exhibit a strong inhibitory activity, many studies suggest that hydrolysates derived from chicken protein have a longer antihypertensive activity due to the variety of peptides in their composition (Jamdar et al., 2012; Onuh, Girgih, Aluko, & Aliani, 2013; Terashima et al., 2010). Jamdar et al. (2012), for example, could not improve the ACE inhibitory activity using poultry viscera protein hydrolysates fractionated by ultrafiltration membranes. Onuh et al. (2015) found that peptides present in the CBSH (chicken breast skin protein hydrolysate) and CTSH (chicken thigh skin protein hydrolysate) reduced the inhibitory activity by approximately 50%, probably due to a loss of synergistic functions, which contributes to strong anti-hypertensive properties. Additionally, Cheng et al. (2008) and Herregods et al. (2011) suggest that the purification of food-derived compounds with ACE inhibitory activity is not profitable and that hydrolysates with high ACE inhibitory activity are preferable in terms of costs and applicability.

Several methods have been devised for estimating potential antihypertensive properties of peptides and the most commons involve *in vitro* ACE or renin activity assays (Aluko, 2015). The method described by Cushman and Cheung (1971) was reported in 5 articles (Cheng et al.,

2008; Nakade et al., 2008; Saiga et al., 2003, 2006, 2008) and uses Hippuryl-L-histidyl-L-leucine (HHL) as ACE substrate, with activity estimated by the amount of hippuric acid released from HHL measured by spectrophotometry (Aluko, 2015). Onuh et al. (2015) and Udenigwe et al. (2017) applied the methodology described by Udenigwe et al. (2009) which is based on the use of FAPGG (N-(3-[2-furyl] acryloyl)-phenylalanylglycylglycine) as ACE substrate and estimates. Mas-Capdevila et al. (2018) reported an alternative fluorometric ACE assay, using o-Abz-Gly-p-Phe(NO2)-Pro-OH as substrate and Onuh et al. (2016) determined the plasma ACE activity plasma according to the spectrophotometric method reported by Girgih, Alashi, He, Malomo & Aluko (2013).

It is known *in vitro* ACEI activity and *in vivo* antihypertensive effects of protein hydrolysates are not always correlated, due to the bioavailability of the ACE inhibitory peptides after oral administration and the fact that peptides may influence blood pressure by mechanisms other than ACE inhibition (Herregods et al., 2011; Mas-Capdevila et al., 2018). Considering this, *in vivo* assays are performed in order to confirm this bioactivity in an animal model.

Table 4 shows the blood pressure reducing effects of different chicken protein-derived protein hydrolysates and/or bioactive peptides after their administration to SHRs. Although differences were observed to the doses of hydrolysates and bioactive peptides, peptide production method and protein sources, the short and/or long-term administration of all bioactive compounds to spontaneously hypertensive rats led to a reduction in the systolic blood pressure of the animals, with different rates of blood pressure–lowering activity. The tested synthetic peptide (Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-Phe) (Saiga et al., 2006) and the peptide YYRA (Nakade et al., 2008) produced a maximum decrease within 25 min and 3 h, respectively, whereas the protein hydrolysates obtained from breast muscle (Saiga et al., 2003; Udenigwe et al., 2017), leg bones (Cheng et al., 2008), feet (Mas-Capdevila et al., 2018),

collagen (Saiga et al., 2008) and skins from the thigh and breast muscles (Onuh et al., 2016, 2015) took 2-6h and 2-6 weeks after short and long-term administration.

It is known that the use of randomized evaluations and blind assessments improve the reliability of scientific works (V. D. O. Silva et al., 2012). However, only 2 article reported randomization of samples (Cheng et al., 2008; Onuh et al., 2016) and none of the studies reported blind evaluation methods.

5. CONCLUSION

Administration of chicken protein-derived hydrolysates and peptides to spontaneously hypertensive rats exhibit a potential antihypertensive activity with likely cardiac protective effects against end-organ damage. The degree of the activity depends mainly on the type of enzyme and on the enzymatic hydrolysis conditions. Protein hydrolysates have several advantages when compared with purified chicken peptides. Chicken hydrolysates have a more prolonged antihypertensive activity than purified peptides because of the synergistic effect of their different peptides. The use of hydrolysates reduces the costs with purification and scaling, providing profitability and viability.

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Table 1. Scientific quality criteria adopted to assess the methodological quality of the published works

Criteria*	Definition	Score
Control group	Studies that related the use of control group	2
	Papers that did not report the use or did not clearly cited	1
Type of control group	The use of both positive (PC) and negative (NC) control groups	2
	The use of only one control group type, articles that did not clearly mentioned or those ones with score 1 in the "control group" criteria.	1
Administration period	Papers studying the long-term effect of the bioactive compound	2
	A short-term effect study	1
Blind assessments	Papers including double or single-blind assessments	2
	When the blind assessment details were not given in the text	1
Randomized studies	Experiments where the groups were randomized	2
	The absence of randomized experimental design or articles in which randomization was not clearly described	1
Sample size	Papers with 5 or more animals/group	2
	Studies with less than 5 animals/group	1
Type of analysis	Articles that evaluated additional variables, such as other cardiovascular and body weight effects, biochemical parameters	2
	Studies that evaluated only the blood pressure of the animals	1

PC: positive control group; NC: negative control group. *Criteria were adopted according to Greenhalgh (1997) and other systematic reviews (ANDRADE, E. F. et al., 2015; FERREIRA et al., 2013; PEREIRA, U. P. et al., 2011; SILVA, V. D. O. et al., 2012)

Table 2. Scores of the selected articles according to the evaluation criteria

	Published works								
Quality criteria	Cheng et al. (2008)	Mas-Capdevila et al. (2018)	Onuh et al. (2016)	Saiga et al. (2008)	Onuh et al. (2015)	Udenigwe et al. (2017)	Nakade et al. (2008)	Saiga et al. (2006)	Saiga et al. (2003)
Control group*	2	2	2	2	2	2	2	2	2
Type of control group**	1	2	1	1	2	2	2	1	1
Administration period***	2	2	2	2	1	1	1	1	1
Blind evaluation****	1	1	1	1	1	1	1	1	1
Randomization•	2	1	2	1	1	1	1	1	1
Sample size••	2	2	2	2	1	1	1	2	2
Type of analysis•••	2	2	2	2	1	1	1	1	1
Total	*12*	*12*	*12*	*11*	*9*	*9*	*9*	*9*	*9*

*control group (score 2), control group unclear or studies without control group (score 1); **positive and negative controls (score 2), one type of control group, control group type unmentioned, or studies with score 1 in the "control group" parameter (score 1); ***studying long-term effect (score 2), short-term (score 1); ****blind assessments (score 2), unclear or studies without blind assessments (score 1); •randomized studies (score 2), unclear or articles without randomization (score 1); ••Samples groups ≥ 5 animals (score 2); less than 5 animals per group (score 1); •••exclusive SBP measurements (score 1), evaluation of additional *in vivo* variables, such other cardiovascular effects, biochemical parameters, body weight effects, etc. (score 2).

Table 3. Summary of the selected articles

	Published works								
Parameters	Cheng et al. (2008)	Mas-Capdevila et al. (2018)	Saiga et al. (2008)	Onuh et al. (2015)	Nakade et al. (2008)	Udenigwe et al. (2017)	Onuh et al. (2016)	Saiga et al. (2006)	Saiga et al. (2003)
Bioactive compound	Hydrolysate (A4)*	Hydrolysate	Hydrolysate	Hydrolysates (CBS, CBSH, CTS, CTSH)**	Peptide (YYRA)***	Hydrolysate (SPH-P; SPH-PPc)♦	Hydrolysates (CSPH)♦♦	Peptide♦♦♦	Hydrolysate
Fractionation and/or purification techniques	No	0.45 µm membrane	Ultrafiltration membrane (3 kDa) and HPLC	Ultrafiltration membranes (1; 3, 5 and 10 kDa)	HPLC	No	No	No	Ultrafiltration membrane (1 kDa) and HPLC
Administration period	Short and long-term	Short-term	Short and long-term	Short-term	Short-term	Short-term	Long-term	Short-term	Short-term
Administration route	Intubation	Intubation	Oral	Gavage	Intubation	Gavage	Unclear	Intravenous	Oral
Blind evaluation	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear
In vivo analysis	Systolic blood pressure; Total weight gain; heart weight and heart/body weight ratio	Systolic blood pressure; aorta and plasma analysis	Systolic blood pressure; blood components analysis	Systolic blood pressure	Systolic blood pressure	Systolic blood pressure	Systolic blood pressure; plasma analysis; metabolomics studies;	Systolic blood pressure	Systolic blood pressure

*HPLC: High Performance Liquid Chromatography; NC: Negative control; PBS: Phosphate buffered saline; PC: positive control; SHR: Spontaneously hypertensive rats; WKY: Wistar-Kyoto rats. *Hydrolysate obtained after hydrolysis with Alcalase for 4 hours; **CBS: Unhydrolyzed defatted chicken breast skin meal; CBSH: chicken breast skin protein hydrolysate from pepsin + pancreatin digestion; CTS: Unhydrolyzed defatted chicken thigh skin meal and CTSH: chicken thigh skin protein hydrolysate from alcalase digestion; ***YYRA: Tyr-Tyr-Arg-Ala; ♦SPH-P: Spent hen hydrolysate from pepsin digestion and SPH-PPc: Spent hen hydrolysate from pepsin–pancreatin digestion; ♦♦CSPH: chicken skin protein hydrolysate; ♦♦♦Synthesized peptide: Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-Phe.*

Table 4 Effects of administration of chicken protein-derived protein hydrolysates and/or bioactive peptides on short-term and long-term changes in the systolic blood pressure (SBP) of spontaneously hypertensive rats

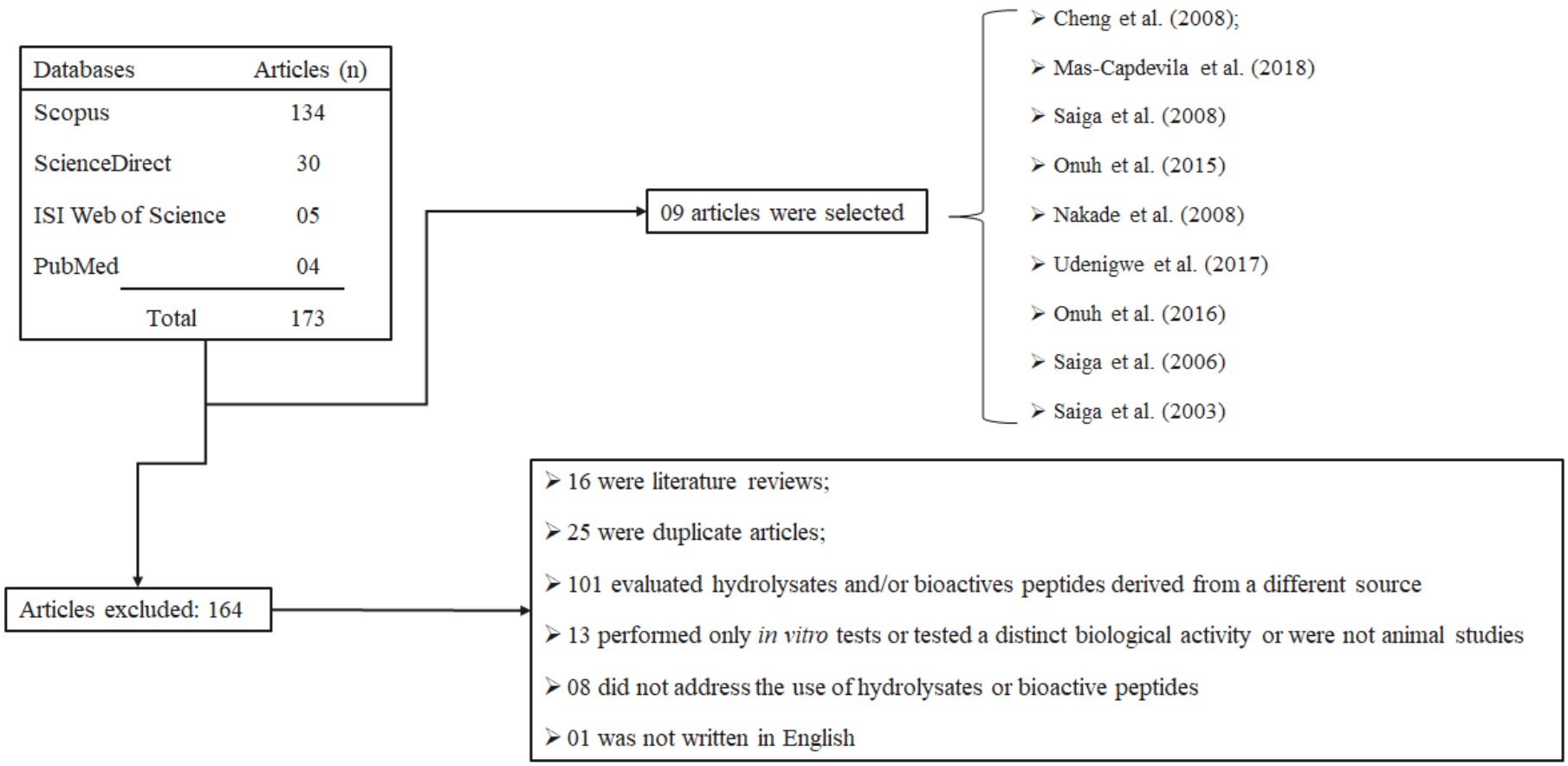
Protein source	Protease	Dose (mg/kg bw)	SBP maximum reduction (mmHg)[‡]	Reference
Leg bones	*Bacillus licheniformis* proteinase	50	- 25 mmHg after 4 h - 20 mmHg after 4 weeks	Cheng et al. (2008)
Feet	Protamex®	85[♦]	- 30 mmHg after 6h	Mas-Capdevila et al. (2018)
Collagen	*Aspergillus oryzae* protease; a mix of proteases[*]; pepsin and trypsin/chymotrypsin	3,000	- 40 mmHg after 6 h - 20 mmHg after 2 weeks	Saiga et al. (2008)
Skins from the thigh and breast muscles	*Bacillus licheniformis* alcalase; porcine pepsin with pancreatin	100	CTSH: - 30 mmHg after 3 h CBSH: - 32 mmHg after 5 h	Onuh et al. (2015)
Bone	Pepsin	10	- 20 mmHg after 3 h	Nakade et al. (2008)
Breast muscle	Pepsin + Pancreatin	200	- 40 mmHg after 2 h	Udenigwe et al. (2017)
Skins from the thigh and breast muscles	Alcalase and pepsin + pancreatin	400[♦]	- 39 mmHg after 6 weeks	Onuh et al. (2016)
-	-	30	- 36 mmHg after 25 min	Saiga et al. (2006)[**]
Breast muscle	*Aspergillus* protease; trypsin/chymotrypsin; porcine small intestinal juice	1,000	- 45 mmHg after 2 h	Saiga et al. (2003)

bw: body weight; CBSH: chicken breast skin protein hydrolysate from pepsin + pancreatin digestion; CTSH: chicken thigh skin protein hydrolysate from alcalase digestion. [♦]*Dose with higher antihypertensive effect;* [*]*Protease FP, protease A amano G, and Protease N;* [**]*Authors synthesized an ACE inhibitory peptide [Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-Phe] found in the extract digested by gastric enzyme in a previous work* (SAIGA et al., 2003). [‡]*Some values are estimates obtained from graphs in the respective publications.*

Figure legends

Fig. 1. Flowchart of the papers search process using the keywords “antihypertensive effect” and “bioactive peptides” and “chicken protein” and “rats”. Adapted from Andrade et al. (2015).

Figure 1


Databases
Articles (n)
Scopus
134
ScienceDirect
30
ISI Web of Science
05
PubMed
04
Total
173
09 articles were selected
Cheng et al. (2008);
Mas-Capdevila et al. (2018)
Saiga et al. (2008)
Onuh et al. (2015)
Nakade et al. (2008)
Udenigwe et al. (2017)
Onuh et al. (2016)
Saiga et al. (2006)
Saiga et al. (2003)
Articles excluded: 164
16 were literature reviews;
25 were duplicate articles;
101 evaluated hydrolysates and/or bioactives peptides derived from a different source
13 performed only in vitro tests or tested a distinct biological activity or were not animal studies
08 did not address the use of hydrolysates or bioactive peptides
01 was not written in English

5 ANTIOXIDANT AND ANGIOTENSIN-CONVERTING ENZYME INHIBITORY PROPERTIES OF Coturnix coturnix Japonica BREAST MUSCLE PROTEIN HYDROLYSATES AND PEPTIDES PROFILE DETERMINATION

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ABSTRACT

This study evaluated the antioxidant and angiotensin-converting enzyme (ACE) inhibitory activities of *Coturnix japonica* protein hydrolysates (CJPH) and its *in vitro* gastrointestinal fractions. CJPH showed to be nontoxic and presented a high 2,2-Diphenyl-1-Picrylhydrazyl radical-scavenging activity (55.07-69.28%) besides a strong ACE inhibition (IC_{50} = 0.10 $mg.mL^{-1}$). Although a low capacity to scavenge the 2,2-azino-bis- (3-ethylbenzothiazoline)-6-sulphonic acid radical (19.97%) has been found, an improvement around 3.33 folds was verified after *in vitro* digestion. The peptide size was crucial for the biological activities, the kinetics studies revealed an uncompetitive ACE inhibition pattern to CJPH and its gastrointestinal fraction (GiPH (3)), and several potential peptides were found in the GiPH(3). The *Coturnix coturnix japonica* breast proteins have potential use to develop hydrolysates that could be applied as an active ingredient of functional foods.

Keywords: Protein hydrolysates, Japanese quail; Radical-scavenging activity; Angiotensin-converting enzyme; Gastrointestinal digestion; Enzymatic kinetics

PRACTICAL ABSTRACT

Quail breast muscle is a nutritious by-product of the poultry industry. Since this protein-rich meat has a lower amount of fat and cholesterol levels, its consumption is proper for a low-fat diet. Besides these nutritional properties, the quail meat can serve as a potential source of different bioactive peptides. In this study, the antioxidant and angiotensin-converting enzyme (ACE) inhibitory effects of *Coturnix japonica* protein hydrolysates (CJPH) was evaluated before and after *in vitro* gastrointestinal digestion. Moreover, the peptide profiles in the gastrointestinal protein hydrolysate with 3 kDa MWCO - GiPH(3) were identified. This hydrolysate could be used for future nutraceutical applications.

1. INTRODUCTION

Researches involving the search for novel natural antihypertensive and antioxidants agents, particularly those obtained from proteins hydrolysis, have attracted considerable attention, due to their potential beneficial effects related to many diseases (Fontoura et al., 2014; Gu et al., 2012). It is known that the dietary proteins have peptides sequences encrypted within their primary structure that may be able to modulate specific physiological functions after their release from the native protein during food processing and gastrointestinal digestion (Aluko, 2015; Fontoura et al., 2014; Phongthai, D'Amico, Schoenlechner, Homthawornchoo, & Rawdkuen, 2018). In this context, the term "bioactive peptides" refers to specific protein fragments that can exhibit diverse biological functions or physiological effects, such as antihypertensive, antioxidant, immunomodulatory, antimicrobial, antithrombotic and mineral binding (Sánchez & Vázquez, 2017).

The hydrolysates prepared from animal proteins hydrolysis can confer nutritional and functional properties to food besides serving as a source of bioactive peptides (Jamdar, Rajalakshmi, & Sharma, 2012). These hydrolysates have been extensively studied and the results suggest that they exhibit longer antioxidant and angiotensin-converting enzyme (ACE) inhibitory activity due to the variety of peptides in their composition (Jamdar et al., 2012; Onuh, Girgih, Aluko, & Aliani, 2014).

The consumption of poultry meat has increased among consumers especially after the increase in demand for quality meat. Quail is one of the leanest types of poultry, for this reason, their meat is known to be proper for a low-fat diet (Fakolade, 2015; Kalafova et al., 2018; Tavaniello et al., 2017). The most common quail breed for human consumption is the *Coturnix coturnix japonica* (Fakolade, 2015). This poultry meat shows high protein content (22.23-23.38%), lower amount of fat (2.21-2.75%), a high degree of unsaturated fatty acids (FA), lower cholesterol levels, is a source of minerals (sodium, potassium, and iron), and it is rich in

essential amino acids with the lysine and methionine levels comprising 12,36% of breast meat protein (Fakolade, 2015; Genchev, Mihaylova, Ribarski, Pavlov, & Kabakchiev, 2008; Kalafova et al., 2018; Tavaniello et al., 2017).

Due to the high nutritional quality of poultry meat, it is of great scientific interest to obtain new protein hydrolysates containing bioactive peptides that, once ingested as part of a diet, could exercise their basic nutritional functions as well as produce metabolic or physiological effects useful in maintaining human health (Jamdar et al., 2012). The radical-scavenging and ACE-inhibitory activities of hydrolysates obtained from poultry protein hydrolysis have already been demonstrated (Jamdar et al., 2012; Mane & Jamdar, 2017; Nie, Xu, Zhao, & Meng, 2017; Onuh et al., 2014), however obtaining quail breast muscle proteins hydrolysates still lacks studies. The present work aims to describe the antioxidant and angiotensin-converting enzyme inhibitory activity of *Coturnix coturnix japonica* breast muscle protein hydrolysates and its digesta fractions, as well identify the peptide profile in this quail hydrolysates after *in vitro* gastrointestinal digestion.

2. MATERIALS AND METHODS

2.1. Preparation of the *Coturnix japonica* protein hydrolysates (CJPH)

The methodology used for the extraction and deproteinization of the Japanese quail breast muscle was according to the proposal by Escudero et al. (2013), with modifications. Twenty frozen quail carcasses, each one weighing approximately 100g, were acquired in a commercial establishment. After deboning, 50 g of the breast muscle (*Pectoralis major*) was cut into cube and homogenized (Scilogex D160 Homogenizer, 30000 rpm, 10 min) with HCl (200 mL; 0.01 N), followed by successive filtration (PTFE; 25 mm x 0.22 µm) and centrifugation steps (12000 g; 20 min; 4ºC) until completely remove the precipitate. After addition of 3 volumes of ethanol (20 min; 4°C), the deproteinized extracts were filtrated (PTFE; 25 mm x 0.22 µm) and centrifugated again (12000 g; 20 min; 4ºC). Ethanol was eliminated by evaporation using a

rotary evaporator. The dried extracts were dissolved in 30 mL of Milli-Q water and stored at -20ºC until use as the *Coturnix japonica* protein hydrolysate (CJPH).

2.2. The *in vitro* gastrointestinal (GI) digestion

The *in vitro* GI digestion of CJPH was carried out according to the method described by Versantvoort, Oomen, Van De Kamp, Rompelberg, and Sips (2005). Briefly, the *in vitro* digestion was started by adding the Japanese quail protein hydrolysates (3.63 mL; 7000 µg.mL^{-1}) in a saliva solution (3 mL; pH 6.8), kept under agitation for 5 min. After that, the solution was adjusted to pH 2 and pepsin (1%) was added. The reaction was carried out for 120 min. The solution was neutralized to pH 8 and then was added pancreatin (1%), remaining under agitation for 60 min. The pH was again adjusted to 8.2 and a solution of bile extract was added, with the incubation carried out for 60 min. All incubations were performed at 37°C under agitation at 100 rpm. Samples were collected at different times (120 and 240 min), representing the gastric and gastrointestinal digestions, respectively, and then were fractionated passing through an ultrafiltration membrane with molecular weight cut-offs (MWCO) of 10 and 3 kDa (Millipore Co., Bedford, MA). The permeates were collected, freeze-dried, reconstituted using Milli-Q water and stored at -20ºC as 3–10 kDa and <3 kDa gastrointestinal ultrafiltrated (UF) membrane fractions until posterior analysis.

2.3. Samples

The CJPH was the *Coturnix japonica* protein hydrolysate (undigested and deproteinized) hydrolysate; GPH(3) and GPH(3-10) were the gastric protein hydrolysates obtained from pepsin hydrolysis of CJPH with <3 and 3–10 kDa of molecular weight (MW), respectively; and GiPH(3) and GiPH(3-10) were the gastrointestinal protein hydrolysates obtained from pepsin + pancreatin hydrolysis of CJPH with <3 and 3–10 kDa MWCO, respectively.

2.4. Antioxidant activities

2.4.1. DPPH radical-scavenging activity assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging effect of CJPH was measured by the method of Brand-Williams, Cuvelier, and Berset (1995), modified by Fukumoto and Mazza (2000). CJPH hydrolysates (22 μL) at three concentrations (2.5, 5.0 and 10.0 $mg.mL^{-1}$) was mixed with a 2,2-Diphenyl-1-picrylhydrazyl (DPPH) solution (200 μL; 25 $mg.mL^{-1}$ of ethanol), previously prepared. The mixtures were incubated at room temperature in the dark for 120 min, and the resulting absorbance of the reduction of DPPH radicals was measured at 595 nm using a Microplate Lector LM-LGC (LGC Biotechnologies Ltd., São Paulo, Brazil). The blank of each concentration was prepared as described above replacing the samples with distilled water. The DPPH radical-scavenging activity (%) was calculated using the following formula:

$$\text{DPPH radical-scavenging activity (\%)} = (A - B)/A \times 100$$

where A is the blank absorbance and B is the absorbance of the sample. The test was carried out in triplicate.

2.4.2. ABTS radical-scavenging activity assay

The 2,2-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) scavenging activity of the *Coturnix japonica* protein hydrolysates were carried out using the ABTS assay method described by Re et al. (1999), with some modifications. A stock solution of ABTS radical (7 mM) with potassium persulfate (140 mM, final concentration) was produced and incubated at room temperature in the dark for 16h before use. An aliquot of ABTS stock solution was diluted in ethanol to an absorbance of 0.7 (± 0.02) at 734 nm, in order to prepare the working solution of ABTS radical. 30 μL of different concentrations (2.5, 5.0 and 10.0 $mg.mL^{-1}$) of CJPH were mixed with 3 mL of the working solution and the absorbance at 734 nm was measured after 06 min. Trolox (6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid) was used to perform a standard curve. The percent scavenging of ABTS radical was calculated using the following equation:

$$\text{ABTS radical-scavenging activity (\%)} = (A - B)/A \times 100$$

Where A is the working solution absorbance and B is the absorbance of the sample. The IC_{50} value, defined as the hydrolysate concentration in $mg.mL^{-1}$ required to produce 50% reduction of the initial ABTS concentration, was calculated by linear regression of the curve showing the ABTS radical-scavenging activity (%) versus hydrolysate concentration ($mg.mL^{-1}$). The test was carried out in triplicate.

2.5. Angiotensin-converting enzyme (ACE) inhibitory activity

The *in vitro* ACE inhibition was assayed according to Cushman and Cheung (1971) adapted by Meira et al. (2012). The reaction started incubating CJPH (20 μL) at three concentrations (2.5, 5.0 and 10.0 $mg.mL^{-1}$) with 40 μL of ACE (0.1 $U.mL^{-1}$, from rabbit lung, Sigma-Aldrich) and 100 μL of a buffered substrate solution (5 mM Hippuryl-histidyl-leucine in 50 mM HEPES-HCl buffer containing 300 mM NaCl; pH 8.3; 37°C; 30 min). To stop the reaction, HCl (150 μL; 1 M) was added to the mixture. The released hippuric acid (HA) was extracted using 1 mL of ethyl acetate and the upper layer (organic phase) was transferred to a glass tube to be evaporated in a sand bath (80ºC). The dried samples were dissolved in distilled water and measured spectrophotometrically at 228 nm. Captopril (1 mM, Sigma-Aldrich) was used as positive control. ACE inhibition (ACEi) (%) was calculated using the following formula:

$$\text{ACEi (\%)} = (A - B)/(A - C) \times 100$$

where A is the HA content of the control without an extract sample, B is the HA content of the control without ACE, C is the HA content of the reaction with an extract sample and ACE. The IC_{50} (the concentration of protein that inhibits 50% of the ACE activity) was calculated by linear regression of the curve showing the inhibitory activity (%) versus hydrolysate concentration ($mg.mL^{-1}$).

2.6. Determination of kinetic parameters of ACE inhibition

The kinetic of ACE inhibition was studied in the presence and absence of three different concentrations (0, 5.0 and 10.0 $mg.mL^{-1}$) of CJPH and GiPH(3) samples with 6, 3.5, 2, 1 and 0.5 mM Hippuryl-histidyl-leucine. A non-regular regression fit of the kinetics data to the Michaelis–Menten equation was used to estimate the kinetic parameters, K_m and V_{max} using the GraphPad Prism version 7.04 (GraphPad Software, Inc., San Diego, USA). Lineweaver—Burk plots were applied to confirm the mode of inhibition of ACE (competitive, non-competitive or uncompetitive). The kinetic experiment was performed in triplicate.

2.7. Peptide profile

In order to verify the profile of the potential peptides formed after the *in vitro* gastrointestinal digestion process, the CJPH and GiPH(3) samples were analyzed by mass spectrometry (MALDI-TOF-MS/MS) using an Autoflex II mass spectrometer (Bruker Daltonics, Bremen, Germany). Initially, samples were mixed to the matrix containing α-cyano-4-hydroxycinnamic acid (10 $mg.ml^{-1}$) in 50% acetonitrile with 0.3% trifluoroacetic acid (1:1 ratio). Subsequently, 1 µL of this mixture was applied to the MALDI plate (MTP ground steel 384, Bruker Daltonics, Bremen, Germany) and dried at room temperature. The positive reflection mode was used to analyze each chromatographic fraction using an acceleration voltage of 19 kV and a laser frequency of 100 Hz. The Peptide Mix solution (Brucker Daltonics) was used for calibration and the ion detection range was m/z 0.4 to 5 kDa.

From the MS spectra, the peaks of interest were chosen to be fragmented using the LIFT DEFAULT method at an acceleration voltage of 6 kV. All data were obtained using Flex Control software, and the spectra were processed using Flex Analysis One software (Version 3.0, Bruker Daltonics). The automatic spectral processing was performed using SearchGui v3.3.13 software (CompOmics, Ghent, Belgium) (http://compomics.github.io/projects/searchgui.html) (Barsnes & Vaudel, 2018). The database search was done through SearchGui software choosing the MS Amanda (Dorfer et al., 2014) as

the search engine. The input parameters settings included: precursor m/z tolerance, 0.5 – 1.5 Da and fragment m/z tolerance, 0.5 Da. The raw data files obtained from the MALDI-TOF-MS/MS analysis of the samples were searched against the *Coturnix* or *Gallus* subdirectory of the SwissProt protein database (UniProt, EBI, UK). The postprocessing of the identifications was performed via PeptideShaker v1.16.38 software (CompOmics, Ghent, Belgium) (http://compomics.github.io/projects/peptide-shaker.html) (Vaudel et al., 2015).

2.8. Potential toxicity of the *Coturnix japonica* protein hydrolysates (CJPH) in *Allium cepa* bioassay

The *Allium cepa* test was performed as described by Fiskejö (1985), with modifications. Healthy, ungerminated and equal sized bulbs (n = 55; Ø 20-30 cm; 30–40 g weight) of common onion, with white external cataphylls, were acquired in a commercial establishment in Recife-Pernambuco. Prior to testing and to reduce the possible effects of budding inhibitors, the bulbs were placed in a refrigerator (4 °C) for 24 hours. After this, the bulbs were randomly positioned for germination during five days at room temperature on sterilized polypropylene tubes (15 ml), with the lower part immersed into solutions containing: dechlorinated water (negative control - NC); copper sulfate 0.6 $\mu g.mL^{-1}$ (positive control for genotoxicity - PC); and CJPH at three concentrations (2.5, 5.0 and 10.0 $mg.ml^{-1}$). All dilutions were carried out in distilled water and the volume of solution absorbed was replenished daily to keep the roots submerged. Five replicates were made for each experimental group to ensure that the results were reproducible.

The mean root growth (MRG) were used to determine the toxic potential of CJPH. This parameter was determined by the average length of the three largest roots for each onion bulb per experimental group. For the cytotoxicity analysis, the roots tips (1-3 mm) from controls and treated samples were excised and fixed immediately in Carnoy´s fluid (ethanol:acetic acid; 3:1; v/v). After 24 hours, root tips were hydrolyzed in 45% HCl (10 min), washed with distillated water and stained with hematoxylin. Samples were placed on a glass slide and the crush smear

technique was used to finalize the histological preparation. For each treatment, five microscope slides were prepared with *Allium cepa* root meristem.

The microscope slides images were captured using the Motic camera (MOTICAM 1000, QUIMIS) attached to an optical microscope (Leica, Wetzlar, Germany) and analyzed using the software MOTIC Image Plus 2.0 at 1000x magnification. 1000 cells were counted from each slide, totalizing 5000 cells per treatment. The antiproliferative capacity (cytotoxicity) of the treatments was verified from the mitotic index (MI), obtained by the ratio between the number of dividing cells (Prophase, Metaphase, Anaphase and Telophase) and the number of cells counted per treatment (Mitotic + interphase cells). For analysis of genotoxicity and mutagenicity were evaluated by counting the chromosomal abnormalities (chromosomal breaks, binucleated cells, nuclear buddings and chromosomal bridges) and the presence of micronuclei in the meristematic cells of the *Allium cepa* root.

2.9. Data Analysis

After testing for normality and homogeneity of variances (Shapiro–Wilk and Levene test, respectively), data were subjected to analysis of variance (ANOVA) using GraphPad Prism version 7.04 (GraphPad Software, Inc., San Diego, USA). The post hoc Tukey test was the parametric alternative used to compare all groups. Data were presented as the mean ± standard error of the mean and the significance was considered at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Antioxidant properties of the *Coturnix japonica* protein hydrolysates

For this analysis, two radical systems (DPPH and ABTS radicals-scavenging activities) were applied to investigate the antioxidant properties of the *Coturnix japonica* protein hydrolysates, since the antioxidant activity of protein hydrolysates may not be attributed to a single mechanism (Gu et al., 2012; Wang et al., 2018). Table 1 presents the mean values of the

DPPH and ABTS radicals-scavenging activities of CJPH for three concentrations (2.5, 5 and 10 mg.mL^{-1}).

3.1.1. DPPH radical-scavenging activity

The CJPH was able to react with free DPPH radicals and terminate the radical chain reaction. It was verified a correlation (R^2= 0.998, $p < 0.05$) between the tested concentrations (2.5, 5.0 and 10.0 mg.mL^{-1}) and the DPPH radical-scavenging activity (56.43, 60.19 and 69.28%), with a significant difference among them (Table 1). DPPH radical is relatively stable and has been extensively used to evaluate the free radical-scavenging potential ability of natural antioxidative substances (Gu et al., 2012; Jamdar et al., 2012; Nie et al., 2017). The DPPH radical activity observed to CJPH was higher than that reported for protein hydrolysate prepared from poultry viscera protein (Jamdar et al., 2012), chicken skin (Onuh et al., 2014), and feather (Callegaro, Welter, & Daroit, 2018). Nie et al. (2017) evaluating chicken bone peptide hydrolysates at three concentrations (2, 4 and 6 mg.mL^{-1}) found a maximum percentage of DPPH radical activity varying between 60-65%, with the activity showing to be dose-dependent and these findings are comparable with the results presented here to the *Coturnix japonica* protein hydrolysates.

3.1.2. ABTS radical-scavenging activity

The ABTS radical-scavenging activity of the CJPH at different concentrations (2.5, 5.0 and 10.0 mg.mL^{-1}) were 5.60, 10.99 and 19.97%, which were significantly different. The ABTS radical activities and concentrations also showed a correlation (R^2= 0.9978, $p < 0.05$) and the IC_{50} value was 25.75. The ABTS radical-scavenging assay is a simple and fast method that has been widely applied to analyze the radical-scavenging activities of hydrophilic and hydrophilic compounds (Ketnawa, Wickramathilaka, & Liceaga, 2018; Phongthai et al., 2018). In this study, CJPH showed less capacity to scavenge ABTS radical (10.8; 5.47 and 3.47 folds) than to DPPH$^{\bullet}$ at the same concentrations (2.5, 5.0 and 10.0 mg.mL^{-1}, respectively). Differences

between DPPH and ABTS radicals-scavenging capabilities are common and could be due to the difference of solubility and diffusivity of radicals and peptides in the reaction system (Callegaro et al., 2018).

3.1.3. Effect of GI digestion and the ultrafiltration (UF) on antioxidant properties of the *Coturnix japonica* protein hydrolysates

In order to verify the resistance of the CJPH against gastrointestinal proteases and the influence of the molecular weight in its antioxidant effects, an *in vitro* gastrointestinal digestion was carried out with the resulting digesta fractionated into groups according to the molecular weight of the water-soluble peptides (3 and 3-10 kDa). In addition, as the concentration of 10 $mg.mL^{-1}$ presented a significantly higher antioxidant activity than the others, it had been chosen to evaluate the antioxidant abilities of the gastric and gastrointestinal extracts. Figure 1 shows the DPPH and ABTS radical activity of the CJPH hydrolysate and its gastrointestinal UF membrane fractions.

It was observed that the treatment of CJPH with gastrointestinal proteases (pepsin and pancreatin) caused a significant decrease ($p < 0.05$) in the DPPH radical-scavenging ability around 1.14 folds (69.28 ±1.00% to 60.77 ±1.58%) (Figure 1A), while the percentage of ABTS radical-scavenging activity increase approximately 3.33 folds along the digestion, reaching 66.40 ±4.85% for the GiPH(3) (Figure 1B). Similar findings were reported by Wang et al. (2018) to Xuanwei ham before and after cooking and *in vitro* simulated gastrointestinal digestion. These authors observed that while the cooked-digested samples showed a significant decrease in the DPPH radical activity, the ABTS radical-scavenging property showed to be higher, increasing around 3 and 4 folds compared to control and cooked Xuanwei ham samples, respectively. This improvement in the ABTS radical activity during simulated gastrointestinal digestion was also verified for microwave-treated protein hydrolysates (Ketnawa et al., 2018) and rice bran dry powder hydrolysates (Phongthai et al., 2018).

The increase on the ABTS radical activity and the slight decrease on the DPPH radical property after hydrolysis with gastric and intestinal enzymes observed here may be explained by changes that possibly occurred in the CJPH during this process. It is known that pepsin and the additional hydrolysis with pancreatin lead to structural changes in the peptide composition of the protein hydrolysates, becoming more hydrophilic and, thus, enhancing the ABTS radicals quenching by the samples. Probably, the peptides bonds may have suffered additional cleavage, leading to the accumulation of new antioxidant short peptides and free amino acids which could more readily react with ABTS radicals rather than DPPH radicals (Ketnawa et al., 2018; Wang et al., 2018).

Upon comparing UF fractions, a significant increase had been observed ($p < 0.01$) in the DPPH radical activity around 1.14 folds from GPH(3-10) to GPH(3) (54.40 ±0.35% to 61.93 ±1.10%) and 1.12 folds from GiPH(3-10) to GiPH(3) (54.20 ±0.34% to 60.77 ±1.58%) (Figure 1A), while, in relation to the ABTS radical activity, the effect of the molecular weight was only observed to the GiPH(3-10) and GiPH(3) (47.65 ±4.01% to 66.40 ±4.85%) (Figure 1B). These results show that the antioxidant properties of *Coturnix japonica* protein hydrolysates are influenced by the peptide molecular weight, probably, as reported by Phongthai et al. (2018), because peptides with lower molecular weight can react with free radicals more easily, having stronger antioxidant activities and are more resistant to digestive enzymes. Some similar findings showing a high DPPH radical-scavenging activity of low molecular weight peptides have been previously reported for thigh skin (Onuh et al., 2014) and chicken blood corpuscle (Zheng, Si, Ahmad, Li, & Zhang, 2018) protein hydrolysates. Ngoh and Gan (2016) also reported the effect of low molecular weight peptides on the radical-scavenging activity. Assessing the ABTS radical activity in five fractions (100, 50, 30, 10 and 3 kDa MWCO) of Pinto bean protein hydrolysates, the one with <3 kDa MWCO presented the highest antioxidant property (42.18 ± 14.34%). Phongthai et al. (2018) showed that rice bran protein hydrolysates

fractions with smaller peptides (<3 kDa and 3-5 kDa MWCO) exhibited a higher ABTS radical-scavenging activity (1.06 and 1.07 folds, respectively) than the fraction with 5-10 kDa MWCO.

The results presented in this work indicate that there is a stability of CJPH throughout *in vitro* digestion. This hydrolysate survived to the gastrointestinal digestion, maintaining or improving its antioxidant activity, then it could exert this effect *in vivo*. According to Zheng et al. (2018), a prerequisite for *in vivo* action of bioactive peptides and their exploitation for functional foods is the resistance against gastrointestinal proteases.

3.2. Angiotensin-converting enzyme inhibitory activity of CJPH

The ACE inhibitory activity of CJPH is presented in Table 1. The results show that the ACE inhibitory activity exhibited a dose-dependent effect at different concentrations (R^2 = 0.9973, $p < 0.05$). The 10.0 $mg.mL^{-1}$ concentration showed the highest ACE inhibition, but no differences were observed between concentrations of 2.5 and 5.0 $mg.mL^{-1}$ (Table 1).

The angiotensin-converting enzyme has an important role in blood pressure regulation. This enzyme is responsible to cleave the decapeptide angiotensin I to form angiotensin II, the center of the pathogenetic role of the renin-angiotensin-aldosterone system in hypertension (Oparil et al., 2018). In this way, the inhibition of this enzyme is considered an important method to suppress increases in blood pressure (Gu et al., 2012). Several studies have shown the ACE inhibitory effect of hydrolysates and/or peptides from various proteins sources, such as poultry viscera (Mane & Jamdar, 2017), buffalo milk (Abdel-Hamid, Otte, De Gobba, Osman, & Hamad, 2017), pig meat (Simonetti, Gambacorta, & Perna, 2016), chicken collagen (Soladoye, 2014), Greek yoghurt (Politis & Theodorou, 2016), casein (Tu et al., 2018) and Xuanwei ham (Wang et al., 2018). And, once these natural compounds have limited negative side effects and low toxicity, they can be used as alternatives to antihypertensive drugs (Aluko, 2015).

The CJPH assayed in this paper exhibited a strong ACE inhibitory activity (IC_{50} = 0.10 $mg.mL^{-1}$) and it was higher than previous studies for hydrolysates obtained from chicken leg protein hydrolyzed by pepsin + trypsin (IC_{50} = 2.58 $mg.mL^{-1}$) (Yuliatmo et al., 2017), poultry viscera hydrolysate prepared by autolytic digestion (IC_{50} = 2.65 $mg.mL^{-1}$) (Mane & Jamdar, 2017), and Kacang goat meat hydrolyzed by Protamex® + Flavourzyme® (approximately 45%, maximum value observed) (Mirdhayati, Hermanianto, Wijaya, Sajuthi, & Arihara, 2016). Probably, the strong activity observed in our research work could be due to the synergistic effects of different peptides released from degradation of Japanese quail breast muscle proteins during the hydrolysate preparation process which could have enhanced its ACE-inhibitory activity.

3.2.1. Effect of GI digestion and the ultrafiltration (UF) on angiotensin-converting enzyme inhibitory activity of the *Coturnix japonica* protein hydrolysates

This study also sought to verify the maintenance of the ACE inhibitory activity of CJPH hydrolysate during the gastrointestinal digestion as well as the influence of the molecular weight of the peptides in this activity. Similar to antioxidant activities, for this comparative analysis, the concentration with the highest ACE inhibitory activity (10.0 $mg.mL^{-1}$) was chosen to evaluate the ability of inhibition of the angiotensin-converting enzyme by the gastrointestinal UF extracts. The percentage of ACE inhibition of CJPH and its GI ultrafiltrated membrane fractions are shown in Figure 2. Despite a slight reduction around 1.09 folds observed in the ACEi of CJPH after gastric digestion (92.95 ±1.10% to 85.06 ±1.44%), this activity showed a significant increase during the digestion with pancreatin, reaching, in the GiPH(3) samples, an inhibition value of 90.87 ±1.10% (Figure 2). These results reveal that the methodology adopted in this study to prepare hydrolysates from the Japanese quail breast muscle protein was enough to obtain a hydrolysate with strong ACE inhibition property, which is capable to resist to functional inactivation by gastrointestinal enzymes.

Regarding the influence of peptide size on the ACE inhibitory activity, GI fractions with low molecular weight peptides (<3 kDa) showed a significant higher ACE inhibitory activity (GPH(3), 85.06 ±1.44%, and GiPH(3), 90.87 ±1.10%) than their respective hydrolysates with a molecular weight of 3-10 kDa (GPH(3-10), 66.39 ±0.72% and GiPH(3-10), 69.71 ±1.10%) ($p < 0.0001$) (Figure 2). These findings are in agreement with those previously reported that the peptide size can be crucial for the ACE inhibition (Aluko, 2015).

It is known that the control of the enzymatic hydrolysis conditions and the proper choice of the enzyme are crucial for the success of obtaining hydrolysates (Abd El-Salam & El-Shibiny, 2017). Depending on the enzyme used in its production process, the hydrolysates should contain peptides with a wide range of chain lengths, as well as distinct amino acids composition that influences directly their absorption rates, nutritional values and biological responses (Aluko, 2015; Jamdar et al., 2012). Furthermore, since the antihypertensive properties of peptides depends on their amino acid sequence and composition, losses of amino acid residues during the digestion process could lead to a reduction in this activity (Aluko, 2015). In this study, CJPH was obtained by an acid extraction process followed by a deproteinization step, an important preliminary cleanup procedure in peptide analysis (Martínez-Maqueda, Hernández-Ledesma, Amigo, Miralles, & Gómez-Ruiz, 2013). This hydrolysate showed to be stable throughout *in vitro* digestion with the ACE inhibitory activity maintained at a high percentage of inhibition levels (up to 80%) (Figure 2). Thus, it seems that the *Coturnix japonica* protein hydrolysate has a potential for *in vivo* activity. Further studies must be done to confirm this activity in an animal hypertension model.

3.3. Kinetic parameters of ACE inhibition

In order to comprehend the mechanism of the Angiotensin-converting enzyme inhibition, Lineweaver–Burk plots were determined in the absence and the presence of CJPH or GiPH(3) samples at 5.0 and 10.0 $mg.mL^{-1}$ concentrations. The double reciprocal plot (Figure 3) showed

that both hydrolysates exhibited an uncompetitive mode of ACE inhibition, i.e. CJPH and GiPH(3) only recognize and bind to the enzyme-substrate complex (ACE-HHL), but not the free enzyme, resulting in the enzyme-substrate-inhibitor complex (ACE-HHL-Hydrolysate). This complex is inactive, which means that the substrate in this condition will not be transformed by the enzyme (Dougall & Unitt, 2015; Stephanopoulos, Aristidou, & Nielsen, 1998).

According to Jakubczyk and Baraniak (2014) and Girgih et al. (2016), when peptides or hydrolysates, that exhibit an uncompetitive mode of ACE inhibition, bind to the ACE-HHL complex, it is reflected in a decrease of K_m and V_{max} values for the inhibited reactions. The results presented here are in agreement with these authors for both hydrolysates. When the CJPH hydrolysates bound to the enzyme-substrate complex, it was observed a reduction in the V_{max} values for the inhibited reactions at 5.0 and 10.0 $mg.mL^{-1}$ concentrations around 1.21 and 1.29 folds, respectively, when compared to the uninhibited reaction (0.399 ±0.009 $A.min^{-1}$). The same effect was observed to the K_m values, decreasing from 0.395 ±0.045 mM for the uninhibited reaction to 0.338 ±0.049 mM and 0.299 ±0.047 mM for CJPH at 5.0 and 10.0 $mg.mL^{-1}$ concentrations, respectively (Table 2). For GiPH(3) samples, the V_{max} values decreased 1.08 and 1.18 folds and K_m values were 0.254 and 0.231 mM at 5.0 and 10.0 $mg.mL^{-1}$ concentrations, respectively (Table 2).

Although previous research works have reported that the Angiotensin-converting enzyme seems to show a preference for competitive inhibitors (Lau, Abdullah, & Shuib, 2013; Mas-Capdevila, Pons, Aleixandre, Bravo, & Muguerza, 2018), uncompetitive inhibitors, described by this paper and other studies (Girgih et al., 2016; Jakubczyk & Baraniak, 2014), as well non-competitive (Deng et al., 2018) and mixed-type (Onuh, Girgih, Malomo, Aluko, & Aliani, 2015; Tu et al., 2018) inhibitors can also be found.

3.4. Peptide profiles of the hydrolysates

The MALDI-TOF-MS analysis of the CJPH detected 62 signals in a range from 2 to 12 kDa (Figure 4A), while in the GiPH(3), 30 peaks were detected at an m/z ranging from 709 to 1241 Da (Figure 4B). Although the MALDI-TOF-MS analysis had been performed to CJPH and GiPH(3) samples, only the gastrointestinal protein hydrolysate with <3 kDa MWCO was selected to MS-MS analysis once this hydrolysate presented higher *in vitro* radicals-scavenging and ACE inhibitory activities. Moreover, since it was simulated the gastrointestinal digestion of the quail breast muscle proteins, it would be possible to identify the potential peptides that would be formed at the end of the digestion process.

MALDI- TOF mass spectrometry of GiPH(3) provided the peptide masses that were used to predict the peptide structures present in these samples, as well the proteins in which they may have been derived (Table 3). As shown in table 3, several potential peptides, released via specific cleavage by pepsin, trypsin or chymotrypsin, were matched with fragments of Japanese quail and chicken proteins. The signals observed by mass spectrometry analysis corresponded to peptide lengths of approximately 8-22 amino acids. According to Lima et al. (2017), antihypertensive and antioxidant peptides are a molecular weight between 756 to 1881 Da, and the majority of the potential peptides found in this paper are in this range. A search of BIOPEP and AHTPDB databases indicated that none of predicted peptide sequences has been reported to possess bioactivity. In this way, the *Coturnix japonica* protein hydrolysate is a promising starting material for purifying novel potentially bioactive peptides.

3.5. Potential toxicity of *Coturnix japonica* protein hydrolysates (CJPH)

Several higher plants, such as *Allium cepa*, *Vicia faba*, *Zea mays*, *Nicotiana tabacum*, *Crepis capillaris* and *Hordeum vulgare*, have been used as test organisms for the detection of genotoxic substances and evaluation of their harmful effects in the human health, since they are similar to mammals in the detection of induced cytogenetic alterations (Cabaravdic, 2010; Khanna & Sharma, 2013; Mangalampalli, Dumala, & Grover, 2018). Within these species, the

test of *Allium cepa* is a bioassay widely used because it is a simple and reliable test. This specie presents large chromosomes in a small number that stain well, high percent of dividing cells and it is easy to cultivate at any time of the year. Additionally, the *Allium cepa* has easily distinguishable genetic endpoints, such as chromosomal aberrations (CAs) and sensitivity for identification of cytotoxicity, genotoxicity and mutagenicity in its root meristematic cells (Francisco et al., 2018; Khanna & Sharma, 2013; Mangalampalli et al., 2018).

Figure 5 shows the results for the mean root growth - MRG (toxicity) of CJPH for three concentrations (2.5, 5 and 10 $mg.mL^{-1}$). The MRG for CJPH showed that the root length of *Allium cepa* did not vary among the 2.5, 5 and 10 $mg.mL^{-1}$ concentrations (2.519 ±0.116; 2.344 ±0.077; 2.276 ±0.170 cm, respectively), neither in relation to negative control group (2.624 ±0.108 cm). Moreover, the negative control and CJPH treatments were significantly higher ($p < 0.05$) than the positive control group (copper sulphate at concentration of 0.6 $\mu g.mL^{-1}$) (0.462 ±0.013 cm) (Figure 1). In this way, CJPH proved to be nontoxic in the *Allium cepa* test. Similar results were found by Macedo, Silva, Batista, Uchoa and Alves (2014) and Silva et al. (2018) for okra and cinnamon extracts, respectively. In none of these articles significant differences were found in the growth of *Allium cepa* roots at the tested concentrations.

To determine the cytotoxicity levels of a substance, the increase or decrease of the mitotic index (MI) in relation to the negative control is always evaluated. It is known that MI is a percentage data that is used to evaluate the rate of cell division, i.e. measures the proportion of cells in mitosis, and their inhibition can be interpreted as cell death or a delay in the kinetics of cell proliferation. This index has been shown to be an important parameter to evaluate the effects that some agents can cause in the cell cycle (Braga & Lopes, 2015; Greenwood et al., 2004). The root cells treated with CJPH did not presented significant alterations (increase or decrease) in the MI in relation to the negative control, indicating that, at these concentrations, CJPH are not capable of increasing the DNA damage in root tip cells (Table 4). The results of

the cytotoxicity test explain why CJPH at 2.5, 5 and 10 mg.mL^{-1} concentrations did not cause reduction in the growth of *Allium cepa* roots tips.

Gayathri, Venkateshvaran and Manekar (2013) studying the cytotoxic and the mitogenic effect of the salivary gland extracts of octopus in *Allium cepa* root tip meristems model reported a different result. The tested extracts increased the mitotic index (MI) when compared to the control system and this effect was time-dependent. Moreover, after partial purification of the extracts, it was observed a new enhancement in the mitosis of the onion root tip meristem without indicating cytotoxicity (Gayathri et al., 2013).

It is known that genotoxic agents can promote repairable damage to the chromosomes or the cell DNA (Melo et al., 2018) and since the *Allium cepa* bioassay also allows the observation of the occurrence of chromosomal abnormalities in the cell cycle, this method can be extremely useful to indicate risks associated with the consumption of different products (Verma & Srivastava, 2018). The optical microscopy results gave a comprehensive view on the impact of the CJPH on *Allium cepa* root tip cells (Figure 6). In the CJPH at 2.5, 5 and 10 mg.mL^{-1} concentrations and negative control group it was observed different mitotic phases and absence of chromosomal abnormalities. Both genotoxic and mutagenic effects were found only in the positive control group and the following chromosomal abnormalities were observed: nuclear budding, binucleated cells, chromosomal breaks, chromosome bridge and micronucleus. The presence of these abnormalities indicates the presence of a clastogenic substance (inductors of chromosomal breaks) and also changes in the mitotic spindle (Braga & Lopes, 2015).

This is the first report on the quantitative and qualitative data of the *Coturnix japonica* protein hydrolysates on the mean root growth and mitotic, chromosomal abnormalities and mutagenic index of onion root tip. The results presented here to CJPH suggest that in none tested concentrations (2.5, 5 and 10 mg.mL^{-1}) the hydrolysate was toxic, cytotoxic, genotoxic or mutagenic. So, CJPH could be safe for human consumption.

4. CONCLUSION

The *Coturnix coturnix japonica* breast muscle is a good source to explore and develop hydrolysates that could be applied as an ingredient of functional food. This research work demonstrated that the Japanese quail breast muscle protein hydrolysate showed to be dose-dependent, it seems to be resistant to the gastrointestinal enzyme, once it was not observed functional inactivation in the bioactivities of the hydrolysate and proved to be safe for consumption. Both CJPH and GiPH(3) possesses a great *in vitro* antioxidant property and strong ACE-inhibitory activity exhibiting an uncompetitive mode of inhibition. Several potential peptides were identified composing the GiPH(3). Since this is the first report of hydrolysate obtained from *Coturnix coturnix japonica* meat proteins, further studies should be carried out to demonstrate its bioactivity *in vivo*.

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Abbreviations

ABTS, 2,2-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid; ACE, angiotensin-converting enzyme; ACEi, Angiotensin-converting enzyme inhibition; *Coturnix japonica*, *Coturnix coturnix japonica*; CJPH, *Coturnix japonica* protein hydrolysate; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; GI, gastrointestinal; GPH(3), Gastric protein hydrolysate with 3 kDa MWCO; GPH(3-10), Gastric protein hydrolysate with 3-10 kDa MWCO; HA, hippuric acid; HHL, Hippuryl-histidyl-leucine; GiPH(3), Gastrointestinal protein hydrolysate with 3 kDa MWCO; GiPH(3-10), Gastrointestinal protein hydrolysate with 3-10 kDa MWCO; MW, molecular

weight; MWCO, molecular weight cut-offs; PTFE, Politetrafluoretileno; Trolox, 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid; UF: ultrafiltrated.

Authors' Contributions

T.S.A. performed the experiments, analysis, interpretation of the experimental results and wrote the manuscript. M.S.F.L., K.M.S.S., F.A.C.S., G.M.G.R. and J.F.S. contributed to the analysis, interpretation of the experimental results and to the discussion. P.N.H., T.C.J. and M.T.H.C. contributed to discussion and reviewed the manuscript. T.S.A., M.T.H.C., and A.L.F.P. conceptualized and designed the study. A.L.F.P. the guarantor of this work.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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Table 1 Radical-scavenging activities (%) and Angiotensin-converting enzyme (ACE) inhibitory activity of *Coturnix japonica* protein hydrolysate

					CJPH concentration[†] (mg.mL^{-1})			
	SS	DF	MS	F value[§]	2.5	5	10	IC_{50} (mg.mL^{-1})
DPPH	310.4	2	155.2	130.9 (***)	56.43^{c} (0.54)	60.19^{b} (0.26)	69.28^{a} (1.00)	-
ABTS$^{\bullet+}$	316.1	2	158.1	38.02 (**)	5.60^{c} (0.83)	10.99^{b} (0.14)	19.97^{a} (1.86)	25.75
ACEi	1625	2	812.5	37.75 (**)	61.00^{b} (1.10)	70.12^{b} (4.37)	92.95^{a} (1.10)	0.10

[§]Significant at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. [†]Mean values (standard error of the mean) of the antioxidant and ACE inhibitory activities of *Coturnix japonica* protein hydrolysate at different concentrations (2.5, 5 and 10 mg.mL^{-1}). ABTS: 2,2′-azino-bis (3-ethyl-benzothiazoline-6sulfonic acid); ACE: Angiotensin-converting enzyme; ACEi: Angiotensin-converting enzyme inhibition; DF: degrees of freedom value; DPPH: 2,2-diphenyl-1-picrylhydrazyl; MS: mean square value; SS: sum of squares value. [*]Different superscript letters at the same parameter are significantly different ($p < 0.05$).

Table 2 Kinetic parameters of Angiotensin-converting enzyme (ACE) inhibitory reaction of *Coturnix japonica* protein hydrolysate (CJPH) and it gastrointestinal ultrafiltrated membrane fraction with molecular weight <3 kDa.

		CJPH ($mg.mL^{-1}$)		GiPH(3) ($mg.mL^{-1}$)	
Kinetic parameter	Control	5	10	5	10
K_m (mM)[a]	0.395 (0.045)	0.338 (0.049)	0.299 (0.047)	0.389 (0.068)	0.370 (0.063)
V_{max}[b]	0.399 (0.009)	0.331 (0.009)	0.309 (0.008)	0.368 (0.013)	0.337 (0.011)

K_m: Michaelis constant; V_{max}: Maximum reaction velocity; CJPH: *Coturnix japonica* protein hydrolysate; GiPH(3): Gastrointestinal protein hydrolysate with 3 kDa MWCO. [a]Hippuryl-L-Histidyl-L-Leucine (HHL) as substrate; [b]$A.min^{-1}$.

Table 3 Observed mass and potential peptides sequences present in the gastrointestinal protein hydrolysate with 3 kDa MWCO (GiPH(3))

Observed mass	Peptide sequence	UniProtKB*	Cleaving protease	Parent protein
728 Da	RAALGAGL	Q5F380	Pepsin	GPI mannosyltransferase 1
850 Da	NPFGQSAVYPFTSTAL	F5GVB9	Pepsin	Basic helix-loop-helix domain containing class B 4 protein
	TTILSHGLNAGIPLHR	G9M9G6	Trypsin	Arginine vasotocin receptor
917 Da	MPVPASPQHPRGYGILL	Q75SP9	Pepsin	Interferon type 1
	NTQQSNQLPKYERVK	Q90XB6	Trypsin	Extracellular sulfatase Sulf-1
935 Da	LEEDDDPGDDEVACSFL	Q9YIC1	Pepsin or Chymotrypsin	Quail calpain
	HITKTEVETQVSLVRK	W0SLN2	Trypsin	Inositol 1,4,5-triphosphate receptor, type 3
975 Da	PYTGKRRNDVRNGFRL	E1C0E1	Pepsin	Uncharacterized protein
	EFSRLEICNLTPDALTK	Q9YIC1	Trypsin	Quail calpain
	KPSYARNRSTRSVSVEL	Q5UAX9	Chymotrypsin	Extracellular sulfatase
1016 Da	IGLAVMGQNL	Q5ZIZ0	Pepsin	6-phosphogluconate dehydrogenase
	IEMEDWNGDKVSALYGGF	B4ZCR2	Pepsin	Fibrinogen beta chain (Fragment)
1089 Da	EEKVEDGGERWSKPHIPAL	A0A1L1RK45	Pepsin	Anion exchange protein
	TYNKETFRDYPDMVHDF	O73632	Pepsin or Chymotrypsin	Acid alpha-glucosidase
	FVFLINQDLITLIKTEAAK	Q9DEV2	Trypsin	Target of Jun3
1122 Da	DNNQISNIPDEYFQGFKTL	Q9DE67	Pepsin	Lumican
	RKSDTVPEELHQMVQEGFL	D7REJ9	Pepsin or Chymotrypsin	FTO isoform 1 (Fragment)
1203 Da	LSFISTNNPSNKTTATTKPTPW	Q85UK0	Chymotrypsin	ATP synthase protein 8
	YNLKERYASWMIYTYSGLF	Q91973	Pepsin	Slow myosin heavy chain 3
1225 Da	IAMVMMKARRCRVQTMKGRAL	Q8JH69	Chymotrypsin	Aromatase (fragment)
	TTTAEREIVRDIKEKLCYVAL	Q9PUB2	Pepsin	Beta-actin (Fragment)
1241 Da	SMSADVPLVVEYKIADMGHEKY	Q9DDF1	Chymotrypsin	Proliferating cell nuclear antigen

*UniProt knowledgebase citable accession number

Table 4 Cells in interphase, number of dividing cells and cytotoxicity (mitotic index) of *Coturnix japonica* protein hydrolysates (CJPH) in *Allium cepa* bioassay.

					Treatments[†]				
							CJPH concentration ($mg.mL^{-1}$)		
	SS	DF	MS	F value[§]	NC	PC	2.5	5	10
Cells in interphase					4590	4752	4605	4611	4615
Number of diving cells					410	248	395	389	385
MI (%)	16.04	4	4.011	66.72 (***)	8.189 (0.104)[a]	4.876 (0.194)[c]	7.898 (0.099)[a]	7.786 (0.084)[b]	7.700 (0.098)[b]

1000 cells were counted from each microscope slide, totalizing 5000 cells per treatment. [†]Mean values (standard error of the mean). NC: negative control (dechlorinated water); PC: positive control (copper sulfate – 0.6 $\mu g.mL^{-1}$); CJPH: *Coturnix japonica* protein hydrolysate; MI: mitotic index. [a-b]Different superscript letters are significant different at $p < 0.05$.

Figure legends

Figure 1 DPPH (A) and ABTS (B) radical-scavenging activities of CJPH and its digesta ultrafiltrated membrane fractions. Means ±SEM with different superscript letters are significantly different at $p < 0.05$.

Figure 2 Angiotensin-converting enzyme (ACE) inhibitory activity of CJPH and its digesta ultrafiltrated membrane fractions. Means ±SEM with different superscript letters are significantly different at $p < 0.05$.

Figure 3 Lineweaver–Burk plot of ACE inhibition by the *Coturnix japonica* breast muscle protein hydrolysate (A) and Gastrointestinal protein hydrolysate with 3 kDa MWCO (B). ACE inhibitory activity was determined in the absence and presence of different hydrolysate concentrations (0, 5.0 and 10.0 $mg.mL^{-1}$). (-●-) without inhibitor; (-■-) 5.0 $mg.mL^{-1}$ and (-▲-) 10.0 $mg.mL^{-1}$. 1/V and 1/[S] represents the reciprocal of HA formation rate and substrate (HHL) concentration, respectively. All data are means of triplicate measurements.

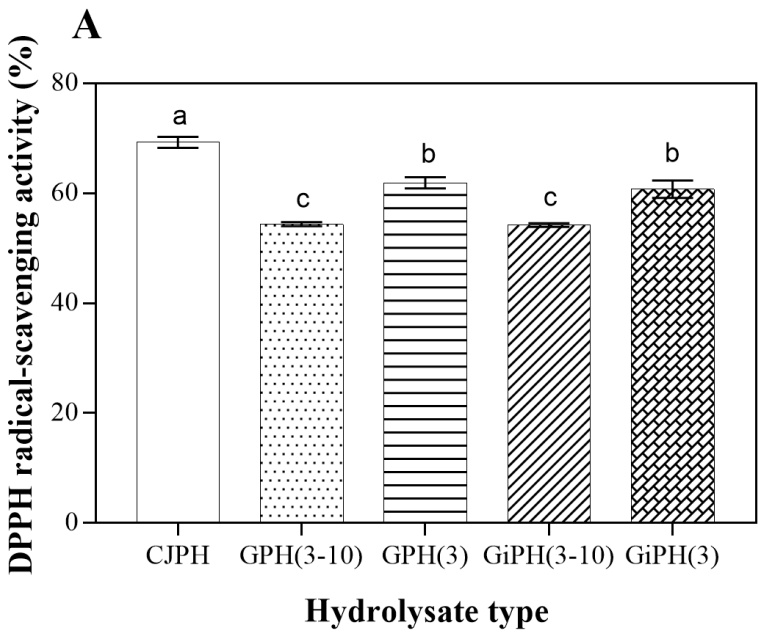
Figure 4 MALDI-TOF-MS mass spectra of the *Coturnix japonica* protein hydrolysate, CJPH (A) and Gastrointestinal protein hydrolysate with 3 kDa MWCO, GiPH(3) (B).

Figure 5 Toxicity (mean root growth) of *Coturnix japonica* protein hydrolysates (CJPH) in *Allium cepa* root meristematic cells. NC: negative control (distillated water); PC: positive control (copper sulphate); $CJPH_{10}$: *Coturnix japonica* protein hydrolysates at 10.0 $mg.mL^{-1}$ concentration; $CJPH_5$: *Coturnix japonica* protein hydrolysates at 5.0 $mg.mL^{-1}$ concentration; $CJPH_{2.5}$: *Coturnix japonica* protein hydrolysates at 2.5 $mg.mL^{-1}$ concentration.

Figure 6 Photomicrographs of *Allium cepa* root meristematic cells at 1000x magnification. Typical stages of mitosis found in the negative control groups and CJPH-treated groups at three different concentrations (2.5, 5.0 and 10.0 $mg.mL^{-1}$) (a-e): prophase (a) – chromosomes were quite visible, metaphase (b) - chromosomes arranged in the equatorial plate of the cell, anaphase (c) - chromosomes movement to opposite poles in a stable way and telophase (d) –

chromosomes organized at opposite poles, awaiting for cytokinesis. Chromosomal aberrations after copper sulphate (positive control) treatment (f-j): binucleated cell (f), nuclear budding (g), chromosomal breaks (h), chromosome bridge (i) and micronucleus (h).

Figure 1


A
DPPH radical-scavenging activity (%)
0
20
40
60
80
a
c
b
c
b
CJPH
GPH(3-10)
GPH(3)
GiPH(3-10)
GiPH(3)
Hydrolysate type

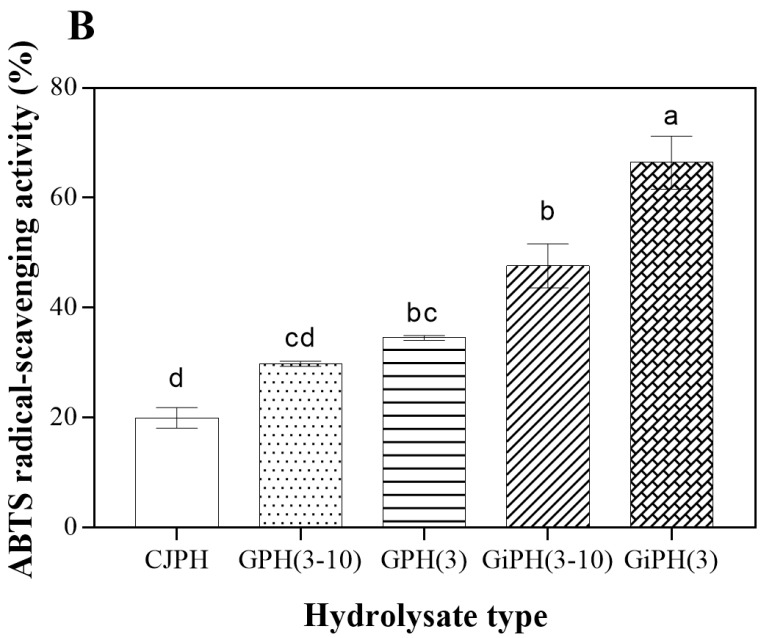

B
ABTS radical-scavenging activity (%)
0
20
40
60
80
d
cd
bc
b
a
CJPH
GPH(3-10)
GPH(3)
GiPH(3-10)
GiPH(3)
Hydrolysate type

Figure 2

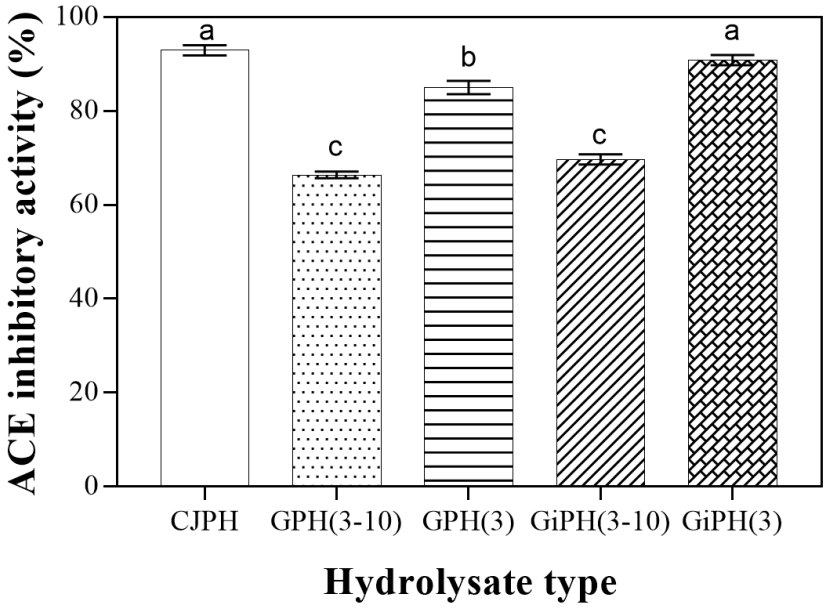
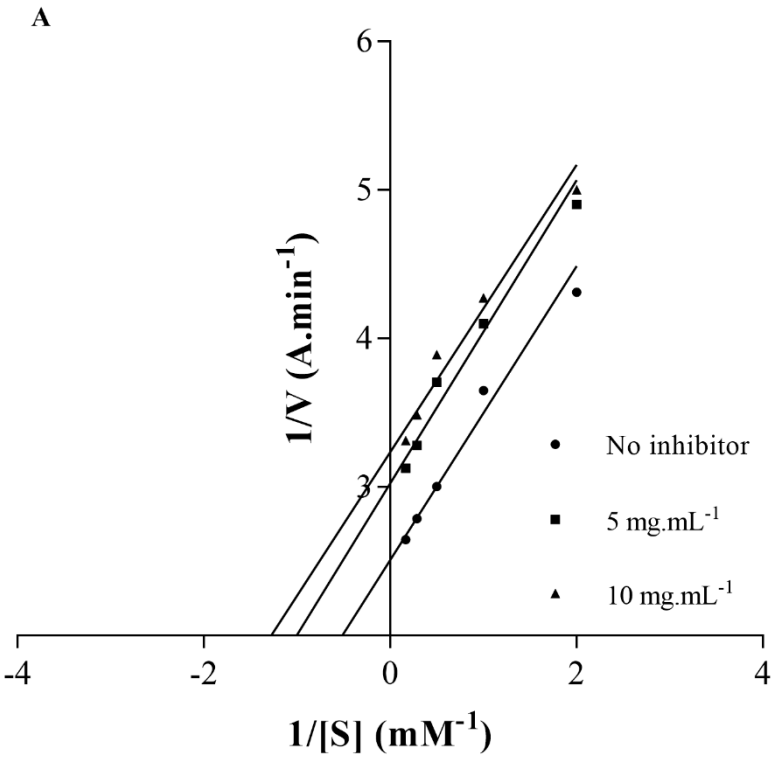

ACE inhibitory activity (%)
100
80
60
40
20
0
a
c
b
c
a
CJPH
GPH(3-10)
GPH(3)
GiPH(3-10)
GiPH(3)
Hydrolysate type

Figure 3


A
6
5
4
3
1/V (A.min-1)
No inhibitor
5 mg.mL-1
10 mg.mL-1
-4
-2
0
2
4
1/[S] (mM-1)

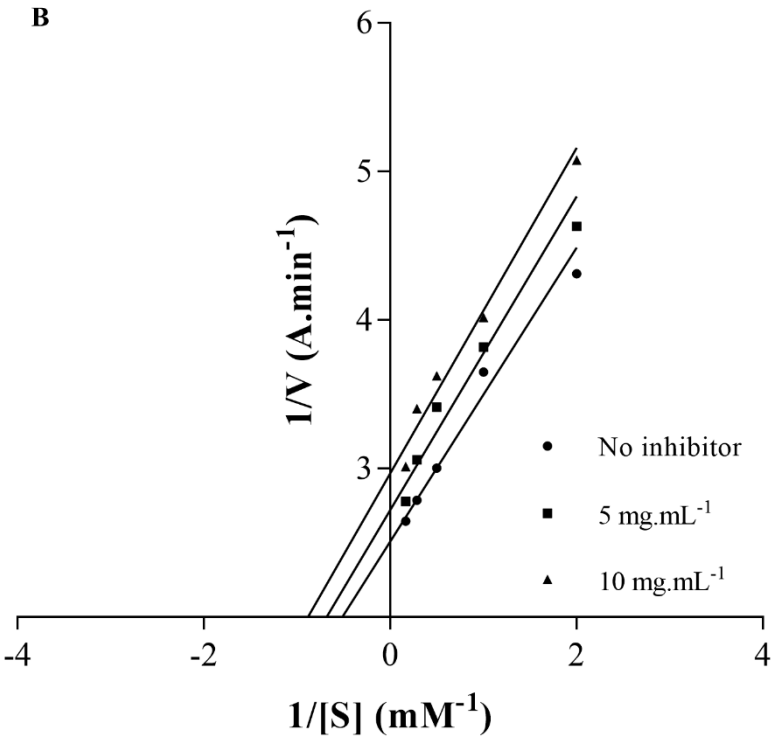

B
6
5
4
3
1/V (A.min-1)
No inhibitor
5 mg.mL-1
10 mg.mL-1
-4
-2
0
2
4
1/[S] (mM-1)

Figure 4

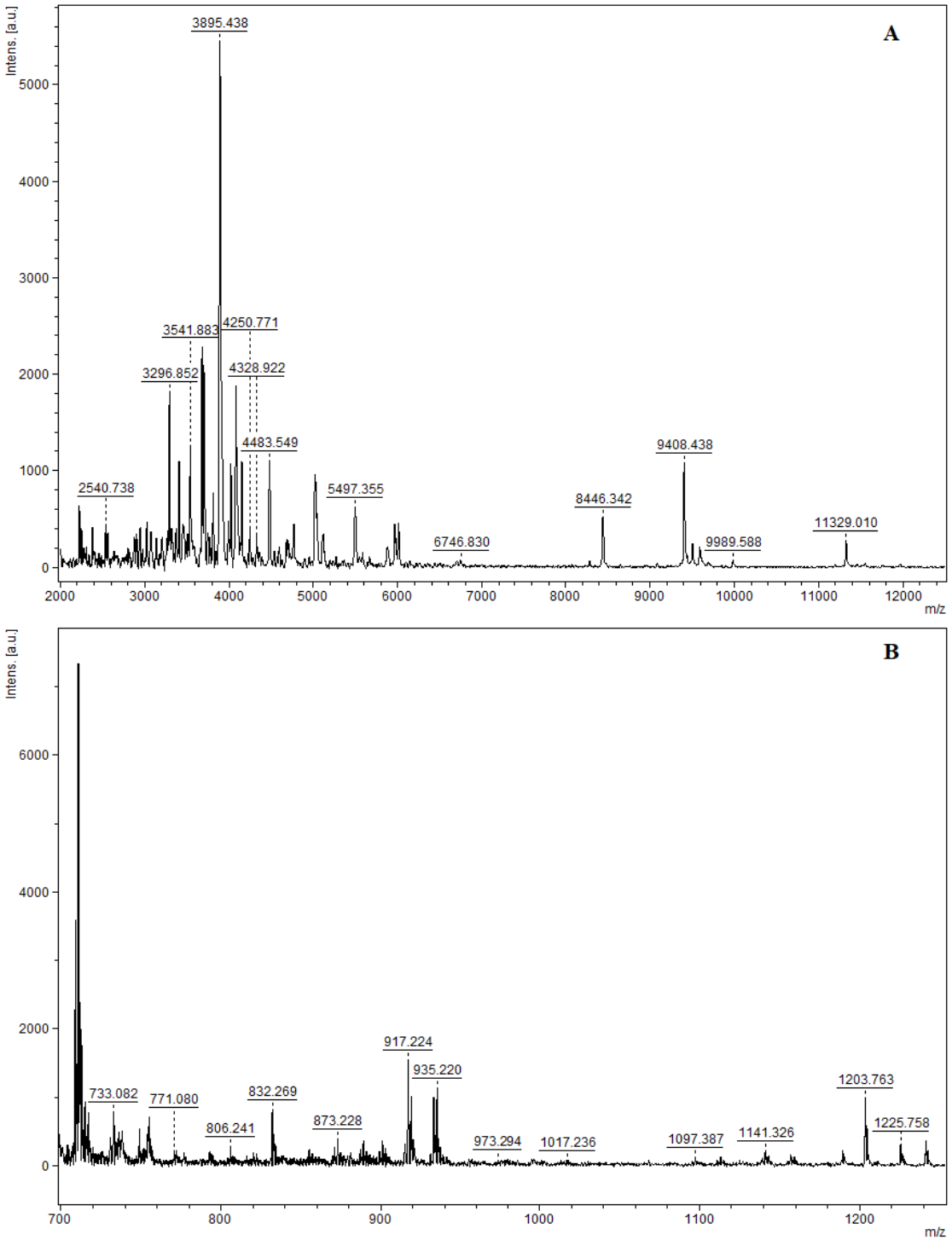

A
Intens. [a.u.]
3895.438
3541.883
4250.771
3296.852
4328.922
4483.549
2540.738
5497.355
6746.830
8446.342
9408.438
9989.588
11329.010
m/z
B
Intens. [a.u.]
733.082
771.080
806.241
832.269
873.228
917.224
935.220
973.294
1017.236
1097.387
1141.326
1203.763
1225.758
m/z

Figure 5

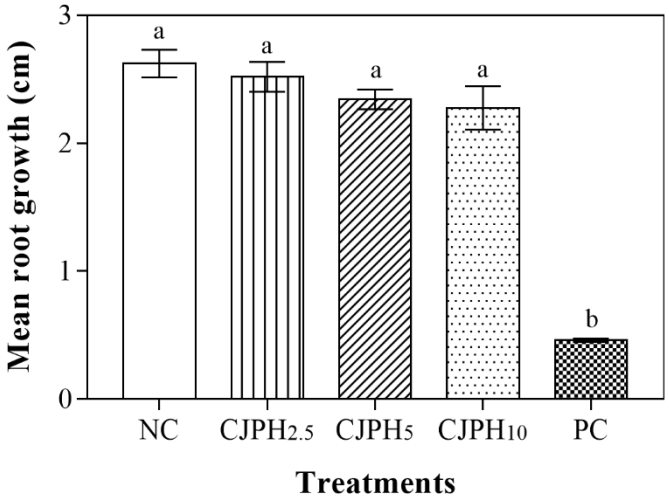
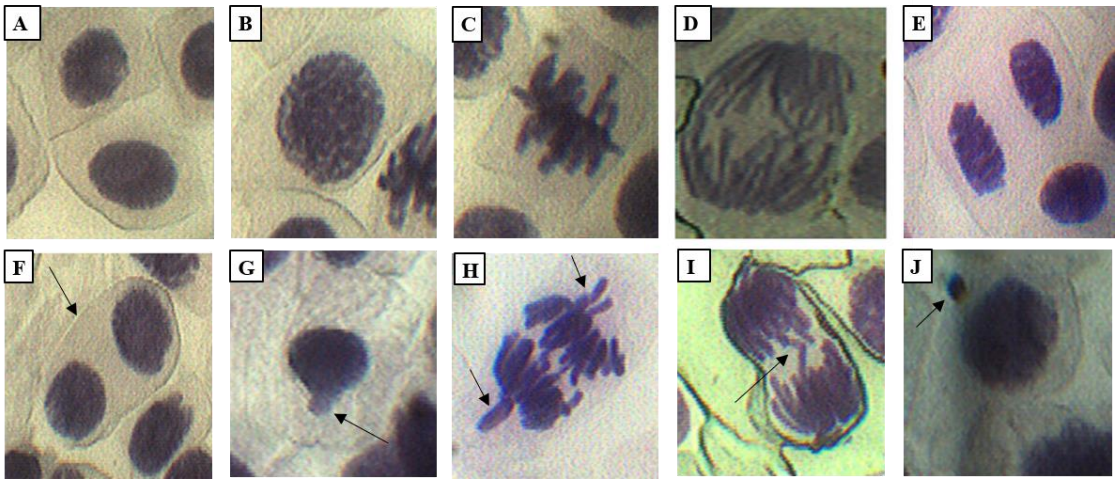

Mean root growth (cm)
3
2
1
0
a
a
a
a
b
NC
CJPH2.5
CJPH5
CJPH10
PC
Treatments

Figure 6



6 CONSIDERAÇÕES FINAIS

Estudos envolvendo a busca de hidrolisados proteicos e peptídeos bioativos de origem das proteínas animais tem crescido ao longo dos anos, uma vez que existe, por parte dos consumidores, cada vez mais conscientes da importância da alimentação na saúde e bem-estar, por alimentos seguros, saudáveis e com elevado valor agregado. Este trabalho teve como principal objetivo estudar as propriedades bioativas de hidrolisados proteicos e peptídeos bioativos de origem das proteínas de frango e codorna, tendo em vista a sua potencial aplicação na indústria alimentar.

A partir dos resultados apresentados foi possível chegar as seguintes conclusões:

1) Hidrolisados proteicos e peptídeos bioativos de frango podem possuir elevada atividade anti-hipertensiva, além de apresentar efeito protetor cardíaco contra danos em órgãos-alvo. Esses compostos bioativos podem ser obtidos utilizando distintas metodologias, as quais influenciam diretamente na atividade do produto final.
2) Esses peptídeos podem ser obtidos a partir da quebra das proteínas da carne crua e dos subprodutos da carne do frango, bem como de produtos residuais do processo de abate dos animais.
3) Os hidrolisados proteicos, por apresentarem em sua composição peptídeos que se distinguem pelo tamanho da cadeia e composição dos aminoácidos, apresentam um efeito anti-hipertensivo mais prolongado que os peptídeos purificados, além de que o seu uso pode reduzir os custos com a purificação e dimensionamento, proporcionando rentabilidade e viabilidade.
4) A codorna japonesa também se mostrou como uma excelente fonte proteica, não apenas por seu valor nutricional, como também pela possibilidade de sua carne poder utilizada para desenvolver hidrolisados que possam ser aplicados como ingrediente em alimentos funcionais.
5) Ambas atividades inibitória da ECA e antioxidante dos hidrolisados do músculo do peito de codornas são dose-dependentes e este composto bioativo mostrou-se ser resistente às enzimas do trato gastrointestinal, por meio de estudos realizados *in vitro*.
6) A excelente inibição da ECA (acima de 80%) bem como a melhora na atividade antioxidante de eliminação dos radicais ABTS, ao final do processo de digestão, pode ser devido ao efeito sinérgico dos vários novos peptídeos que poderão ser formados após

a ingestão de hidrolisados do músculo do peito de codorna japonesas por ação das enzimas digestivas.

7) Uma vez que não foram verificados efeitos tóxicos em células de cebola, o hidrolisado de codorna pode ser seguro para consumo

Futuros estudos relacionados com a identificação, isolamento e caracterização dos peptídeos presentes no hidrolisado afim de compreender quais são os responsáveis pela atividade inibidora da ECA e pela capacidade antioxidante precisam ser realizados, bem como a realização de ensaios *in vivo* (ratos) para confirmar se estes compostos bioativos são capazes de apresentar as mesmas atividades em um modelo vivo.

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ANEXO A - TRENDS IN FOOD SCIENCE & TECHNOLOGY: AUTHOR GUIDELINES (ARTIGO 1)

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Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop.

- **Submission**

Our online submission system guides you stepwise through the process of entering your article details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail. Authors must provide and use an email address unique to themselves and not shared with another author registered in EES, or a department.

- **Referees**

Authors are required to submit, with the manuscript, the names, addresses and e-mail addresses of five potential referees. Note that the editor retains the sole right to decide whether or not the suggested reviewers are used.

- **Peer review**

This journal operates a single blind review process. All contributions will be initially assessed by the editor for suitability for the journal. Papers deemed suitable are then typically sent to a minimum of two independent expert reviewers to assess the scientific quality of the paper. The Editor is responsible for the final decision regarding acceptance or rejection of articles. The Editor's decision is final. More information on types of peer review.

Suggested Reviewers

As of 1 June 2017, authors are required to submit with their articles, the names, complete affiliations (spelled out), country and contact details (including current and valid (preferably business) e-mail address) of four potential reviewers. Email addresses and reviewer names will be checked for validity. Your potential reviewers must not be from your institute, and at least two should be from a different country from where you are based. Authors must not suggest reviewers with whom they have collaborated within the past two years. Your submission will be rejected if these are not supplied. Names provided may be used for other submissions on the same topic. Reviewers must have specific expertise on the subject of your article. Briefly state the appropriate expertise of each reviewer. For each reviewer you suggest you must include details of two recent relevant research or review papers authored by the potential reviewer which have appeared in good quality scientific journals. Full details to be provided in the

covering letter with your submission. Authors cited in your paper can be useful suggested reviewers, provided that they have published in the field over the last few years.

Use of word processing software

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.
To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Article structure

Follow this order when typing manuscripts: Title, Authors, Affiliations, Abstract, Main text, Future Trends or Conclusion (indicating current hypotheses, potential applications and likely future trends and research needs), References, Figure Captions, Figures and then Tables. Do not import the Figures or Tables into your text.

- **Essential title page information**

• **Title.** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• **Author names and affiliations.** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
• **Corresponding author.** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. This responsibility includes answering any future queries about Methodology and Materials. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**
• **Present/permanent address.** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract
Structured Abstracts - a new requirement for submissions as of 1 April 2015

Review and Commentary articles for TIFS should contain a structured abstract, subdivided into sections entitled "*Background*"; "*Scope and Approach*"; and "*Key Findings and Conclusions*". This structured abstract should provide the context or background for the review and why it was carried out. It should emphasize any new and important findings of the review. **Structured abstracts should not exceed 250 words**.

Here is an example of a structured abstract for a review article.

Background
Food poisoning related to sanitary problems is among the most widespread illnesses in the world. The non-hygienic preparation and handling of food in households accounts for a large number of **foodborne disease** outbreaks. These outbreak cases could be avoided if preventive behaviors were adopted during food preparation. Educational actions offer a preventive information strategy for reducing the cases of foodborne diseases in households.
Scope and Approach
In this review the importance of good practices for **food handling** in Brazilian households in order to control foodborne diseases are described. This study emphasizes the importance of foodborne disease control, starting with changing food handlers' habits to ensure that they use best practices in their own homes. Food handlers are the consumers and employees that are most aware of the activities linked to the food production chain.
Key Findings and Conclusions
Educational actions, in the form of a program of good **food preparation** practices in households, are of paramount relevance. Such programs should be developed with methodologies consistent with the target audience and should take into account the causes of possible failures. Furthermore, the school place is the ideal setting for early intervention in **hygiene education**, assuming that childhood is the best time for learning. Children are more open to changes in attitude and can take these habits into households, encouraging hygienic behaviors throughout the family. Dissemination of knowledge as early as possible in the schools is a concrete form of risk communication because it builds trust and credibility.

Keywords - new requirement for submissions as of 1 April 2015

Immediately after the abstract, provide a maximum of 6 keywords, avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes. Examples of keywords are in **bold** in the sample structured abstract above.

Highlights

Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). You can view example Highlights on our information site.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List

here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Formatting of funding sources

List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, please include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors can build footnotes into the text, and this feature may be used. Otherwise, please indicate the position of footnotes in the text and list the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

- **Artwork**

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.
• Embed the used fonts if the application provides that option.
• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Size the illustrations close to the desired dimensions of the published version.
• Submit each illustration as a separate file.

A detailed guide on electronic artwork is available. **You are urged to visit this site; some excerpts from the detailed information are given here.**

Formats

If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format. Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS (or PDF): Vector drawings, embed all used fonts.
TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.
TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.
TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.

Please do not:

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article**. Please indicate your preference for color: in print or online only. Further information on the preparation of electronic artwork.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

- **Tables**

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be

sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules and shading in table cells.

- **References**

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

Data references

This journal encourages you to cite underlying or relevant datasets in your manuscript by citing them in your text and including a data reference in your Reference List. Data references should include the following elements: author name(s), dataset title, data repository, version (where available), year, and global persistent identifier. Add [dataset] immediately before the reference so we can properly identify it as a data reference. The [dataset] identifier will not appear in your published article.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software

Most Elsevier journals have their reference template available in many of the most popular reference management software products. These include all products that support Citation Style Language styles, such as Mendeley. Using citation plug-ins from these products, authors only need to select the appropriate journal template when preparing their article, after which citations and bibliographies will be automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide. If you use reference management software, please ensure that you remove all field codes before submitting the electronic manuscript. More information on how to remove field codes from different reference management software.

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Reference style

Text: Citations in the text should follow the referencing style used by the American Psychological Association. You are referred to the Publication Manual of the American Psychological Association, Sixth Edition, ISBN 978-1-4338-0561-5, copies of which may be ordered online or APA Order Dept., P.O.B. 2710, Hyattsville, MD 20784, USA or APA, 3 Henrietta Street, London, WC3E 8LU, UK.

List: references should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples:

Reference to a journal publication: Van der Geer, J., Hanraads, J. A. J., & Lupton, R. A. (2010). The art of writing a scientific article. *Journal of Scientific Communications, 163,* 51–59. https://doi.org/10.1016/j.Sc.2010.00372.
Reference to a journal publication with an article number: Van der Geer, J., Hanraads, J. A. J., & Lupton, R. A. (2018). The art of writing a scientific article. *Heliyon, 19,* e00205. https://doi.org/10.1016/j.heliyon.2018.e00205.
Reference to a book: Strunk, W., Jr., & White, E. B. (2000). *The elements of style.* (4th ed.). New York: Longman, (Chapter 4).
Reference to a chapter in an edited book:
Mettam, G. R., & Adams, L. B. (2009). How to prepare an electronic version of your article. In B. S. Jones, & R. Z. Smith (Eds.), *Introduction to the electronic age* (pp. 281–304). New York: E-Publishing Inc.
Reference to a website: Cancer Research UK. Cancer statistics reports for the UK. (2003). http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/ Accessed 13 March 2003.
Reference to a dataset: [dataset] Oguro, M., Imahiro, S., Saito, S., Nakashizuka, T. (2015). *Mortality data for Japanese oak wilt disease and surrounding forest compositions*. Mendeley Data, v1. https://doi.org/10.17632/xwj98nb39r.1.
Reference to a conference paper or poster presentation: Engle, E.K., Cash, T.F., & Jarry, J.L. (2009, November). The Body Image Behaviours Inventory-3: Development and validation of the Body Image Compulsive Actions and Body Image Avoidance Scales. Poster session presentation at the meeting of the Association for Behavioural and Cognitive Therapies, New York, NY.

Reviews should not contain more than 100 references; Commentaries or Features should not exceed 50 references.

- **Data visualization**

Include interactive data visualizations in your publication and let your readers interact and engage more closely with your research. Follow the instructions here to find out about available data visualization options and how to include them with your article.

- **Supplementary material**

Supplementary material such as applications, images and sound clips, can be published with your article to enhance it. Submitted supplementary items are published exactly as they are received (Excel or PowerPoint files will appear as such online). Please submit your material together with the article and supply a concise, descriptive caption for each supplementary file. If you wish to make changes to supplementary material during any stage of the process, please make sure to provide an updated file. Do not annotate any corrections on a previous version. Please switch off the 'Track Changes' option in Microsoft Office files as these will appear in the published version.

Data in Brief

You have the option of converting any or all parts of your supplementary or additional raw data into one or multiple data articles, a new kind of article that houses and describes your data. Data articles ensure that your data is actively reviewed, curated, formatted, indexed, given a DOI and publicly available to all upon publication. You are encouraged to submit your article for *Data in Brief* as an additional item directly alongside the revised version of your manuscript. If your research article is accepted, your data article will automatically be transferred over to *Data in Brief* where it will be editorially reviewed and published in the open access data journal, *Data in Brief*. Please note an open access fee of 500 USD is payable for publication in *Data in Brief*. Full details can be found on the Data in Brief website. Please use this template to write your Data in Brief.

- **Online proof correction**

Corresponding authors will receive an e-mail with a link to our online proofing system, allowing annotation and correction of proofs online. The environment is similar to MS Word: in addition to editing text, you can also comment on figures/tables and answer questions from the Copy Editor. Web-based proofing provides a faster and less error-prone process by allowing you to directly type your corrections, eliminating the potential introduction of errors. If preferred, you can still choose to annotate and upload your edits on the PDF version. All instructions for proofing will be given in the e-mail we send to authors, including alternative methods to the online version and PDF.

We will do everything possible to get your article published quickly and accurately. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. It is important to ensure that all corrections are sent back to us in one communication. Please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility.

- **Offprints**

The corresponding author will, at no cost, receive a customized Share Link providing 50 days free access to the final published version of the article on ScienceDirect. The Share Link can be used for sharing the article via any communication channel, including email and social media. For an extra charge, paper offprints can be ordered via the offprint order form which is sent once the article is accepted for publication. Both corresponding and co-authors may order offprints at any time via Elsevier's Webshop. Corresponding authors who have published their article gold open access do not receive a Share Link as their final published version of the article is available open access on ScienceDirect and can be shared through the article DOI link.

ANEXO B - TRENDS IN FOOD SCIENCE & TECHNOLOGY: COMPROVANTE DE SUBMISSÃO (ARTIGO 1)

Manuscript Details

Manuscript number	TIFS_2019_172
Title	Hydrolysates and bioactive peptides generated from chicken protein hydrolysis: a systematic review of antihypertensive effects in rats
Article type	Review Article

Abstract

Background: In recent years, obtaining protein hydrolysates and peptides from food proteins has been extensively studied in order to better understand not only their nutritional and functional properties, but also the possibility of their use as ingredients in functional foods. It is known that many dietary proteins have, within their primary structure, peptide sequences which upon release can be absorbed by enterocytes and modulate specific physiological functions, as well as provide essential amino acids and contribute to energy metabolism Scope and approach: To obtain a better insight into chicken protein hydrolysates and bioactive peptides with antihypertensive activity, a systematic review was conducted to evaluate the efficacy of these bioactive compounds in controlling the blood pressure in rats. In addition, the methodological quality of the published articles was evaluated. Key findings and conclusions: Searches in Scopus, ScienceDirect, ISI Web of Science and PubMed databases were focused on studies about the use of hydrolysates and peptides obtained from the enzymatic hydrolysis of various chicken tissues in rats. It was possible to verify that the positive antihypertensive effects were mainly dependent on the enzyme and enzymatic hydrolysis conditions and the monitoring of vital organ functions is essential to the regulation of blood pressure. Due to their efficacy and low cost, chicken protein hydrolysates can be an interesting alternative for biotechnological applications. Administration of chicken protein-derived hydrolysates and peptides to spontaneously hypertensive rats (SHR) exhibits a potential antihypertensive activity with likely cardiac protective effects against end-organ damage.

Keywords	protein hydrolysates, bioactive peptides, chicken protein, antihypertensive activity, enzymatic hydrolysis, spontaneously hypertensive rats
Manuscript region of origin	South America
Corresponding Author	Ana Lúcia Figueiredo Porto
Corresponding Author's Institution	Laboratory of Technology of Bioactive Product (LABTECBIO). Department of Morphology and Animal Physiology, Federal Rural University of Pernambuco
Order of Authors	Thiago Araújo, Maria Carolina Wanderley, José Manoel Duarte Neto, Wendell Albuquerque, Carolina F Prezotto, Polyanna Herculano, maria taciana soares, Ana Lúcia Figueiredo Porto
Suggested reviewers	Jose Teixeira, Teresa Neves-Petersen, Lorenzo Pastrana, Rotimi Aluko

Submission Files Included in this PDF

File Name [File Type]

cover_letter.doc [Cover Letter]

Hightlights.docx [Highlights]

Abstract.docx [Abstract]

New_Capitulo1.docx [Manuscript File]

Capitulo 1_Fluxograma.tif [Figure]

ANEXO C - JOURNAL OF FOOD BIOCHEMISTRY: AUTHOR GUIDELINES (ARTIGO 2)

1. SUBMISSION

Authors should kindly note that submission implies that the content has not been published or submitted for publication elsewhere except as a brief abstract in the proceedings of a scientific meeting or symposium.

Once the submission materials have been prepared in accordance with the Author Guidelines, manuscripts should be submitted online via the **journal online editorial system**.

Data Protection

By submitting a manuscript to or reviewing for this publication, your name, email address, and affiliation, and other contact details the publication might require, will be used for the regular operations of the publication, including, when necessary, sharing with the publisher (Wiley) and partners for production and publication. The publication and the publisher recognize the importance of protecting the personal information collected from users in the operation of these services, and have practices in place to ensure that steps are taken to maintain the security, integrity, and privacy of the personal data collected and processed. You can learn more at **https://authorservices.wiley.com/statements/data-protection-policy.html**.

2. AIMS AND SCOPE

The *Journal of Food Biochemistry* publishes fully peer-reviewed original research and review papers on the effects of handling, storage, and processing on the biochemical aspects of food tissues, systems, and bioactive compounds in the diet.

Researchers in food science, food technology, biochemistry, and nutrition, particularly based in academia and industry, will find much of great use and interest in the journal. Coverage includes:

- Biochemistry of postharvest/postmortem and processing problems
- Enzyme chemistry and technology
- Membrane biology and chemistry
- Cell biology
- Biophysics
- Genetic expression
- Pharmacological properties of food ingredients with an emphasis on the content of bioactive ingredients in foods

Examples of topics covered in recently-published papers on two topics of current wide interest, nutraceuticals/functional foods and postharvest/postmortem, include the following:

- Bioactive compounds found in foods, such as chocolate and herbs, as they affect serum cholesterol, diabetes, hypertension, and heart disease
- The mechanism of the ripening process in fruit
- The biogenesis of flavor precursors in meat
- How biochemical changes in farm-raised fish are affecting processing and edible quality

3. MANUSCRIPT CATEGORIES AND REQUIREMENTS

- **Original Papers –** reports of new research findings or conceptual analyses that make a significant contribution to knowledge.
- **Commentaries –** evidence-based opinion pieces involving areas of broad interest and invited commentaries.

- **Comprehensive Reviews –** critical reviews of the literature, including systematic reviews and meta-analyses.
- **Editorial articles –** are usually commissioned but unsolicited material may be considered. Please approach the Editor-in-Chief before submitting this material.
- **Letters to the Editor –** are welcomed.
- **Special Issues** and **Special Sections** on topics of interest are also regularly published.

- Authors are required to recommend at least three (3) reviewers eligible to peer review the manuscript submission. Authors will be asked for the names and email addresses of suggested reviewers during the submission process.
- Authors are required to submit a Practical Application Abstract in addition to the Abstract. The Practical Application Abstract should be included in the manuscript along with the Abstract.
- The overall composition, style, and English language quality are subject for immediate rejection of a manuscript.
- *Journal of Food Biochemistry* will not review research papers that focus on non-food products, including fatty acid or lipid composition research.

4. PREPARING THE SUBMISSION

Parts of the Manuscript

The manuscript should be submitted in separate files: main text file; figures.

Main Text File

The text file should be presented in the following order:

i. A short informative title that contains the major key words. The title should not contain abbreviations and should not be in all capital letters (see Wiley's **best practice SEO tips**);
ii. A short running title of less than 40 characters;
iii. The full names of the authors;
iv. The author's institutional affiliations where the work was conducted, with a footnote for the author's present address if different from where the work was conducted;
v. Abstract and keywords;
vi. Main text;
vii. Acknowledgments;
viii. References;
ix. Tables (each table complete with title and footnotes);
x. Figure legends;
xi. Appendices (if relevant).

Figures and supporting information should be supplied as separate files.

Authorship

Please refer to the journal's Authorship policy in the **Editorial Policies and Ethical Considerations** section for details on author listing eligibility.

Acknowledgments

Contributions from anyone who does not meet the criteria for authorship should be listed, with permission from the contributor, in an Acknowledgments section. Financial and material support should also be mentioned. Thanks to anonymous reviewers are not appropriate.

Conflict of Interest Statement

Authors will be asked to provide a conflict of interest statement during the submission process. For details on what to include in this section, see the 'Conflict of Interest' section in the **Editorial**

Policies and Ethical Considerations section below. Submitting authors should ensure they liaise with all co-authors to confirm agreement with the final statement.

Abstract

Please provide an abstract of 150 words containing the major keywords summarizing the article.

Keywords

Please provide four to six keywords.

Main Text

- The main text should include the introduction, materials and methods, results, and discussion. Please include section headers with numbers (ex: 1. Introduction, 2. Materials and Methods)
- The journal uses US spelling; however, authors may submit using either option, as spelling of accepted papers is converted during the production process.

Materials and Methods

If a method or tool is introduced in the study, including software, questionnaires, and scales, the author should state the license that is available under and any requirement for permission for use. If an existing method or tool is used in the research, the authors are responsible for checking the license and obtaining the permission. If permission was required, a statement confirming permission should be included in the Materials and Methods section.

References

References should be prepared according to the Publication Manual of the American Psychological Association (6th edition). This means in text citations should follow the author-date method whereby the author's last name and the year of publication for the source should appear in the text, for example, (Jones, 1998). The complete reference list should appear alphabetically by name at the end of the paper.

A sample of the most common entries in reference lists appears below. For more information about APA referencing style, please refer to the **APA FAQ**. Note that for journal articles, issue numbers are not included unless each issue in the volume begins with page one, and a DOI should be provided for all references where available.

Journal article

Beers, S. R. , & De Bellis, M. D. (2002). Neuropsychological function in children with maltreatment-related posttraumatic stress disorder. *The American Journal of Psychiatry*, *159*, 483–486. doi:10.1176/appi.ajp.159.3.483

Book

Bradley-Johnson, S. (1994). *Psychoeducational assessment of students who are visually impaired or blind: Infancy through high school* (2nd ed.). Austin, TX: Pro-ed.

Internet Document

Norton, R. (2006, November 4). How to train a cat to operate a light switch [Video file]. Retrieved from **http://www.youtube.com/watch?v=Vja83KLQXZs**

Footnotes

Footnotes should be placed as a list at the end of the paper only, not at the foot of each page. They should be kept to a minimum. Keep footnotes brief; they should contain only short comments tangential to the main argument of the paper and should not include references. They should be numbered in the list and referred to in the text with consecutive, superscript Arabic numerals.

Tables

Tables should be self-contained and complement, not duplicate, information contained in the text. They should be supplied as editable files, not pasted as images. Legends should be concise but comprehensive and should not be written in all capital letters – the table, legend, and footnotes must be understandable without reference to the text. All abbreviations must be defined in

footnotes. Footnote symbols: †, ‡, §, ¶, should be used (in that order) and *, **, *** should be reserved for P-values. Statistical measures such as SD or SEM should be identified in the headings.

Figure Legends

Legends should be concise but comprehensive and should not be written in all capital letters – the figure and its legend must be understandable without reference to the text. Include definitions of any symbols used and define/explain all abbreviations and units of measurement.

Figures

Although authors are encouraged to send the highest-quality figures possible, for peer-review purposes, a wide variety of formats, sizes, and resolutions are accepted.

Click here for the basic figure requirements for figures submitted with manuscripts for initial peer review, as well as the more detailed post-acceptance figure requirements.

Color Figures

Figures submitted in color may be reproduced in colour online free of charge. Please note, however, that it is preferable that line figures (e.g. graphs and charts) are supplied in black and white so that they are legible if printed by a reader in black and white.

Additional Files

Appendices

Appendices will be published after the references. For submission they should be supplied as separate files but referred to in the text.

Graphical Table of Contents

The journal's table of contents will be presented in graphical form with a brief abstract.

The table of contents entry must include the article title, the authors' names (with the corresponding author indicated by an asterisk), no more than 80 words or 3 sentences of text summarizing the key findings presented in the paper and a figure that best represents the scope of the paper (see the section on abstract writing for more guidance).

Table of contents entries should be submitted to Scholar One in one of the generic file formats. When uploading, please designate the image as Graphical Abstract Image.

The image supplied should fit within the dimensions of 50mm x 60mm, and be fully legible at this size.

Supporting Information

Supporting information is information that is not essential to the article, but provides greater depth and background. It is hosted online and appears without editing or typesetting. It may include tables, figures, videos, datasets, etc.

Click here for Wiley's FAQs on supporting information.

Note: if data, scripts, or other artefacts used to generate the analyses presented in the paper are available via a publicly available data repository, authors should include a reference to the location of the material within their paper.

General Style Points

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