NATÁLIA ELIZABETH GALDINO ALVES

EFEITO DO ARMAZENAMENTO DE FEIJÕES CARIOCA (*Phaseolus vulgaris* L.) SOBRE A COMPOSIÇÃO NUTRICIONAL E FITOQUÍMICA E PROPRIEDADES ANTI-INFLAMATÓRIA E ANTI-ATEROSCLERÓTICA DE SEUS HIDROLISADOS PROTÉICOS EM CÉLULAS DE MACROFÁGOS HUMANOS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência da Nutrição, para obtenção do título de *Doctor Scientiae*.

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RESUMO

ALVES, Natália Elizabeth Galdino, D.Sc., Universidade Federal de Viçosa, fevereiro de 2017. **Efeito do armazenamento sobre a composição nutricional de feijões comuns (*Phaseolus vulgaris* L.) e ação anti-inflamatória e anti-aterosclerótica de seus hidrolisados protéicos em células de macrofágos humanos**. Orientadora: Hércia Stampini Duarte Martino. Coorientadores: Sônia Machado Rocha Ribeiro e Leandro Licursi de Oliveira.

O presente trabalho teve como objetivo avaliar o efeito do armazenamento comercial sobre a composição nutricional e fitoquímica de feijões comuns da variedade carioca (*Phaseolus vulgaris* L.) (Artigo 1) e determinar o efeito do armazenamento sobre o potencial anti-inflamatório (Artigo 2) e anti-aterosclerótico (Artigo 3) de seus hidrolisados proteicos em macrófagos humanos THP-1. Foram utilizados dois genótipos de feijão comum da variedade carioca, contrastantes para endurecimento e escurecimento ao longo do armazenamento. Os feijões BRSMG Madrepérola (MP) (mais estável ao armazenamento) e BRS Pontal (PO) foram estocados em temperatura ambiente, (22 ± 3 ^{0}C; < 65% de umidade relativa), acondicionados em embalagens lacradas de polipropileno, por até 180 dias, e coccionados nos tempos zero, e após três e seis meses de estocagem, quando foram realizadas as analises (Artigo 1 e 2: 0, 3 e 6 meses; Artigo 3: 0 e 6 meses). Os grãos foram analisados quanto à composição centesimal, fibra alimentar total e frações, conteúdo de minerais, fitato, compostos fenólicos (Artigo 1) e hidrolisados proteicos (Artigos 2 e 3). O potencial anti-inflamatório e anti-aterosclerótico dos hidrolisados proteicos foi avaliado *in vitro*, utilizando células de macrófagos THP-1, com inflamação induzida por lipopolissacarídeo (LPS) (Artigo 2) ou lipoproteína de baixa densidade oxidada (LDL-ox) (Artigo 3). |O armazenamento comercial por até 180 dias, em temperatura ambiente, não afetou a qualidade nutricional e fitoquimica dos grãos, visto que somente foi observada redução do conteúdo de lipídio total ($p = 0.007$). Os hidrolisados dos feijões carioca reduziram os níveis de marcadores inflamatórios mesmo quando utilizados em baixas concentrações, de forma independente do tempo de armazenamento, embora tenham sido observadas diferenças entre os cultivares (0.1 mg/mL: redução de TNF-alfa pelos hidrolisados PO 0, 3 e 6, e por MP 6; redução de IL-1 β por MP 0 e PGE-2 por MP 0 e 6). Com relação ao potencial anti-aterosclerótico, observou-

se que os hidrolisados dos feijões armazenados reduziram a expressão do receptor de LDL-ox (LOX-1), de metaloproteína-9 (MMP-9), de molécula de adesão intracelular-1 (ICAM-1), e a expressão de 10 citocinas relacionadas ao processo aterosclerótico. Conclui-se, portanto, que as condições de armazenamento utilizadas no presente estudo foram adequadas para preservação da qualidade nutricional e fitoquímica dos feijões. Além disso, a inibição de marcadores inflamatórios e ateroscleróticos indica que o armazenamento dos feijões por seis meses, independentemente de suas características de resistência ao armazenamento, não alterou as propriedades fisicoquímicas e biológicas de seus hidrolisados proteicos.

ABSTRACT

ALVES, Natália Elizabeth Galdino, D.Sc., University Federal of Viçosa, February, 2017. **Effect of storagem on nutritional compositiiton of common bean (*Phaseolus vulgaris* L.) and anti-inflammatory and anti-atherosclerotic potential of its protein hydrolysates on human macrophages**. Adviser: Hércia Stampini Duarte Martino. Co-adivsers: Sônia Machado Rocha Ribeiro and Leandro Licursi de Oliveira.

The aim of this study was to evaluate the effect comercial time of storage (six monhts) on nutritional and phytochemical composition of common bean, carioca cultivar (*Phaseolus vulgaris* L.) (Manuscript 1) and to determinate thestorage effect on anti-inflammatory (Manuscript 2) and anti-atherosclerotic effect (Manuscript 3) of their protein hydrolysates on human macrophages. Two common bean carioca genotypes were used. The common beans BRSMG Madreperola (MP) (more stable to storage) and BRS Pontal were stored under room temperature (22 ± 3 ^{0}C; < 65% relative umidity), in propilen package, up to 180 days, and cooked at zero, three and six months of storage, when analysis of moisture contente, dietary fiber, minerals, phytate, phenolic compounds (Manuscript 1) and protein hydrolysates (Manuscripts 2 e 3) were performed. Anti-inflammatory and anti-atherosclerotic potential of protein hydrolysates was evaluated *in vitro*, using THP-1 human macrophages, wherein inflammation was induced by lipopolysacarides (Manuscript 2) or oxydized low density lipoprotein (ox-LDL) (Manuscript 3). According to results, we can affirm that comercial storage up to 180 days, at room temperature, did not affected nutritional and phytochemical quality of the carioca beans, since it was observed only slight reduction on total lipid contente ($p = 0.007$). Carioca protein hydrolysates reduced the levels of inflammatory markers even under lowest concentrations, independently of storage time, although few diferences were observed beteween cultivars (0.1 mg/mL: TNF-alfa reductions by hydrolysates from PO 0, 3 and 6, and by MP 6; IL-1 β reductions by MP 0 and PGE-2 by MP 0 and 6). Regarding to anti-atherosclerotic potential it was observed that hydrolysates from stored beans decreased teh expressio of ox-LDL receptor 1 (LOX-1), metaloprotein-9 (MMP-9), intercellular adhesion molecule-1 (ICAM-1), and reduced the expression of 10 citokines related to atherosclerotic process. In conclusion, the storage conditions used in the presente study was suitable to preserve nutritional and phytochemical quality of carioca beans. In adition, the

inhibition of inflammatory and atherosclerotic markers indicate that the storage by six months did not change physicochemical and biological properties of carioca bean hydrolysates, , independently fo their characteristics to resit to storage.

1. INTRODUÇÃO GERAL

O consumo de leguminosas tem sido associado à redução de risco para doenças crônicas não transmissíveis (DCNTs), incluindo doenças cardiovasculares (DCV), que são a principal causa de morte em muitos países. A aterosclerose está entre os fatores de risco mais significantes para o desenvolvimento de DCV. O processo aterosclerótico envolve a diferenciação de macrófagos e proliferação na intima arterial, com subsequente formação de células espumosas carregadas de lipídios, as quais induzem a liberação de citocinas e fatores de crescimento pró-inflamatórios (CAM; DE MEJIA, 2012).

Nesse sentido, estratégias eficazes de prevenção são necessárias. Existem evidências, de que compostos bioativos de leguminosas podem atuar na redução de importantes mediadores inflamatórios (DE MEJIA; DIA, 2010), principalmente devido à presença de polifenóis (LIU, 2013) e peptídeos bioativos (CAM; DE MEJIA, 2012). Embora grande parte dos estudos avalie o efeito da soja sobre esses marcadores, outras leguminosas, como o feijão comum (*Phaseolus vulgaris* L.), podem atuar na redução de fatores de risco para DCV (BAZZANO et al., 2011).

O feijão comum (*Phaseolus vulgaris* L.) se destaca entre as leguminosas por ser culturalmente importante na nutrição humana e amplamente consumido, principalmente na América Latina (MARTINO et al., 2012). É fonte apreciável de proteínas, carboidratos complexos, fibra alimentar, vitaminas e minerais, além de outros compostos com propriedades funcionais já reconhecidas (CÁRDENAS; ROSA; COSTA, 2010).

Ressalta-se a importância dessa leguminosa como fonte proteica, não somente por seu papel construtivo e energético, mas também porque suas proteínas são precursoras de peptídios ativos biologicamente, os quais possuem variadas funções fisiológicas. Os peptídeos bioativos de feijões podem ser obtidos durante a digestão gastrointestinal, por meio de fermentação ou hidrólise enzimática (WANG; DE MEJIA, 2005). Hidrolisados ou peptídeos de feijões têm demonstrado atividades antioxidante, anti-inflamatória, anti-hipertensiva e anticancerígena (LUNA-VITAL *et al.*, 2015). A presença de compostos fenólicos também merece destaque por suas propriedades anti-inflamatórias e anti-ateroscleróticas, as quais são explicadas pela capacidade desses fitoquímicos em sequestrar

radicais livres e modular a sinalização celular durante o processo inflamatório (GARCÍA-LAFUENTE et al., 2009).

O Brasil é o maior produtor mundial de feijão com produção anual de 3,5 milhões de toneladas (MAPA, 2016), com liberação aprovada do cultivo comercial de variedades geneticamente modificadas que possuem vantagens econômicas, ambientais e nutricionais diferenciadas (EMBRAPA, 2012). No País, existem cerca de 40 variedades de feijões, cuja produção e consumo variam conforme os hábitos regionais e condições climáticas locais (MAPA, 2016). O feijão Carioca lidera as preferências dos consumidores brasileiros e novos cultivares têm sido desenvolvidos para resistir às alterações tecnológicas, principalmente aspectos do grão (coloração e dureza) durante o armazenamento (TEIXEIRA; SIQUEIRA; BASSINELLO, 2011). As principais alterações em feijões Carioca estão relacionadas ao rápido escurecimento e endurecimento dos grãos, o que afeta a qualidade sensorial e nutricional, e prejudica a comercialização (SIQUEIRA *et al.*, 2016). O tempo de armazenamento por seis meses em condições ambientais favorecem o fenômeno HTC- *hard-to-cook* (duro de cozinhar), aumentando o tempo de cocção e reduzindo a digestibilidade de proteínas (Nyakuni et al., 2008). Outras alterações nutricionais associadas ao período de armazenamento são: perda de fitato, taninos, compostos fenólicos e lipídios (CHIARADIA; GOMES, 1987).

Contudo, apesar do número considerável de variedades disponíveis, há escassez de estudos acerca do efeito do tempo de armazenamento sobre as características nutricionais e funcionais. Tampouco é desconhecido o efeito do tempo de armazenamento sobre as características físico-químicas e propriedades bioativas dos hidrolisados proteicos de feijão Carioca, justificando a realização do presente estudo. Durante o armazenamento proteínas maiores podem sofrer hidrolise enzimática natural, formando pequenos poliptídeos (HOHLBERG; STANLEY, 1987), o que pode alterar as propriedades funcionais dos hidrolisados proteicos. Assim, espera-se observar diferenças de composição nutricional e fitoquímica entre genótipos de feijão Carioca armazenado por seis meses, especialmente pelas características distintas de estabilidade ao armazenamento, o que poderá determinar diferentes efeitos de seus hidrolisados proteicos sobre marcadores inflamatórios e ateroscleróticos em células de macrófagos humanos.

1. OBJETIVOS

1.1. Objetivo geral

Investigar o efeito do armazenamento de feijões carioca (*P. vulgaris* L.) sobre sua composição nutricional e fitoquímica, e propriedades anti-inflamatórias e anti-ateroscleróticas de seus hidrolisados proteicos em células de macrófagos humanos.

1.2. Objetivos específicos

- Comparar o efeito do armazenamento pelo período de seis meses sobre a composição centesimal, conteúdo mineral e fitoquímicos de feijões comunstipo carioca (*P. vulgaris* L.);
- Caracterizar as propriedades físico-químicas e perfil peptídico dos hidrolisados proteicos dos feijões Cariocaobtidos por digestão gastrointestinal simulada *in vitro;*
- Avaliar as propriedades anti-inflamatórias dos hidrolisados proteicos dos feijões Carioca, armazenados por seis meses, sobre em macrófagos humanos (THP-1), com inflamação induzida por lipopolissacarídeo (LPS);
- Avaliar as propriedades anti-ateroscleróticas dos hidrolisados proteicos dos feijões Carioca, armazenados por seis meses, em macrófagos humanos (THP-1), com aterosclerose induzida por LDL-oxidada

2. REFERENCIAL TEÓRICO

2.1. Feijão e alimentação humana

O feijão comum (*Phaseolus vulgaris* L.) é uma importante leguminosa para a nutrição humana. Trata-se de um dos mais antigos alimentos da história. A existência de feijões domésticos data de 10000 anos antes de Cristo. Muitos historiadores atribuem a difusão da semente pelo mundo às guerras, uma vez que foi componente fundamental em muitas batalhas. Atualmente, essa leguminosa é consumida em grande escala no México, América Central e do Sul e países da África e Ásia (CONAB, 2013; CASTRO GUERRERO *et al.*, 2016). A composição química de feijões é amplamente variável, em função do cultivar, da estação de crescimento, da localização geográfica e do estresse ambiental. Alguns cultivares podem apresentar de 15 a 30 % de proteína, 60 a 70 % de carboidrato total, e 0,7 a 2% de lipídios, em base seca (MARTINO *et al.*, 2012; CASTRO GUERRERO *et al.*, 2016). Essa leguminosa é a fonte importante de proteína, minerais, vitaminas e fibra alimentar para muitas populações devido ao baixo custo, podendo prover de 10 a 20 % da quantidade recomendada dessesnutrientes para um indivíduo adulto (MARTINO *et al.*, 2012).

O Brasil é o maior produtor e consumidor de feijões do mundo. Na safra de 2014/2015 estimou-se produção de 3,3 toneladas (CONAB, 2016), apenas 5,2% inferior à safra anterior (2011/2012) (CONAB, 2013). Respondemos por 20,4 % do consumo total mundial (CONAB, 2013; 2016), o que demonstra a preferência do brasileiro, sendo que o consumo de lentilha e soja, por exemplo, responde por menos de 1 % do consumo de leguminosas (POF, 2010). O consumo de feijão cozido no Brasil é de 183 g/dia, a média da população urbana é de 117,9 g/dia e da população rural de 208,1 g/dia (MAPA, 2013). Contudo, têm-se observado redução no consumo de feijão ao longo dos anos, devido a melhora da situação sócio-econômica da população que resultou em mudanças no padrão alimentar e maior procura por alimentos que demandam menor tempo de preparo (IBGE, 2010; VELÁSQUEZ-MELÉNDEZ *et al.*, 2012).

O gênero Phaseolus inclui todas as espécies conhecidas de feijão, sendo a *Phaseolus vulgaris* L., a variedade mais encontrada. Aproximadamente 40 cultivares são consumidos no Brasil, entre os quais os feijões Carioca, Preto, Roxo e Mulatinho são os mais difundidos (MESQUITA *et al.*, 2006). A cultivar Carioca é a mais aceita pela população brasileira, correspondendo a 52% da área cultivada de feijão (BRASIL, 2010).

O desenvolvimento de cultivares geneticamente melhorados (não modificados geneticamente) tem sido realizado no Brasil pela EMBRAPA, com aprovação da Comissão Técnica Nacional de Biossegurança. Esses cultivares resultam de mais de 10 anos de pesquisas realizadas pela EMBRAPA Arroz e feijão (Goiânia, GO) e EMBRAPA Recursos genéticos e biotecnologia (Brasília, DF). São econômica e ambientalmente vantajosos, pois apresentam maior rendimento, maior produtividade, e menor necessidade de químicos prejudiciais ao ambiente (BRASIL, 2010). Tais modificações podem melhorar a qualidade nutricional das sementes,, possibilitando o desenvolvimento de cultivares ricos em vitaminas e minerais essenciais ao metabolismo humano (BRASIL, 2010).

O desenvolvimento desses novos cultivares é favorável em aumentar a produção e qualidade dos grãos, que é aspecto importante para comercialização e satisfação do consumidor. Para os produtores e consumidores o melhoramento genético é favorável, pois pode aumentar a qualidade das sementes ao longo do armazenamento, reduzindo o escurecimento e endurecimento.

2.2. Fitoquímicos presentes em feijões

O feijão contém substâncias anteriormente denominadas fatores antinutricionais, como: ácido fítico, lectinas, compostos fenólicos e taninos, saponinas, inibidores enzimáticos, glicosídeos cianogênicos e glucosinolatos. Esses fatores foram assim denominados por sua capacidade de reduzir a biodisponibilidade de certos nutrientes, prejudicando o crescimento (SHAHIDI, 1997; MESSINA, 2014). No entanto, os efeitos à saúde humana e animal são dependentes da concentração e interação com outros nutrientes, e em algumas situações podem atuar favoravelmente na redução de risco de doenças crônicas.

Do ponto de vista antinutricional, a baixa digestibilidade proteica é um dos grandes problemas nutricionais observados em feijões comuns, o que é, em sua maior parte, atribuído aos inibidores de tripsina, taninos, lectinas e ácido fítico (REYES-MORENO; PAREDEZ-LÓPEZ, 1993).

Os inibidores de tripsina, além de reduzirem a razão de eficiência proteica (HERNÁNDEZ-INFANTE *et al.*, 1998), prejudicam o crescimento (HYMOWITZ, 1986; ANAYA *et al.*, 2015). Os taninos podem contribuir também para a redução da ingestão alimentar, da taxa de crescimento e da biodisponibilidade de ferro (GUZMÁN-MALDONADO; ACOSTA-GALLEGOS; PAREDES-LÓPEZ, 2000; JARAMILLO *et al.*, 2015; DELIMONT; HAUB; LINDSHIELD, 2017). Lectinas puras podem produzir ulceração e necrose intestinal em ratos (OLIVEIRA; VIDAL; SGARBIERI, 1989) e vegetais contendo lectinas estão associadas à patogênese de várias doenças intestinais, como: doença inflamatória intestinal, diabetes, artrite reumatoide e doença celíaca (GONG *et al.*, 2017). Contudo, os procedimentos de cocção podem inativar parcialmente inibidores de tripsina, lectinas e taninos em feijões comuns (HERNÁNDEZ-INFANTE *et al.*, 1998; HAILESLASSIE; HENRY; TYLER, 2016) e aumentar a biodisponibilidade de nutrientes e compostos bioativos (LÓPEZ-MARTÍNEZ *et al.*, 2017).

Grande parte dos benefícios desses fitoquímicos são descritos para a soja, principalmente quanto à atividade inibitória da carcinogênese atribuída à inibidores de tripsina, taninos e a lectina (DE MEJÍA *et al.*, 2003; SAHIN, 2014). Os benefícios potenciais dos feijões à saúde, embora tenham sido ignorados, são atualmente investigados. A seguir são detalhadas as propriedades antinutricionais e funcionais de fitoquímicos presentes em feijões comuns.

2.2.1. Fitato

Os fitatos são compostos complexos naturalmente formados durante a maturação de sementes e grãos. O fitato é denominado como ácido hexafosfórico mio-inositol ou 1,2,3,4,5,6 hexaquis (diidrogênio fosfato) mio-inositol. Durante a estocagem, fermentação, germinação e digestão de grãos e sementes, o ácido fítico pode ser parcialmente

desfosforilado para produzir compostos pentafosfato (IP5), tetrafosfato (IP4), trifosfato (IP3) e possivelmente inositol difosfato (IP2) e monofosfato (IP1), por ação de fitases endógenas (BURBANO *et al.*, 1995; SILVA; BRACARENSE, 2016). Contudo, somente as formas IP5 e IP6 apresentam efeito prejudicial na biodisponibilidade de minerais como: ferro, zinco e cálcio (HARLAND; NARULA, 1999; SILVA; BRACARENSE, 2016).

Em feijões comuns a concentração de ácido fítico pode variar de 0,1 a 2,7% (HARLAND; NARULA, 1999; MESSINA, 2014), em função do cultivar ou métodos de determinação (REYES-MORENO; PAREDEZ-LÓPEZ, 1993). A presença de fitato pode estar relacionada, além da menor biodisponilidade de minerais, à baixa qualidade de proteínas de feijões, devido à capacidade de ligação a esse macronutriente, diminuindo a proteólise, ou por ligação direta à enzimas digestivas (proteolíticas ou amilolíticas) (SATHE, 2002; PARMAR *et al.*, 2017).

Dentre os efeitos benéficos, o fitato pode contribuir para normalização dos níveis de glicemia e lipídios séricos, aumento da capacidade antioxidante, redução de cálculos renais e redução do risco de alguns tipos de cânceres (YONEKURA; SUZUKI, 2003; MESSINA, 2014). O inositol hexafosfato (IP6) está presente em quase todas as células de mamíferos, ainda que em pequena quantidade, onde atua como regulador de funções vitais celulares, como transdução de sinal, proliferação e diferenciação celular (VUCENIK; SHAMSUDDIN, 2006).

A redução do risco de câncer é determinada pela atuação de fitatos no controle do crescimento e progressão de tumores e metástases, e sua atuação no sistema imune e suas propriedades antioxidantes, também contribuem para destruição de células tumorais (VUCENIK; SHAMSUDDIN, 2006; MESSINA, 2014). A redução da progressão de tumores pode estar relacionada à supressão de vias de sinalização e proliferação celular, além da supressão da ciclooxigenase-2 (COX-2) associada à inflamação, como observado para ácido fítico de farelo de arroz (SAAD; ESA; ITHNIN, 2013). Em sua revisão Silva e Bracarense (2016) destacam vários estudos *in vitro* e *in vivo* (ratos, camundongos e humanos) que explicitam os mecanismos anti-neoplásicos do IP6, bem como sua ação protetora em diversas condições patológicas, como doença de Alzheimer's, dislipidemia, doenças cardiovasculares, diabetes, úlceras, inflamação intestinal, dentre outras. Especificamente em humanos, a ingestão de IP6 (6 g/dia, divididas em duas doses) preveniu citopenia, queda de leucócitos e

plaquetas, além de melhorar a qualidade de vida e capacidade funcional de pacientes em tratamento quimioterápico para câncer de mama (n= 7) (BACIC *et al.*, 2010). Curhan e colaboradores (2004) em estudo cohort com mulheres jovens (n = 96.245) observaram que a ingestão de fitato dietético pode ser uma nova e segura abordagem para prevenção de cálculos renais, uma vez que houve forte associação inversa entre ingestão de fitato e risco de formação de cálculos, possivelmente pelo efeito inibitório do fitato sobre a formação de cristais de oxalato de cálcio na urina.

2.2.2. Polifenóis

A ação adversa dos compostos fenólicos se dá pela formação de complexos entre polifenóis e proteínas, o que contribui para a baixa digestibilidade de leguminosas em animais e humanos, por formar complexos insolúveis, tornando a proteína parcialmente indisponível, ou por meio da inibição de enzimas digestivas (SATHE, 2002). Os compostos fenólicos podem também inibir a biodisponibilidade de certos minerais como o zinco (SHAHIDI, 1997) e ferro (TAKO *et al.*, 2014; HART *et al.*, 2015).

Esses compostos são classificados como ácidos fenólicos, derivados taninos e flavonóides. Os feijões comuns (*Phaseolus vulgaris* L.) apresentam importante composição de polifenóis, cujos teores podem variar conforme o estado da matéria-prima (*in natura* e processado) e variedade (**Tabela 1**). A variedade Carioca pode ainda apresentar os seguintes ácidos fenólicos: caféico, p-cumárico, sinápico e ferúlico (GARCIA *et al.*, 1998; MOJICA *et al.*, 2015) .

Tabela 1 - Concentrações de compostos fenólicos em feijões comuns (*Phaseolus vulgaris* L.)

Classe	Composto	Conteúdo*
Flavonóis	(+)-Catequina	5,07
	(-)-Epicatequina	0,14
	(-)-Epigalocatequina	0,05
	Procianidina dímero B1	1,22
	Procianidina dímero B2	0,12
	Procianidina dímero B3	0,82
	Campferol 3-O-glicosídeo	30 (30,0; 54,5)
	Campferol 3-O-xilosil-glicosídeo	11,5 (0; 17,0)
	Campferol 3-O-acetil-glicosídeo	16,40
Isoflavonóis	Genistein	0,20

*Concentrações expressas por média ou média (min; max) (quando disponível) (mg/100 g de feijões crus).

Fonte: *Phenol Explorer: Data base on poliphenol content in foods* (2013)

Os taninos são classificados em taninos hidrolisáveis e condensados. Os primeiros são facilmente hidrolisados em açúcares, ácidos carboxílicos e compostos fenólicos simples. Os taninos condensados são os mais comuns e consistem de catequinas ou leucoantocianidinas e resíduos flavonóides que produzem tipicamente antocianidinas na degradação ácida (CARMONA *et al.*, 1996). As antocianinas de *P. vulgaris* são delfinidinas 3-glicosídeo e 3,5-diglicosídeo ou uma mistura de pelargonidina e cianidina 3-glicosídeo e 3,5-diglicosídeo (CHIARADIA; GOMES, 1997). Em feijões os taninos são fortes inibidores da atividade de tripsina, quimotripsina e alfa-amilase (CARMONA *et al.*, 1996; HAILESLASSIE; HENRY; TYLER, 2016) e pode afetar a biodisponibilidade de ferro (DELIMONT; HAUB; LINDSHIELD, 2017).

O efeito sobre a biodisponibilidade de ferro de feijões parece depender do tipo de composto fenólico. Por exemplo, em estudo com feijões preto (*Phaseolus vulgaris* L.), os fenólicos catequina, ácido 3,4-diidroxibezóico, campferol e campferol 3-glicosídeo

promoveram maior absorção de ferro em células CACO-2, enquanto a micertina, micertina 3-glicosídeo, quercetina e quercetina 3-glicosídeo inibiram a absorção (HART *et al.*, 2015).

Os efeitos benéficos à saúde têm superado as propriedades antinutricionais de polifenóis, uma vez que taninos e outros polifenóis (antocianinas e flavonoides) são considerados fatores de proteção contra radicais livres em doenças como o câncer e a aterosclerose (CARBONARO, 2006). Foi observado também efeito anti-inflamatório de casca de feijão comum, ao inibir as enzimas COX-1 e COX-2, de maneira dependente do conteúdo de fenólicos e da atividade antioxidante, a qual pode ser afetada pelo cultivar e solvente de extração utilizado (DAVE; AMÉLIE; PARTHIBA, 2010).

2.2.3. Peptídeos bioativos

2.2.3.1.Obtenção de hidrolisados e peptídeos bioativos dietéticos

Os peptídeos bioativos de fontes alimentares são peptídeos de origem vegetal ou animal que apresentam efeitos regulatórios fisiológicos, além de contribuir para uma alimentação adequada e equilibrada (HARTMANN; MEISEL, 2007). Os peptídeos são produzidos a partir de proteínas durante a digestão gastrointestinal, fermentação de material alimentar ou por hidrólise enzimática (WANG; DE MEJIA, 2005).

Esses peptídeos estão encriptados na estrutura primária de proteínas animais e vegetais, e podem ser liberados por proteólise *in vitro, in vivo*, ou combinação de ambos. A liberação *in vivo* envolve a digestão gastrointestinal com enzimas como pepsina, tripsina, quimiotripsina e peptidases da borda em escova intestinal, e também enzimas derivadas da microbiota intestinal. A produção de peptídeos *in vitro* inclui hidrólise enzimática de proteínas alimentares por enzimas endógenas presentes na matriz alimentar, e proteólise durante o processamento ou amadurecimento do alimento, por meio da ação de enzimas de culturas iniciadoras ou por enzimas isoladas de microorganismos proteolíticos (por exemplo:

Lactobacillus helveticus, *Lactobacillus delbrueckii subsp. bulgaricus* and *L. delbrueckii subsp. Lactis*) (ESPECHE TURBAY *et al.*, 2012).

Para avaliar peptídeos naturalmente produzidos durante a digestão gastrointestinal empregam-se digestões *in vitro*, utilizando proteinases como pepsina, tripsina, quimiotripsina e pancreatina. Utiliza-se também hidrólise enzimática realizada com enzimas microbiais ou de plantas, como alcalase, flavorenzima, papaína e bromelina. A germinação de sementes também pode ser utilizada eficientemente como um processo hidrolítico natural, por meio da geração de enzimas intrínsecas (BAMDAD *et al.*, 2009).

Geralmente a obtenção de hidrolisados ou peptídeos bioativos por meio de hidrólise enzimática ou digestão gastrointestinal simulada compreendem as seguintes etapas: extração de proteínas, determinação do grau de hidrólise, filtração, separação e identificação dos peptídeos. A extração ou isolamento de proteínas é opcional, e irá depender do objetivo da geração de peptídeos. Nos casos em que a digestão gastrointestinal simulada for realizada para simular o processo digestivo humano, a realização da hidrólise enzimática da matriz alimentar completa será mais realista, e a extração (ou isolamento) de proteínas não será necessária.

O isolamento de proteínas será aplicável nos casos em que se pretende aumentar a concentração de peptídeos ou outras situações a depender do objetivo de estudo ou aplicação dos hidrolisados ou peptídeos obtidos. A determinação do grau de hidrólise, embora opcional, é uma importante ferramenta para predizer o potencial bioativo dos hidrolisados ou peptídeos, uma vez que peptídeos menores possuem maior potencial biológico, devido a maior biodisponibilidade e capacidade de interação com membranas celulares.

Posteriormente à produção do hidrolisado proteico realiza-se a filtração e separação de peptídeos que pode ser realizada por diafiltração, cromatografia por exclusão de tamanho, eletroforese bidimensional, cromatografia de alta eficiência e eletroforese de capilaridade. Prossegue-se, então, com a identificação estrutural dos peptídeos por meio de ESI/MS/MS, MALDI-TOF-MS ou TANDEM MS (SAAVEDRA *et al.*, 2013).

Em posse da sequência peptídica pode-se utilizar ferramentas virtuais para caracterizar os peptídeos quanto às suas propriedades físico-químicas (tamanho molecular, estrutura química, carga líquida e ponto isoelétrico; PepDraw®), bem como seu potencial bioativo (BIOPEP®) e proteínas parentais (BLAST®). Na base de dados do BIOPEP® pode-se

investigar a bioatividade da sequencia peptídica desde que essa sequência tenha sido validada previamente em estudos celulares, experimentais ou clínicos. Essa ferramenta fornece o potencial bioativo (antioxidante, anti-hipertensivo, anti-trombótico, anti-diabético), a dupla ou tripla sequencia responsável pela bioatividade referida, e referência literária em que o potencial foi comprovado. A validação do potencial biológico requer a síntese de estruturas análogas à sequência identificada (de interesse), e avaliação *in vitro* ou *in vivo* da bioatividade. A síntese de peptídeos é realizada por empresas específicas, e muitas vezes pode ser de alto custo.

A avaliação da hidropaticidade permite predizer o potencial de interação com membranas celulares e pode ser mensurada diretamente (cromatografia de interação hidrofóbica) (DIA; BRINGE; GONZÁLEZ DE MEJÍA, 2014) ou estimada virtualmente (ProtParam Expansy®) (PROTPARAM, 2015).

O estudo *in silíco*, é uma abordagem interessante a ser utilizada previamente aos estudos de potencial biológico em modelos celulares e humanos. Esse tipo de estudo consiste em uma técnica ou simulação computacional que coloca uma pequena molécula (ligante) no sítio de ligação de seu alvo macromolecular (receptor) e calcula a sua afinidade de ligação (YURIEV; AGOSTINO; RAMSLAND, 2011), permitindo avaliar a força de interação de sequencias peptídica com alvos moleculares, diminuindo o número de testes de bioatividade. O estudo *in silíco* é também chamado de engenharia reversa do genoma (*reverse genome engineering*), considerado uma rápida, econômica e efetiva estratégia para a descoberta de novos peptídeos bioativos (SAAVEDRA *et al.*, 2013).

Pode-se, ainda, avaliar diretamente a atividade antioxidante do hidrolisado ou frações peptídicas por meio de testes como ABTS (2,2-azinobis (*3-ethyl-benzothiazoline-6-sulfonic acid*), DPPH (*2,2-diphenyl-1-picrylhydrazyl*) e ORAC (*oxygen radical absorption capacity*) (LUNA-VITAL *et al.*, 2015).

Os resultados de geração de peptídeos e bioatividade variam altamente conforme as condições de extração de proteínas, pré-tratamento e especificidade das enzimas, dentre outros fatores. A enzima alcalase é uma endoprotease que apresenta alta probabilidade para gerar peptídeos, a qual resulta em elevado grau de hidrólise (TORRUCO-UCO *et al.*, 2009).

A digestão gastrointestinal simulada é um modelo de digestão bastante utilizado para obtenção de peptídeos bioativos a partir de feijões comuns. Nesse processo utiliza-se as

enzimas pepsina e pancreatina, as quais representam a combinação de várias enzimas gástricas, resultando em uma ampla especificidade para proteínas (LUNA-VITAL *et al.*, 2015).

Similarmente à digestão gastrointestinal, a combinação de proteases tem sido utilizada para proteínas derivadas de feijão comum, resultando em maior grau de hidrólise quando comparado ao uso de uma única enzima (BETANCUR-ANCONA et al.,2014). O uso de proteases com ampla especificidade enzimática resultará em maior liberação de peptídeos pequenos, aumentando o número de resíduos aminoácidos terminais nos hidrolisados que podem ser clivadas por exoproteases.

O uso de diferentes variedades de feijão comum influencia os peptídeos gerados. A faseolina, principal proteína de reserva de feijão comum, é altamente variável à proteólise de diferentes cultivares (MONTOYA *et al.*, 2008). Por exemplo, sobas mesmas condições de hidrólise usando subsequentemente pepsina/tripsina/alfa-quimiotripsina, nove variedades de feijão comum apresentaram diferenças no grau de hidrolise (RUI *et al.*, 2012).

Outra condição que pode resultar em diferenças nos peptídeos gerados é o tratamento térmico, uma vez que o aquecimento causa desnaturação parcial, aumentando a exposição de resíduos hidrofóbicos de proteínas globulares, como a faseolina (MONTOYA *et al.*, 2008), e aumenta a sucetibilidade à hidrólise.

Segundo Luna-Vital e colaboradores (2015), a combinação de enzimas, tempo de hidrólise e pré-tratamento térmico, dentre outras condições, devem ser consideradas para melhorar o processo de hidrólise, reduzindo os custos operacionais e otimizando o tempo para obtenção de elevado grau de hidrólise, que irá resultar em elevada quantidade de peptídeos biologicamente eficientes.

2.2.3.2.Bioatividade de hidrolisados proteicos e peptídeos dietéticos

Os peptídeos bioativos apresentam, dentre outras, as seguintes propriedades: antimicrobianas, anti-hipertensivas, hipocolesterolêmicas, antitrombóticas e antioxidantes, aumento da absorção/biodisponibilidade de minerais, efeitos cito ou imunomoduladores e

atividades opiódes. Determinados peptídeos são multifuncionais e podem exercer mais de um desses efeitos (HARTMANN; MEISEL, 2007).

Dentre as leguminosas, observa-se na literatura científica um grande número de estudos sobre peptídeos bioativos da soja, os quais podem contribuir para a redução do risco de doenças crônicas (WANG; DE MEJIA, 2005). Para feijões comuns (*Phaseolus vulgaris* L.), os estudos disponíveis sugerem atuação de peptídeos no controle da saciedade (SUFIAN *et al.*, 2007), ação antifúngica (YE; NG, 2001; WONG; NG, 2005), anti-HIV (YE; NG, 2001; WONG; NG, 2005), anticancerígena (WONG; NG, 2005), anti-hipertensiva (RUI *et al.*, 2013) e anti-inflamatória (OSEGUERA-TOLEDO *et al.*, 2011).

Foi demonstrado efeito de peptídeos de feijão (cultivar Country Beans – *Dolichos lablab*) sobre a maior liberação de colecistoquinina por células entero-endócrinas (SUFIAN *et al.*, 2007), o que sugere influência no controle da saciedade. Peptídeos da variedade Pinto (*P. vulgaris*) resultaram em potente atividade antifúngica e inibição da transcriptase reversa do HIV (YE; NG, 2001). Peptídeos de feijões brancos (*P. vulgaris*), além do efeito anti-HIV e antifúngico, promoveram inibição da proliferação de células de leucemia e câncer de mama (WONG; NG, 2005). Proteínas de feijão vermelho (*P. vulgaris*) são sugeridas como boa fonte de peptídeos inibidores da enzima conversora de angiotensina (ECA) e, portanto, podem apresentar ação anti-hipertensiva (RUI *et al.*, 2013).

Com relação ao efeito anti-inflamatório, Oseguera-Toledo e colaboradores (2011) sugeriram que feijões parcialmente hidrolisados podem fornecer compostos úteis no combate à oxidação e inflamação associadas à doenças crônicas devido à elevada capacidade antioxidante dos hidrolisados, os quais inibiram a expressão de COX-2 e produção de prostanglandina E2 em macrófagos previamente estimulados com lipopolissacarídeos (LPS). Nesse estudo foram utilizados feijões da espécie *Phaseolus vulgaris* L., das variedades Pinto Durango e Negro. De acordo com os autores pelas características e tamanho dos peptídeos eles poderiam ser facilmente absorvidos.

Os estudos anteriormente citados são provenientes de ensaios *in vitro* e poucos estudos avaliam o efeito de hidrolisados proteicos de feijão comum *in vivo*. A maioria avalia frações peptídicas ou peptídeos bioativos específicos, e não o hidrolisado completo. Para hidrolisados proteicos da variedade Carioca não há estudos *in vivo* publicados até o momento. Em ratos espontaneamente hipertensos foi comprovado o efeito anti-hipertensivo de

hidrolisados proteicos de feijão da China (*Vigna radiate*; 600 mg/kg de peso corporal por via oral) (LI *et al.*, 2005) e de frações peptídicas de feijão Azufrado Higuera (*Phaseolus vulgaris* L.; 4 mg/kg de peso corporal, via intraperitoneal) (ARIZA-ORTEGA *et al.*, 2014). O uso de derivados isolados de feijão *Phaseolus vulgaris* contendo o inibidor de alfa-amilase 1 e/ou fitohemaglutinina têm demonstrado efeitos positivos sobre a indução da perda de peso, maior controle glicêmico, redução do apetite geral e para alimentos palatáveis (CARAI *et al.*, 2009). O BeanBlock® (Indena SpA, Milan, Italy), patenteado como um supressor natural do apetite, derivado de feijão vermelho (*kidney bean; Phaseolus vulgaris*) promoveu redução da ingestão alimentar e maior controle glicêmicos em animais (CARAI *et al.*, 2011; LOI *et al.*, 2013). Em homens com sobrepeso (1 cápsula de 50 mg, duas vezes ao dia, por 12 semanas), o produto promoveu significativa perda de peso, redução do perímetro da cintura e do estresse oxidativo, e maior controle da saciedade (LUZZI *et al.*, 2014). Resultados semelhantes quanto à perda de peso e o controle glicêmico foram observados para outro produto derivado de feijão comum branco (*kidney bean*; *Phaseolus vulgaris*), denominado *Phase 2* (BARRETT; UDANI, 2011).

A atividade antioxidante de peptídeos é favorecida pela presença de aminoácidos hidrofóbicos (GUO; YONEKURA, 2009), tamanho molecular (inferior a 1 kDa) (LUNA-VITAL *et al.*, 2015), maior grau de hidrolise e sinergismo entre peptídeos (VANDENBORRE; SMAGGHE; VAN DAMME, 2011). A atividade antioxidante para hidrolisados completos é maior que para frações proteicas (CARRASCO-CASTILLA *et al.*, 2012), provavelmente devido ao sinergismo entre peptídeos e à presença de compostos fenólicos associados principalmente aos aminoácidos aromáticos e básicos (SARMADI; ISMAIL, 2010), os quais também contribuem para as propriedades anti-inflamatórias e anti-ateroscleróticas (GARCÍA-LAFUENTE *et al.*, 2009).

2.3.Efeito do armazenamento sobre a qualidade de feijões

Considerando que a variedade de feijão mais utilizada pela população brasileira é o feijão carioca, responsável por 70 % do consumo total de feijão, destaca-se a importância de modificações genéticas destinadas à melhora de sua qualidade.

Um dos principais problemas relacionados à produção do feijão Carioca é o seu rápido escurecimento. Isso é economicamente desfavorável para os agricultores, pois impede o armazenamento por longos períodos, uma vez que reduz a qualidade dos grãos. Nesse sentido, novos cultivares de feijões carioca foram e estão sendo desenvolvidos para adiar o escurecimento (FARIA *et al.*, 2004) e, ou para reduzir o endurecimento ao longo do tempo, que é outra característica economicamente importante (TEIXEIRA; SIQUEIRA; BASSINELLO, 2011).

A aceitação e a comercialização de um novo cultivar dependem das exigências dos consumidores, para os quais interessam aspectos como: tamanho, forma, tempo de cocção e coloração (BASSINELLO *et al.*, 2003). O escurecimento é considerado pelos consumidores como uma característica de envelhecimento e endurecimento. Contudo, nem todos os feijões velhos são endurecidos e, ou escuros, principalmente porque as qualidades tecnológicas e nutricionais são determinadas pelo genótipo e influências do armazenamento durante o crescimento da planta e desenvolvimento dos grãos (TEIXEIRA; SIQUEIRA; BASSINELLO, 2011).

Elevadas temperaturas e umidade relativa em presença de luz e oxigênio prejudicam a absorção de água e modificar o conteúdo fenólico (RIOS; ABREU; CORRÊA, 2003). Temperaturas superiores a 25 ºC e umidade relativa maior que 65% podem favorecer o escurecimento e contribuir para o fenômeno “hard-to-cook” (HTC) (JUNK-KNIEVEL; VANDENBERG; BETT, 2007). Esse fenômeno é caracterizado por defeito textural que resulta em necessidade de maior tempo de cocção para obtenção de textura adequada, aumentando os gastos de energia para cocção e as perdas nutricionais (BRESSANI, 1983).

O tempo de armazenamento é importante para preservar a qualidade dos grãos, uma vez que o envelhecimento de feijões pode aumentar o escurecimento e endurecimento por defeitos de hidratação (RIOS; ABREU; CORRÊA, 2003), por perda de fitatos (HINCKS;

STANLEY, 1986; NASAR-ABBAS *et al.*, 2008), e mudanças no metabolismo de compostos fenólicos que resultam em lignificação (HINCKS; STANLEY, 1986; NASAR-ABBAS *et al.*, 2008).

A redução da capacidade de absorção de água e digestibilidade dos cultivares é intensificada pelo tempo de armazenamento, ao passo que pode ocorrer aumento do conteúdo de compostos fenólicos (RIOS; ABREU; CORRÊA, 2003). Após longo período de armazenamento (cinco anos) Martin-Cabrejas *et al.* (1997) observaram redução do conteúdo de fenólicos totais, de taninos e de ácido fítico, enquanto que o conteúdo de proteínas foi ligeiramente aumentado, sugerindo alterações fisiológicas pós-colheitas. Em curto período de armazenamento (seis meses), Rios, Abreu e Corrêa (2002) observaram aumento do conteúdo de fenólicos e escurecimento. Segundo os autores, a perda de qualidade de feijões ao longo do tempo de armazenamento manifesta-se pelo aumento do grau de dureza do grão, maior tempo de cocção, mudança do sabor e da coloração (RIOS; ABREU; CORRÊA, 2002).

A variação nas concentrações de taninos pode ser atribuída a fatores como tamanho dos grãos, coloração natural do grão, condições de manuseio do material e metodologia utilizada na determinação. A presença de antocianinas é outro fator interferente, pois são mais propensas à oxidação que taninos condensados poliméricos, segundo Iaderoza *et al.* (1989), citado por Rios, Abreu e Corrêa (2002).

Outras alterações nutricionais durante armazenamento prolongado incluem redução de lipídios (CHIARADIA; GOMES, 1987) e alterações proteícas. No caso de proteínas os resultados são controversos, com estudos mostrando aumento do conteúdo proteico (MARTIN-CABREJAS *et al.*, 1997; RIOS; ABREU; CORRÊA, 2003), redução, ou nenhuma alteração (DONADEL; PRUDENCIO-FERREIRA, 1999). Além disso, proteínas maiores podem sofrer hidrólise enzimática natural originando pequenos polipeptídeos durante o armazenamento (HOHLBERG; STANLEY, 1987), o que pode alterar suas propriedades funcionais.

Com realação ao aspecto tecnológico, em estudo que avaliou cinco tipos de feijão Carioca (BRS Requinte, BRS Pontal, Pérola, CNFC 10467 e BRS Madrepérola), foi observado variação na coloração entre os genótipos e todos sofreram escurecimento durante o armazenamento (180 dias). As variações de cor foram observadas também no início do armazenamento, indicando que a variabilidade de cor é influenciada pelas características

genéticas (TEIXEIRA; SIQUEIRA; BASSINELLO, 2011). Contudo, alguns cultivares podem apresentar níveis menores de escurecimento, o que está associado à presença de um gene responsável pela produção de proantocianidina (BASSETT, 1996).

Dentre os genótipos analisados por Teixeira, Siqueira e Bassinello (2011) o BRS Madrepérola se destacou pelo maior valor de luminosidade, de coloração amarela e de índice de amarelamento, ou seja, houve menor escurecimento, enquanto o BRS Pontal apresentou resultados inferiores. O endurecimento foi avaliado pelo tempo de cocção, que não diferiu no tempo inicial de armazenamento.

Os genótipos Pérola, CNFC 10467 e BRS Madrepérola apresentaram maior estabilidade até os 180 dias de armazenamento e menores tempos de cocção. Já os genótipos BRS Requinte e BRS Pontal mostraram-se estáveis até os 90 dias de armazenamento. Porém, após esse período o tempo de cocção foi bruscamente elevado. Cruzando os dados de escurecimento e endurecimento, os genótipos Madrepérola e CNFC 10467 foram os que menos escureceram e que mantiveram o menor tempo de cocção, enquanto o BRS Pontal apresentou maior escurecimento e endurecimento ao longo do armazenamento. Observa-se, portanto, uma tendência de escurecimento e endurecimento durante o armazenamento. Contudo, esses dois eventos ocorrem em intensidades diferentes, dependendo do genótipo (TEIXEIRA; SIQUEIRA; BASSINELLO, 2011).

2.4. Processo inflamatório e aterosclerose

A inflamação aguda é uma rápida resposta a infecções microbianas ou injúrias teciduais, que envolve o recrutamento e a ativação de neutrófilos. Se a inflamação aguda é resolvida, o tecido normal é restaurado ou forma-se uma cicatriz de tecido conjuntivo. Se o estímulo não é adequadamente eliminado, o processo inflamatório persiste e evolui (POBER; SESSA, 2007).

O processo inflamatório participa em várias doenças crônicas, como diabetes, câncer e doença cardiovascular (OBERYSZYN, 2007). Este processo pode ser mensurado por meio da avaliação de diversos marcadores como interleucinas, fator nuclear kapa B (NF-κB), fator

de necrose tumoral-alfa (TNF-alfa), cicloxigenases (COX), óxido nítrico (NO), prostaglandinas (PG), dentre outros. O NF-κB induz a transcrição de mediadores pró-inflamatórios, como a óxido nítrico-sintase indúzivel (*Inducible nitric oxide synthase*, iNOS), cicloxigenase 2 (COX-2), TNF-alfa e interleucinas (IL) (OSEGUERA-TOLEDO *et al.*, 2011).

Os macrófagos têm papel chave no processo inflamatório. Em resposta a um estímulo infeccioso os monócitos circulantes migram para o compartimento extra-vascular e se diferenciam em macrófagos nos tecidos. Mediante diferenciação essas células perdem a capacidade de replicação e suas propriedades antibacterianas são marcadamente aumentadas, permitindo que participem ativamente da resposta inflamatória. O processo de diferenciação é complexo e controlado pela expressão ou ativação de vários fatores de transcrição (TAKASHIBA *et al.*, 1999).

Os lipopolissacarídeos (LPS), derivados de bactérias gram-negativas são considerados um dos mais potentes ativadores da resposta secretória de macrófagos. O fator de necrose tumoral alfa (TNF-α) é o principal mediador inflamatório secretado por macrófagos quando estimulados por LPS *in vitro* e *in vivo*. Esse fator não é secretado de reservas intracelulares, mas sintetizado de novo em resposta a um estímulo efetivo, que age via fatores nucleares, sendo o NF-κB um regulador potencial da transcrição do gene de TNF- α. O NF-κB, um heterodímero de proteínas p65 e p 50 da família Rel, é um fator de transcrição induzível, presente no citoplasma de muitas células eucariotas. Muitos estímulos, incluindo LPS, induzem a fosforilação do IkB (proteína regulatória que inibe o NF-κB), com subsequente liberação e ativação desse fator nuclear. Após a ativação ele é translocado do citoplasma para o núcleo, onde se liga a sítios específicos na região promotora do TNF- α ativando a transcrição de seu gene (TAKASHIBA *et al.*, 1999).

A proliferação de macrófagos na intima arterial e sua progressão para células espumosas carregadas de lipídios induz a liberação crônica de múltiplas citocinas pro-inflamatórias e fatores de crescimento como MCP-1, IL-1, IL-3, IL-6, IL-8, IL-18 e TNF- α (CAM; DE MEJIA, 2012). Durante o processo, grande quantidade de óxido nítrico e prostaglandina E2 (PGE2) são gerados pelas enzimas iNOS e COX-2, respectivamente. A iNOS é expressa em resposta ao interferon-γ (IFN-γ), LPS e várias citocinas pró-inflamatórias (CHAVEZ;

APAN; MARTINEZ-VAZQUEZ, 2005). A COX-2 converte ácido araquidônico à prostaglandina (WILLIAMS; MANN; DUBOIS, 1999).

Esse processo inflamatório crônico é a base fisiológica da aterosclerose. A inflamação crônica induz a agregação de macrófagos que expressam integrinas $\alpha V\beta 3$ nas lesões ateroscleróticas, e esse receptor induz a liberação das citocinas inflamatórias citadas anteriormente. Existem evidências de que peptídeos da soja são capazes de interagir com integrinas $\alpha V\beta 3$, inibindo a inflamação mediada pelo receptor $\alpha V\beta 3$ e as vias NFkB mediadas por Akt (ou proteína quinase B) (CAM; SIVAGURU; GONZALEZ DE MEJIA, 2013).

A LDL-oxidada (LDL-ox) apresenta um importante papel no processo aterosclerótico. A interação entre LDL-ox e seu principal receptor (LOX-1 - *lectin-like oxidized low-density lipoprotein receptor 1*), conduz a expressão de moléculas de adesão, como por exemplo ICAM (*intracellular adhesion molecule*), responsável pela adesão de monócitos no endotélio (LIBBY; DICARLI; WEISSLEDER, 2010). Em seguida, a MCP-1 (*monocyte chemo-attractant protein-1*) promove a migração dos monócitos do endotélio para a intima (DUNN *et al.*, 2008). A interação entre LDL-ox e LOX-1 também resulta em aumento de espécies reativas de oxigênio (EROS) e da atividade de metaloproteinases de matriz (MMPs - *matrix metalloproteinases*) (SZMITKO *et al.*, 2003). O aumento de EROS inativa a óxido nítrico sintetase endotelial (eNOS - *endothelium nitric oxide syntethase*), e a expressão aumentada de MMPs está relacionada a algumas doenças crônicas, incluindo inflamação e aterosclerose (VERMA; HANSCH, 2007).

Essas alterações podem contribuir para a desestabilização e ruptura de placas ateroscleróticas, levando à formação de trombos (SZMITKO *et al.*, 2003). Peptídeos dietéticos com sequencias bioativas podem apresentar potencial anti-inflamatório, anti-aterosclerótico e anti-trombótico, atuando na redução da expressão de proteínas relacionadas a processos inflamatórios, como interleucinas (IL), MCP-1, TNF-alfa, e do recepetor LOX-1 (MONTOYA-RODRIGUEZ *et al.*, 2014).

Considerando o papel de compostos bioativos alimentares na redução de fatores de risco associados à DCVs, torna-se relevante elucidar os efeitos sobre o estado inflamatório e da aterosclerose, caracterizando os efetores envolvidos, de maneira a esclarecer sobre os alvos

moleculares e os mecanismos pelos quais peptídeos bioativos de feijão podem atuar na redução do risco de DCVs.

3. METODOLOGIA GERAL

O estudo foi realizado nos laboratórios de Nutrição Experimental e de Análise de Alimentos do Departamento de Nutrição e Saúde (DNS), e também o laboratório de Espectrofotometria de Absorção Atômica do Departamento de Solos, pertencentes à Universidade Federal de Viçosa (UFV – Viçosa, Minas Gerais). Parte das análises foi concluída no doutorado Sanduíche (CAPES-PDSE) na Universidade de Illinois Urbana-Champaing (UIUC), EUA, em laboratórios coordenados pela Professora Pesquisadora Dra. Elvira Gonzalez de Mejia.

3.1. Delineamento experimental

O efeito do tempo de armazenamento sobre a composição nutricional, propriedades anti-inflamatórias e anti-ateroscleróticas de dois genótipos de feijão comum (*Phaseolus vulgaris* L.) da variedade Carioca, denominados BRS Pontal (PO) e BRSMG Madrepérola (MP), foi avaliado por meio de delineamento inteiramente casualizado (DIC), sem repetição. Após a colheita, os feijões PO e MP foram armazenados por até seis meses, à temperatura ambiente (aproximadamente 25 ºC). No tempo zero e após três e seis meses de armazenamento os feijões foram cozidos em calor úmido sob pressão e moídos para obtenção da farinha integral, a qual foi armazenada à -18ºC até posteriores análises de composição centesimal e fitoquímica, obtenção e caracterização dos hidrolisados proteicos, e avaliação do potencial anti-inflamatório e anti-aterosclerótico dos hidrolisados proteicos em macrófagos humanos.

3.2.Matéria-prima

3.2.1. Amostras

Os genótipos de feijão comum (*Phaseolus vulgaris*, L) da variedade Carioca cedidos pela EMBRAPA, BRS Pontal e BRS Madrepérola, são contrastantes para escurecimento e endurecimento. Os dois cultivares foram seletivamente melhorados pela EMBRAPA arroz e feijão. Programa de melhoramento genético (Santo Antônio de Goiás, Brasil) por cruzamento entre 2 linhagens (BRSMG Madreperóla: AN 512666-0 e AN 730031; BRS Pontal: BZ3836//FEB 166/NA910523) (DEL PELOSO *et al.*, 2004; CARNEIRO *et al.*, 2012). As melhores linhagens foram selecionadas após seis gerações com base no potencial de produtividade e resistência as doenças. Além disso, os aspectos dos grãos também foram considerados para o BRSMG Madreperóla, resultando em um cultivar com escurecimento e endurecimento tardios ao longo do armazenamento. As sementes foram multiplicadas no mesmo campo e sob a mesma administração de colheita adotando desenho inteiramente casualizado com três repetições.

Após a colheita, os feijões foram enviados ao Departamento de Nutrição e Saúde da Universidade Federal de Viçosa, onde foram armazenados em temperatura ambiente (aproximadamente 25 ºC), sem controle de umidade, por zero, três e seis meses, em embalagens de polipropileno, lacradas à vácuo. Os procedimentos de armazenamento foram assim determinados de maneira a reproduzir o método de armazenamento comercial.

3.2.2. Cocção e Processamento

Os feijões foram processados para obtenção da farinha integral dos mesmos de acordo com procedimentos propostos por Dias e colaboradores (2015), a qual foi utilizada para análise da composição química e fitoquímica, e ensaios *in vitro*. Resumidamente, os feijões

foram pesados após catação, lavados e submetidos à cocção por calor úmido sob pressão na proporção de 1:2 (p/v) até atingir consistência macia, própria para consumo, e com mínimo teor de água de cocção residual. Foram pesados juntamente com a água de cocção e secos em estufa com circulação de ar (modelo MA 035, Marconi®, Piracicaba, São Paulo, Brasil) por 8 horas à 60ºC. Os feijões secos foram processados em moinho (MA 090 CFT) à 2000 rpm, provido de peneira (600 µm, 30 mesh). A farinha integral obtida foi acondicionada em embalagens à vácuo, rotulada, e armazenada à -18 ºC até o momento das análises.

3.3.Análise da composição química e fitoquímica

3.3.1. Composição química centesimal

A composição centesimal foi avaliada utilizando os procedimentos analíticos preconizados pela Association of Official Analytical Chemists – AOAC (2002), nos laboratórios de Análise de Alimentos e Nutrição Experimental (DNS/UFV). Resumidamente, a umidade foi determinada por secagem em estufa à 105 ºC, por gravimetria utilizando 10 gramas de farinha integral de feijão. Para a determinação do conteúdo de cinzas, 2,5 g foram submetidas à calcinação em mufla. O método micro Kjeldahl foi utilizado para a determinação do conteúdo protéico por meio da quantificação de nitrogênio total (fator de conversão igual a 5,75) (BRASIL, 2001). Para essa análise foram pesados em papel manteiga 50 mg de cada amostra. Dez gramas de cada amostra foram utilizados para determinação do conteúdo de lipídios por extração em aparelho Soxhlet, utilizando-se éter de etílico como extrator, por 8 horas sob-refluxo. O conteúdo de carboidratos foi calculado por meio da diferença entre o total da amostra e os conteúdos de proteínas, lipídios, fibra alimentar, umidade e cinzas. O valor energético foi determinado utilizando os fatores de conversão 4, 4 e 9 kcal/g de alimento para carboidratos, proteínas e lipídios, respectivamente (FRARY; JOHNSON, 2005).

3.3.1.1.Fibra alimentar

O conteúdo de fibra alimentar total, fração solúvel e insolúvel foram determinados por meio do método enzimático gravimétrico (AOAC. 2011), no laboratório de Nutrição Experimental (DNS/UFV). Para hidrólise enzimática foram utilizadas α-amilase termoresistente, protease e amiloglicosidase (*Total dietary fiber assay kit*, Sigma®). Para a filtração foram utilizados cadinhos de vidro com placa de vidro sintetizado com porosidade número 2 (ASTM 40-60mesh) e celite como auxiliar de filtração. O teor de fibra alimentar total foi obtido por meio da soma entre as frações solúvel e insolúvel.

3.3.1.2. Amido resistente

O conteúdo de amido resistente foi determinado por kit enzimático da Megazyme®, segundo os procedimentos analíticos 32-40.01 da AOAC (2002). As amostras (0,1 g) foram incubadas com α-amilase pancreática e amiloglicosidase no banho metabólico (Marconi®, modelo MA 093, Piracicaba, Brasil) a 37ºC, durante 16 horas, sob agitação (200 rpm/min) para a digestão do amido não resistente. A inativação das enzimas e extração dos componentes diferentes do amido resistente (exemplo: amido) foi realizada com etanol 50% e 99%. O amido resistente das amostras foi dissolvido com KOH 2 M, neutralizado com acetato de sódio (pH 3,8) e hidrolisado com amiloglicosidase e a D-glicose resultante foi mensurada com o reagente de Glucose Determination (GOPOD) por leitura em espectrofotômetro (Thermo Scientific®, Evolution 60S, EUA) a 510 nm. O branco e o padrão foram preparados utilizando-se água e glicose, respectivamente. O cálculo do conteúdo de amido resistente foi realizado na planilha disponível em www.megazyme.com.

3.3.2. Conteúdo mineral

Para análise do conteúdo de minerais (ferro, cálcio, zinco, cobre, manganês, magnésio, sódio, potássio e fósforo), foram utilizados os procedimentos descritos por Gomes e Oliveira (2011). Foi pesado 1 g de amostra em triplicada em tubo de digestão e adicionados 10 mL de ácido nítrico concentrado. Posteriormente, as amostras foram aquecidas em bloco digestor com exaustão, com temperatura inicial de 80 ºC e aumento progressivo até 160 ºC, temperatura à qual as amostras foram submetidas durante 16 horas até a formação de solução límpida. Os tubos foram resfriados em temperatura ambiente. O conteúdo foi transferido para balão volumétrico de 50 mL, o tubo lavado com água deionizada e agitado em vórtex, para evitar perdas, e seu conteúdo vertido no balão volumétrico até que completar o volume. Essa solução foi utilizada para leitura do conteúdo dos minerais em equipamento de espectrofotometria de emissão de plasma (Perkin Elmer – Optima 3300 DV, Norwalk, USA), no laboratório de Espectrofotometria de Absorção Atômica (Departamento de Solos/UFV). Todas as vidrarias utilizadas foram previamente desmineralizadas em solução de ácido nítrico 10 % durante 12 h e secas em estufa de circulação de ar.

3.3.3. Ácido fítico

O teor de fitato foi determinado por cromatografia de troca iônica e espectrofotometria, segundo método de Latta e Eskin (1980), com modificações propostas por Ellis e Morris (1986). Para a extração dos fitatos, pesou-se 0,1 g de RH e adicionou-se 5 mL de HCl 2,4%. Prosseguiu-se com agitação horizontal, em agitador rotativo, por 12 horas à 250 rpm. Centrifugou-se o extrato à 3000 rpm por 15 minutos. O sobrenadante foi filtrado à vácuo em funil de büchnner e purificado utilizando coluna de troca iônica, com fase estacionária constituída por resina Dowex- AGX-4. A coluna foi pré-condicionada com NaCl 2 M e o extrato obtido das etapas anteriores foi aplicado cuidadosamente à mesma. Os fósforos inorgânicos foram eluídos com NaCl 0,05 M, seguido da eluição dos fitatos retidos com NaCl 2 M. O fitato foi determinado colorimetricamente, com base na coloração rósea do reagente de Wade, formada a partir da reação entre o íon férrico e o ácido sulfossalicílico, que apresenta absorbância máxima à 500 nm. Na presença de fitato, o ferro é sequestrado e indisponível para reagir com o ácido sulfossalicílico, resultando em uma redução da intensidade da cor. Uma curva analítica utilizando padrão de ácido fítico ($C_6H_{18}O_{24}P_6$ x

Na.yH2O, Sigma®), nas concentrações de 10 a 100 µg.mL $^{-1}$ foi elaborada, por meio da equação de regressão linear (y = -0.0038x + 0.4963; R^2 = 0.994) para expressar o teor de fitatos em miligramas de ácido fítico por 100 gramas de RH.

3.3.5. Compostos fenólicos

3.3.5.1.Taninos condensados

O conteúdo de taninos totais foi avaliado por meio do método de reação vanilina, segundo Burns (1971), com modificações de Maxson e Rooney (MAXSON; ROONEY, 1972) e Price, Van Scoyoc e Butler (1978). Foram utilizadas alíquotas de 200 mg de farinha das variedades de feijões, em 10 mL de solução a 1 % de HCl em metanol. Os tubos foram colocados em agitador automático à 80 rpm durante 20 minutos para a extração dos taninos mantendo temperatura à 30 ºC. Em seguida, foram centrifugados a 3.000 rpm, por 20 minutos. Alíquotas de 1 mL do sobrenadante foram adicionadas a 2,5 mL de solução a 1 % de vanilina em metanol e 2,5 mL de solução a 8 % de HCl em metanol. Os tubos foram mantidos em repouso por 20 minutos e as absorbâncias foram medidas à 500 nm em espectrofotômetro (UV-1601, Shimadzu®) contra o branco. Uma curva-padrão de catequina foi usada para expressar os resultados em mg equivalente de catequina por 100 mg de amostra (y = 0,5002x + 0,0052; R^2 = 0,9995).

3.3.5.2. Fenólicos totais e identificação de compostos fenólicos

O teor de fenólicos totais foi determinado por meio do método de Folin–Ciocalteu (SINGLETON; ORTHOFER; LAMUELA-RAVENTÓS, 1999). Brevemente, os compostos fenólicos foram extraídos por agitação horizontal (100 rpm, 20 min) em solução metanólica

60%, seguido por filtração à vácuo (3 μm de porosidade). Alíquotas de 100 μL do extrato foram vortexadas com 1 mL de Folin–Ciocalteu (0,25 N) por 10 segundos. Após 3 minutos foi adicionado 1 mL de carbonato de sódio à 7,5%. Após 7 minutos, adicionou-se 5 mL de água destilada, e manteve-se a solução sobre agitação por 30 minutos, em temperatura ambiente. A absorvância foi lida em espectofotometro (Thermo scientific, Evolution 60S, USA) à 765 nm. Os resultados foram expressos em mg equivalentes de ácido gálico por grama de amostra (mgEAG/g), utilizando uma curva padrão obtida por diferentes concentrações de ácido gálico ($y = 30.763x + 0.0818$; $R^2 = 0.996$).

Para a identificação dos compostos fenólicos, cada amostra de farinha integral de feijão (400 mg) (tempos 0, 3 e 6) foi colocada em tubo Falcon® e 10 mL da solução metanol/água/ácido clorídrico (50:48:2) foi adicionada. O material foi sonicado por 1 hora. O extrato foi centrifugado a 8000 rpm e uma alíquota (1,5 mL) foi retirada, filtrada em membranas de politetrafluoretileno (PTFE) de 0,22 μm, e colocada em *vials* para análise por cromatografia líquida de alta eficiência acoplado à espectrometria Cromatografia Líquida de Alta Eficiência acoplado a espectrometria de massas com analisador hibrido quadrupolo-tempo de voo (HPLC-QTOF-MS), utilizando o sistema Acquity (Waters®), acoplado a um sistema de quadrupolo/Tempo de Voo (QtoF, Waters) pertencente a Empresa Brasileira de Pesquisa Agropecuária – EMBRAPA Agroindústria Tropical. As corridas cromatográficas foram realizadas em uma coluna Waters Acquity UPLC BEH (150 x 2,1 milímetros, 1,7 μm), temperatura fixa de 40 ◦C, fases móveis água com 0,1 % de ácido fórmico (A) e acetonitrila com 0,1 % de ácido fórmico (B), gradiente variando 0-15 min (2-95 %) de B; (15,1-17) min (100 %) de B; (17,1-19,1) min (2 %) de B, fluxo de 0,4 mL / min e volume de injeção de 5 μL. O modo ionização por eletrospray (*electrospray ionization,* ESI foi adquirido na faixa de 110-1180 Da, temperatura da fonte fixa a 120 ºC, temperatura de dessolvatação 350 ºC, fluxo do gás dessolvatação de 350 L/h, cone de extração de 0,5 V, voltagem capilar de 2,6 kV. Em baixo scan, a tensão de cone foi 35 V, energia de colisão de 5 eV (trap). Em alto scan, a tensão do cone foi de 35 V, com uma rampa de energia de colisão de 20-40 rampa eV (trap). Leucina encefalina foi utilizada como *lock mass*. O modo de aquisição foi o MS^E. O instrumento foi controlado pelo software Masslynx 4.1 (Waters Corporation). Os dados cromatográficos foram analisados por meio do software MarkerLynx (Waters) para discriminação de potenciais marcadores químicos. Os seguintes parâmetros foram utilizados:

tempo de retenção 1,2 a 7,0 minutos; alcance de massa entre 120 a 1200 Da; e eliminação do ruído no nível 5. Os marcadores foram identificados por meio da correlação entre o tempo de retenção (t_R) e razão massa/carga (*m/z*) resultante da ordem de eluição descrita no cromatograma e normalizados baseado na intensidade de cada íon. A escala utilizada foi centrado na média para a formação da análise de componente principal (PCA). Por meio da comparação entre cada amostra foi gerado a Análise discriminante com calibração multivariada por mínimos quadrados parciais ortogonal (*Orthogonal Parcial Least Square Discriminant Analysis* - OPLS-DA) e distinção de potenciais marcadores, que apresenta os íons de variação em cada genótipo e por tempo de armazenamento.

3.4. Peptídeos bioativos

3.4.5. Obtenção do Hidrolisado proteico

Para avaliação do potencial anti-inflamatório utilizou-se o hidrolisado completo, ou seja, nenhuma etapa de extração foi realizada anteriormente à hidrólise enzimática. Para o estudo de potencial anti-aterosclerótico o isolado proteico foi extraído por 1 hora à 35 ºC após ajuste de pH para 8.0 com NaOH (0,1 M). A mistura foi centrifugada à 5000 *g* por 15 min à 4 ºC. O precipitado foi extraído novamente sob as mesmas condições anteriores e os extratos foram combinados. O pH foi ajustado para 4,3 com HCl diluído, e o precipitado proteico foi coletado por centrifugação (10000*g* por 20 min, 4 ºC). O sobrenadante foi descartado e o sedimentado foi liofilizado (Lab Conco Freeze Dryer 4.5; Kansas, Mo., U.S.A.) e utilizado na hidrólise enzimática. Os hidrolisados foram obtidos por meio de digestão gastrointestinal simulada com pepsina e pancreatina em pH e temperatura fisiológicos, conforme descrito por Megías *et al.* (2004), com modificações. Brevemente, a farinha integral de feijão foi diluída em água destilada (1: 20, p:v) e o pH ajustado para 2 com solução de ácido clorídrico (1 N). Em seguida, adicionou-se pepsina (1:20, enzima: substrato, p/p) e prosseguiu-se a digestão por 2 horas. O pH foi ajustado para 7,5 com hidróxido de sódio (1 N), adicionou-se

pancreatina (1:20, enzima: substrato, p/p) e prosseguiu-se a digestão por mais 2 horas. Para as duas enzimas a digestão foi realizada sob agitação, à 37 ºC e o pH foi aferido e ajustado para pH ótimo de cada enzima, se necessário, a cada 30 minutos. A hidrólise foi interrompida com aquecimento à 75 ºC por 20 minutos em banho-maria. O hidrolisado resultante foi centrifugado (20.000 g, 15 min, 4 °C), dialisado para remoção de sais (membrana de acetato de celulose, tamanho do poro: 500 Da) e liofilizados (Lab Conco FreeZone Freeze dry system; Kansas City, MO, USA). As amostras foram armazenadas à -20ºC até posteriores análises.

3.4.6. Determinação do grau de hidrólise

O grau de hidrólise foi determinado conforme metodologia descrita por Cabra *et al.* (2007). A porcentagem de proteína dissolvida após precipitação com 0,2 N de ácido tricloroacético (TCA) foi comparada com o total de proteínas da farinha de feijão comum (100 %), obtido após hidrólise completa com 2 N de ácido sulfúrico a 100 °C por 4 horas. O conteúdo proteico foi determinado pelo método *DC protein assay* (detergente compatível; #500-0112; Bio-Rad ®, Hércules, CA, EUA), segundo instruções do fabricante, utilizando uma curva padrão de albumina do soro bovino, e a leitura realizada à 630 nm.

3.4.7. Avaliação da capacidade antioxidante

A capacidade antioxidante dos hidrolisados foi avaliada por meio do método de capacidade de absorção dos radicais oxigenados (*Oxygen Radical Absorbance Capacity* – ORAC), proposto por Prior *et al*. (2003). O ensaio ORAC foi determinado pela mensuração da fluorescência dos hidrolisados na presença de radicais gerados pelo dicloridrato de 2',2'-Azobis (2-amidinopropano) (AAPH). O ensaio foi carreado em placas escuras de 96 poços,

de fundo claro. Os reagentes foram adicionados na seguinte ordem: tampão fosfato 75 mM pH 7,4; 20 µL de padrão trólox ou amostra, 120 µL de solução fluorescente (concentração final de 70 nM) e 60 µL de AAPH (concentração final de 12 mM). As placas foram colocadas em um leitor de placas de fluorescência (FL 800x), conjunto com filtro de excitação de 485 nm e 520 nm de emissão, e automaticamente incubadas por 15 min à 37 ºC antes da adição do AAPH. A leitura foi realizada a cada 2 min por 3 h. As medidas de fluorescência foram expressas em relação ao valor inicial e os resultados calculados com base na diferença da área abaixo da curva de decaimento de fluorescência entre o branco e a amostra, e expresso como equivalentes de trolox (mM). O análogo da vitamina E (padrão trolox), foi utilizado como padrão.

A capacidade de eliminação de radicais de óxido nítrico (NO) pelos hidrolisados foi determinada utilizando o nitroprussiato de sódio (SNP) como o doador de NO (GREEN *et al.*, 1982). Brevemente, a solução SNP (10 mM) foi incubada com hidrolisados (1 mg de hidrolisado/mL em PBS) à 25 °C. Após 150 min, 0,5 mL de solução incubada foi misturado com 0,5 mL de reagente de Griess (1 % sulfanilamida, dicloreto de etilenodiamina 0,1% e 3 % de ácido ortofosfórico). A absorbância foi imediatamente mensurada em 546 nm. Os resultados foram expressos em % de inibição em relação ao PBS utilizado como controle.

3.4.8. Separação de proteínas – SDS-PAGE

Para analisar e visualizar o padrão de proteínas das amostras foi utilizada a técnica SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) (Mojica *et al.*, 2015). Amostras da farinha integral dos feijões e dos hidrolisados nos tempos 0, 3 e 6 meses de armazenamento foram diluídas com tampão de tricina (BioRad) em β-mercaptoetanol e aquecidos por 5 min antes do carreamento. Foi utilizado gel pronto Tris-HCL 4-20 % (BioRad) e um padrão de proteínas pré-corado (*Precision Plus Protein™ prestained standards*). O gel foi corrido à 200 V por 35 minutos e corado com *Simply Blue Safe Stain*,

por 12 horas (overnight). Posteriormente foram lavados 3 vezes (5 minutos cada) com água destilada. Os géis foram visualizados no *GL 4000 Pro Imaging System* (Carestream Health Inc., Rochester, USA).

3.4.9. Separação e Identificação de peptídeos

Os hidrolisados foram submetidos à cromatografia de exclusão (SEC) e os peptídeos foram separados com base na massa molecular, usando uma coluna de filtração em gel (30 cm x 10 mm, 13 µm granulometria média) carregado com resina Superdex 30, cujo limite de exclusão de tamanho é de 10 kDa (GE Healthcare Life Sciences, Pittsburgh, PA, EUA) (DIA, BRINGE e DE MEJIA, 2014). Os hidrolisados (20 mg/mL) foram eluídos usando o tampão Tris-HCl (pH 7,5; 20 mM). A taxa de fluxo foi definida em 0,5 mL/min. Os picos foram detectados usando o sistema Prime AKTA GE plus (GE Healthcare Life Sciences, Pittsburgh, PA, EUA) à 280 nm. As frações foram coletadas a cada 3 min por meio de um coletor de fração automatizado ligado ao sistema.

As frações peptídicas dos hidrolisados de pepsina-pancreatina obtidos após a filtração de gel foram analisados por cromatografia líquida de alta eficiência associada a espectrometria de massa com ionização por *electrospray* (CLAE-EM/EM ou HPLC-ESI-MS – *liquid chromatography–electrospray-ionization-mass spectrometry*), usando um Ultima Q-tof espectrômetro de massa (Waters, Milford, U.S.A.), equipado com um sistema *Aliance 2795 HPLC*, de acordo com Mojica *et al.* (2015). Somente picos com intensidade superior a 60 % e as sequências com mais de 70 % de probabilidade de identidade foram analisados utilizando o software MassLynx 4.1V (Waters, Milford, Mass., U.S.A.). A presença de sequencias bioativas presentes nas principais proteínas de feijão comum foram confirmadas por meio da base de dados eletrônica *UniProt database BLAST® tool*. O termo "*Phaseolus vulgaris* L" foi utilizado para filtrar os dados por espécie de interesse (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi, acessado em 12 de março de 2015). Dados com cobertura inferior à 60% não foram considerados. O potencial biológico das sequencias peptídicas foi investigado na base de dados BIOPEP (http://www.uwm.edu.pl/biochemia,

acessado em 12 de março de 2015). A estrutura química e propriedades fisico-químicas foram obtidas na base de dados *PepDraw tool* (http://www.tulane.edu/~biochem/WW/PepDraw/, acessado em 20 de março de 2015). Os aminoácidos foram apresentados segundo a nomenclatura de uma letra.

A hidropaticidade (H) dos hidrolisados (50 mg/ml) foi determinada de acordo com Dia, Bringe, e de Mejia (2014), usando uma coluna de alto desempenho de 1 mL fenil Sepharose (0,7 x 2,5 cm; tamanho de partícula médio de 34 µm) e coluna de cromatografia de interação hidrofóbica (GE Healthcare Life Sciences, Pittsburgh, PA, EUA). Os peptídeos angiotensina (H = 0,36, RT = 3,82 min), Bowman Birk inhibitor (derivado da soja) (H = - 0,159; RT = 3,41 min) e um peptídeo sintético de feijão comum (KKSSG; H = - 1.96; RT = 2,95 min) foram usados para construir uma curva padrão de hidropaticidade (y = 2,6919 x - 9,72; LINEAR = 0,926). A ferramenta virtual *ProtParam Expasy* ® (http://web.expasy.org/protparam/, acessado em 13 de fevereiro de 2015) foi utilizada para calcular a grande hidropaticidade média (Grand Average of Hydropathicity – GRAVY), que indica a solubilidade das proteínas de acordo com a soma dos valores de hidropaticidade de todos os aminoácidos na sequência dividido pelo comprimento da proteína. Resultados positivos indicam mais aminoácidos hidrofóbicos e valores negativos sugerem mais aminoácidos hidrofílicos (KYTE; DOOLITTLE, 1982).

3.4.10. Análise *in silico* da inibição do receptor LOX-1

Para a avaliação *in silico*, ou docking computacional, da inibição do receptor de LDL (receptor LOX-1), foram utilizados os peptídeos identificados nos hidrolisados obtidos a partir do isolado proteico, os quais foram utizados nos ensaios de avaliação do potencial anti-aterosclerótico. A análise *in silico* foi realizada para explorar os mecanismos estruturais pelos quais os peptídeos poderiam inibir o receptor LOX-1. Os cálculos de *docking*, ou cálculos de ancoragem, foram realizados no programa DockingServer.17. Previamente, os peptídeos

foram desenhados no programa *Instant MarvinSketch* (ChemAxon Ltd). O campo de força MMFF94 foi considerado como energia de minimização de ligação de moléculas, peptídeos e sinvastatina usando DockingServer. Cargas foram adicionadas aos átomos do ligante. A estrutura cristalizada do receptor LOX-1 foi obtida no banco de dados *Protein Data Bank* (PDB: 1YXK). Encaixe de simulações foram realizadas utilizando o algoritmo genético *lamarckista* (LGA). Cada experimento de encaixe foi derivado de 100 corridas diferentes que foram definidas para encerrar após um máximo de 2.500.000 avaliações de energia.

3.5. Potencial anti-inflamatório e anti-aterosclerótico dos hidrolisados

O efeito anti-inflamatório e anti-aterosclerótico dos hidrolisados dos feijões Pontal e Madrepérola foram avaliados em linhagens de macrófagos THP-1 (linhagem de células derivadas de monócitos humanos), que se diferenciam em macrófagos aderentes após estímulo com PMA (*phorbol 12-myristate 13-acetate*) em macrófagos THP-1.

3.5.5. Viabilidade celular

A eficiência de crescimento das células THP-1 na presença dos hidrolisados em diferentes concentrações foi avaliada por kit específico e avaliação da proliferação celular (*CellTiter 96 Aqueous One Solution Proliferation Assay Kit*). Trata-se de um método colorimétrico para determinação do número de células viáveis em ensaios de proliferação celular ou de citotoxicidade. O kit contém o composto *tetrazolium [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt*; MTS] e um reagente de acoplamento de elétrons (*phenazine ethosulfate*; PES). O PES tem uma estabilidade química considerável, o que permite que ele seja combinado com o MTS para formar uma solução estável. O MTS é bioreduzido pelas células em um produto formazan, cromofóro que é solúvel em meio de cultura de tecidos. Essa conversão é permitida pela

produção de NADPH ou NADH por enzimas desidrogenases de células metabolicamente ativas. A quantidade de formazan mensurado pela absorvância à 490 nm é proporcional ao número de células vivas na cultura. Resumidamente, 5 x 10^4 células foram semeadas em placa de 96 poços e o volume ajustado para 200 µL com meio de crescimento por 24 h (à 37 °C; 5 % CO_2/95 % ar). Após adesão completa, o meio foi removido e os hidrolisados foram administrados dissolvidos em PBS na concentração de 0,1; 1; 2,5 e 5 mg/mL para avaliação do potencial anti-inflamatório, e 0,01 mg/mL; 0,05 mg/mL e 0,1 mg/mL para avaliação do potencial anti-aterosclerótico. Essas concentrações foram estabelecidas conforme os testes de viabilidade celular. Células tratadas com PBS foram usadas como controle. Após 24 horas de tratamento, o meio foi substituído por 100 µL de meio fresco e 20 µL de MTS/PES. A placa foi incubada por 2 horas à 37 ºC e a leitura de absorvância realizada à 515 nm. A porcentagem de células viáveis foi calculada em relação às células controle tratadas com PBS.

3.5.6. Cultivo de células e tratamentos

3.5.6.1.Potencial anti-inflamatório

As células foram cultivadas em meio de cultivo Dulbecco's Modified Eagle's (DMEM) suplementado com penincilina (1%) e estreptomicina (1%), e soro fetal bovino (10%) à 37 °C em 5 % CO_2/95 % de ar em incubadora CO_2 Jacketed (NuAIRE DH Autoflow, Plymouth, MN, USA). Acetato miristato de forbol (Phorbol-12-miristato-13-acetato – PMA) foi utilizado para promover a diferenciação das células THP-1 em macrófagos maduros (TAKASHIBA *et al.*, 1999). A diferenciação foi permitida por 24 horas e confirmada em microscópio óptico pela morfologia celular e completa adesão à placa. As células foram cultivadas em densidade de 1 x 10^6 células por 2 mL em placa de 6 poços. As células foram tratadas com lipopolissacarídeo (LPS) (1 µg/mL), para indução de inflamação, e com os hidrolisados (0,1; 1; 2,5 e 5 mg/mL) por 24 h. Células tratadas com lipopolissacarídeo (LPS)

e tampão fosfato salino (PBS - *phosphate buffered saline*) foram usadas como controle positivo e células tratadas somente com PBS foram consideradas como controle negativo. Após 24 horas de tratamento o meio de cultivo foi coletado e armazenado à -80 ºC até análise de marcadores inflamatórios.

Resumidamente, para prostaglandina-E2 (PGE2) foi utilizado o kit número 514010 da Cayman Chemical (Ann Arbor, MI, USA) o meio coletado foi diluído com o tampão de diluição na concentração de 1: 500 (amostra: tampão) e 50 µL foram plaqueados em placa de 96 poços revestida com anticorpo policlonal anti-mouse IgG. Foi adicionado 50 µL de PGE2-acetilcolinesterase conjugada e 50 µL do anticorpo monoclonal PGE2, sendo a placa includa por 18 horas à 4 ºC. Após a incubação, a placa foi lavada usando o tampão de lavagem fornecido pelo kit. A coloração foi formada pela adição de 200 µL de reagente de Ellman e por agitação da placa por 60-90 minutos no escuro. A quantidade de PGE2 foi calculada usando uma curva padrão de PGE2 (PGE-2: $y = -1,1 \ln(x) + 4,2078$, $R^2=0,998$).

Os níveis de IL-1 beta foram avaliados por meio do kit número 583311 da Cayman Chemical (Ann Arbor, MI, USA), após diluição de 1: 50 (amostra: tampão) e 100 µL foram pipetados em placa de 96 poços revestida com anticorpo. Em seguida foram pipetados 100 µL do anticorpo conjugado com acetilcolinesterase (IL-1b (human) AChE Fab’ Conjugate) e a incubação ocorreu por 12 horas à 4 ºC. Os poços foram lavados 5 vezes com o tampão de lavagem e o reagente de Ellman foi adicionado (200 µl). A placa foi coberta e absorvância lida à 405-420 nm após 90 minutos de incubação. A concentração foi calculada por meio de uma curva padrão de IL-1β ($y=0,0066x + 0,0249$, $R^2=0,999$).

O TNF- α foi avaliado usando o kit número ab46087 da Abcam (Cambridge, MA, USA), usando a diluição de 1:100 (amostra: tampão) e 100 µL foram pipetados em placa de 96 poços pré-revestida. Foi adicionado 50 µl de TNF- α mAb conjugado com biotina e 100 µl da solução streptavidin-HRP foi adicionado e a placa incubada por 3 horas à 25 ºC. A placa foi lavada por três vezes e 100 µL do substrato TMB adicionado e incubado por 10-15 min à 25ºC ao abrigo da luz. A reação foi interrompida com 100 µL da solução de paragem e lida à 450 nm usando leitor Ultra Microplate Reader. A concentração de TNF- α foi calculada usando uma curva padrão de TNF- α ($y=0,7991x – 2,0792$, $R^2 = 0,998$).

3.5.6.2.Potencial anti-aterosclerótico

Para avaliação do potencial anti-aterosclerótico as células THP-1 foram cultivadas e diferenciadas conforme metodologia descrita no item anterior. Após a diferenciação, as células foram tratadas com os hidrolisados (0,01; 0,05 e 0,1 mg/mL) duas horas antes da incubação com LDL-ox (10 µg/mL). A incubação ocorreu por 48 horas. As células tratadas somente com PBS foram consideradas como controle negativo. As células tratadas com LDL-ox e PBS foram utilizadas como controle positivo. A sinvastatina (10µM) foi utilizada como controle farmacológico. Após o tratamento por 48 horas o meio de cultivo e os lisados de células foram coletados e armazenados a -80 ºC até posteriores análises.

No meio celular foram avaliados os níveis de PGE-2 (diluição de 1:250; amostra: tampão) e TNF-alfa (sem diulição), usando os kits de ELISA descritos no item anterior. As concentrações foram obtidas por meio das curvas padrões de TNF-alfa ($y = 0,9776x – 2,532$; $R^2 = 0,997$) e PGE-2 ($y = -1,067\ln(x) + 3,6711$; $R^2 = 0,997$).

Foi realizado um tratamento independente em placa de 96 para o ensaio de inibição de EROS, usando o kit *Cellular Reactive Oxygen Species Detection Assay Kit* (Abcam®, ab113851). As células de THP-1 ($2,5 \times 10^4$ células/poço) foram diferenciadas (48 horas com a PMA; 162 nM), seguido por 48 horas de tratamento, conforme descrito a seção anterior. Uma hora antes da conclusão do tratamento, foi adicionado o *2',7'- dichlorofluorescin diacetate 2'* (DCFDA) (volume de 50 µM/total). Após este período a placa foi transferida para o leitor de microplacas sem etapa de lavagem e a leitura realizada à 485 nm de excitação e emissão à 535 nm. Os resultados foram expressos em intensidade de fluorescência.

A expressão de LOX-1, MMP-9 e ICAM foi avaliada por *Western blot*. Após os tratamentos as células foram lavadas uma vez com DMEM gelado (1 mL) e duas vezes com PBS gelado (1 mL) e lisados com 200 µL de tampão de Laemmli (Biorad) contendo 5 % b-Mercaptoetanol. Os lisados celulares foram sonicados por 30 segundos e ebulidos por 5 min. O conteúdo de proteínas foi quantificado usando ensaio RCDC (BioRad ®) e 20 µg proteína foi carregada em géis Tris-HCL 4-20 % (BioRad) para separação de proteínas. A proteína foi transferida para uma membrana PVDF (Millipore, Billerica, MA) e o bloqueio foi realizado com 3 % leite desnatado em TBST (0,1 %) durante 1 h a 4 °C. As membranas foram lavadas

com 0,1 % TBST (5 vezes 5 min cada) e incubadas com o anticorpo primário respectivo para LOX-1 (ab60178, Abcam ®), MMP-9 (ab76003, Abcam ®) ou ICAM (710278, Lifetechnologies ®) em 1 % de leite desnatado em 0,1% TBST (1: 500) à 4 °C por 12 hroas. As membranas foram lavadas novamente e incubadas com anticorpo secundário conjugado (*anti-rabbit horseradish peroxidase conjugate secondary antibody*) em 1% de leite desnatado em 0,1 % TBST (1:2500) por 3 h em temperatura ambiente. Após a incubação e lavagens, a expressão das proteínas foi visualizada usando um reagente quimioluminescente (GE Healthcare), seguindo as instruções do fabricante. As imagens foram geradas com um sistema *GL 4000 Pro Imaging* (Carestream Health Inc. ®, Rochester NY). A intensidade das bandas foi normalizada usando GAPDH, e seu anticorpo primário diluído em 1% de leite desnatado em 0,1% TBST (1: 500).

O kit *human cytokine antibody array* (#ab133996, Abcam) foi utilizado, em duplicate para analisar a expressão de 23 proteínas no lisados celulares do controle negativo (PBS), controle positivo (PBS+LDL-ox), controle farmacológico (sinvastatina), e hidrolisados obtidos dos feijões PO e MP armazenados por seis meses, na concentração de 0,1 mg/mL, de acordo com instruções do fabricante.

3.6. Análise estatística

Os resultados da composição nutricional, potencial anti-inflamatório e anti-aterosclerótico foram analisados por meio da ANOVA com duas fontes de variação (cultivares de feijões e tempo de armazenamento) e a interação Cultivar*Tempo. Para resultados que apresentaram interação significativa a 5 % de probabilidade, a mesma foi desmembrada e nos efeitos que demonstraram variação significativa ($p<0,05$) com o tempo e equações de regressão foram ajustadas. A ANOVA foi considerada conclusiva para a variável qualitativa – Cultivar – ($p \leq 0,05$), uma vez que foram avaliados dois cultivares. A análise dos resultados foi realizada utilizado o software estatístico Statistical Analysis System (SAS – Institute Inc., North Carolina, USA, 1989), versão 9.2, licenciado pela Universidade Federal de Viçosa.

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4. ARTIGOS

4.3. ARTIGO 1

Tipo de artigo: artigo original.

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Commercial post-harvest storage did not impair nutritional composition of Carioca beans (*Phaseolus vulgaris* L.) and Glycosylated kaempferol can be a possible chemical marker

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Abstract

Most ‘carioca’ beans (*Phaseolus vulgaris L.*) have accelerated fast darkening during post-harvest storage, but little is known about the effect of commercial storage time on its nutritional composition. The aim of this study was to evaluate the effect of storage time (0, 3 and 6 months) on proximal and phytochemical composition of two carioca bean genotypes, fast darkening (BRS Pontal – PO) and slow darkening (BRSMG Madreperola – MP). The beans were stored in sealed polypropylene packaging at room temperature (22 ± 3 ^{0}C), without humidity control (< 65% of relative humidity). After six months, only lipids content was affected by storage for both cultivars (PO: 1.4±0.03 to 1.2±0.4; MP: 1.3±0.1 to 1.2±0.2 g/100 g) ($p = 0.007$). Glycosylated kaempferol was identified as a chemical marker for differentiation along time of storage for PO and MP beans. Thus, commercial time of storage (six months) was suitable to preserve macronutrients, dietary fiber, minerals, total phenolics and antioxidant capacity of the evaluated carioca beans.

Keywords: post-harvest storage, common bean, proximal composition, glycosylated kaempferol.

1. Introduction

Common beans are widely appreciated in developing countries for their affordability, long shelf life, and protein content, which is comparable to animal protein (CASTRO GUERRERO *et al.*, 2016). Brazil is the largest common bean (*Phaseolus vulgaris* L.) producer and consumer of the world (MAPA, 2016). Approximately 40 cultivars of beans are cultivated in Brazil, including Carioca, Preto, Roxo and Mulatinho (MESQUITA *et al.*, 2006). Among these cultivars, the Carioca variety leads the consumer's preference, and accounts for 52% of the total cultivated area (MAPA, 2016). This type of bean has a cream background with tan stripe and presents fast darkening, which prevents storage for long periods, since the bean quality may be affected. In this sense, new Carioca cultivars has been developed to postpone the darkening (FARIA *et al.*, 2004) and hardening over time (TEIXEIRA; SIQUEIRA; BASSINELLO, 2011).

The new cultivars are environmentally advantageous due to higher yield, productivity and less use of harmful chemicals (BRASIL, 2010). The acceptance and commercialization of a new cultivar depends on consumer's demands, such as size, shape, cooking time and color of grains (BASSINELLO *et al.*, 2003). Although not all old beans are dark or hard to cook, consumers consider the darkening as a characteristic of aging and hardening. The agronomical and nutritional quality are determined by the genotype and by storage conditions during the growing and development of beans (TEIXEIRA; SIQUEIRA; BASSINELLO, 2011).

The loss of quality during storage is characterized by increase of degree of hardness, higher cooking time, and changes on flavor and color (RIOS; ABREU; CORRÊA, 2002). High temperature and relative humidity, in the presence of light and oxygen, impair the water absorption and modify the phenolic content (RIOS; ABREU; CORRÊA, 2003). Temperatures higher than 25 ºC and relative humidity above 65% contribute to hard-to-cook phenomenon (HTC) (JUNK-KNIEVEL; VANDENBERG; BETT, 2007), which is characterized by textural defect, resulting in longer time of cooking to obtain suitable texture, thus increasing cost of energy to cook as well as nutritional losses (BRESSANI, 1983).

The time of storage can impair the water absorption and digestibility of cultivars and increase the phenolic content, which is followed by darkening over time (RIOS; ABREU; CORRÊA, 2003). Therefore, time of storage is important to preserve the grain quality, since aging of beans can increase the darkening and hardening by hydration defects (RIOS; ABREU; CORRÊA, 2003), loss of phytate (HINCKS; STANLEY, 1986; NASAR-ABBAS *et al.*, 2008), and changes in phenolic compound metabolism, resulting in lignification (HINCKS; STANLEY, 1986; NASAR-ABBAS *et al.*, 2008).

Nutritionally, storage time is related to loss of phytate, tannins, phenolics and lipids (CHIARADIA; GOMES, 1987). Large protein molecules can suffer natural enzymatic hydrolysis, forming small polypeptides during storage, which may alter the peptides functional properties (HOHLBERG; STANLEY, 1987). It has been demonstrated that after six months of storage under environmental conditions, common beans may present HTC defects, increased cooking time, and reduced *in vitro* protein digestibility (NYAKUNI *et al.*, 2008). For most carioca beans, only 2 months of storage would be enough to observe hardness, even under controlled conditions (5 °C) (COELHO *et al.*, 2013).

There are studies that have evaluated the effects of temperature and relative humidity during storage on the physicochemical and technological properties of common beans. However, there is little information about the effect of storage period on nutritional quality of carioca beans. In this sense, the aim of the study was to evaluate the effect of commercial time of storage (up to six months) at natural conditions (room temperature without humidity control) on nutritional and phytochemical composition of two carioca beans with contrasting darkening and hardening resistance during storage.

2. Methods

2.1. Carioca bean processing

BRS Pontal (PO) and BRSMG Madreperola (MP) are Common bean (*Phaseolus vulgaris* L.) genotypes of carioca commercial type. Beans were cultivated and harvested

in Brazil in the Fall of 2013, and provided by EMBRAPA Rice and Beans (Santo Antônio de Goiás, GO, Brazil). These beans were selectively bred by crossing 2 lineages (MP: AN 512666-0 and AN 730031, and for PO: BZ3836/FEB 166/NA 910523) (DEL PELOSO *et al.*, 2004; CARNEIRO *et al.*, 2012). The best lineages were selected after six generations based on productivity potential and resistance to diseases. For BRSMG Madreperola (MP) the grain aspects were also considered, resulting in a cultivar with slow darkening and late hardening during storage. After harvesting, drying and cleaning procedures, beans grains were stored for three and six months, in sealed polypropylene packaging at room temperature (22 ± 3 ºC) and < 65% of relative humidity, simulating conventional conditions of storage. The storage procedure aimed to reproduce commercial storage period usually found or indicated by Brazilian market. After storage, the beans were cooked in a pressure cooker (1:2 bean: water; 120 ºC, 50 min), dried (air-circulating oven for 8 h/60 °C) and ground (sieve of 600 µm, size 30 mesh; Grinder Vertical Rotor MA 090 CFT, Marconi Equipment, Piracicaba, SP, BR) to obtain the whole flour, which was packed under vacuum and stored at - 20 ºC until analysis.

2.2. Proximal composition

The proximal composition was evaluated using the analytical procedures recommended by the Association of Official Analytical Chemists – AOAC (2011).

2.2.1. Moisture content, ash and macronutrients

All analyses were performed in triplicate using the whole bean flour. Moisture content was determined at 105º C, by gravimetry using 10 g of sample. Ash content was measured using 2.5 g of sample by calcination in muffle furnace. Micro Kjeldahl method was used to determine protein content through total nitrogen quantification (conversion factor for vegetable proteins: 5.75 (BRASIL, 2001), using 50 mg of sample. Ten grams were used to determine lipid in Soxhlet, using ethyl ether as a solvent, by 8 h under reflux.

Carbohydrate content was calculated by the difference between total of the sample and the sum of content of protein, lipid, dietary fiber, water and ash. For energy value the conversion factors of 4, 4 and 9 kcal/g for carbohydrates, proteins and lipids, respectively were considered (FRARY; JOHNSON, 2007).

2.2.2. Resistant starch

The resistant starch (RS) content was determined according to the AACC (2001) and AOAC (2000) using the RS assay kit supplied by Megazyme International Ireland Ltd., Wicklow, Ireland. Briefly, enzymatic hydrolysis of non-resistant starch (NRS) was performed through the simultaneous action of pancreatic α-amylase (10 mg/mL) and amyloglucosidase (3 U/mL) by incubating the sample for 16 h at 37 °C. Subsequently, the NRS was separated by centrifugation, and the pellet containing the RS was purified with ethanol and solubilized with 2 mol/L KOH. The concentration of RS was measured at 510 nm, and the content was expressed as g/100 g common bean flour on a dry weight basis. The results were obtained in duplicate and were presented as the mean ± the standard deviation.

2.2.3. Dietary fiber

The levels of total dietary fiber, soluble and insoluble fraction were determined through the enzymatic gravimetric method (method 900.02) (AOAC, 2011), in duplicate. For enzymatic hydrolysis α-amylase, protease and thermoresistant amyloglucosidase were used (Total dietary fiber assay kit, Sigma®). The total dietary fiber content was obtained through the sum between the soluble and insoluble fractions.

2.2.4. Mineral content

The contents of iron, calcium, zinc, copper, manganese, magnesium, sodium, potassium and phosphorus were determined according to Gomes and Oliveira (2011), in triplicate. All glassware used was previously demineralized in 10% nitric acid solution for 12 h and dried in air circulation oven. Samples (1 g) were weighed in triplicate in digestion tubes and 10 mL of concentrated nitric acid was added. Subsequently, samples were heated in digester block with exhaustion at initial temperature of 80 ºC. Temperature was increased progressively up to 160° C, and exposed to this temperature for 16 h, until a clear solution formed. Tubes were cooled at room temperature and the content transferred to a 50 mL volumetric flask tube, washed with deionized water and agitated in vortex to avoid losses. This solution was used for reading of mineral content using plasma emission spectrophotometry (Perkin Elmer-Optima DV 3300, Norwalk, USA).

2.2.5. Phytate

Phytate content was determined in triplicate by ion exchange and spectrophotometry according to Latta and Eskin (1980), with modifications by Ellis and Morris (1986). The results were expressed in mg /100 g of flour (mg/100g), using a standard curve obtained by different concentrations of phytic acid ($y = -0.0038x + 0.4963$; $R^2 = 0.994$).

2.2.5. Phenolic compounds

Total Phenolics

The total phenolics were determined using Folin–Ciocalteu reagent (SINGLETON; ORTHOFER; LAMUELA-RAVENTÓS, 1999). Phenolic compounds were extracted by horizontal agitation (100 rpm, 20 min) in 60% methanol solution (1 g of sample in 20 mL of solution) and filtered in vacuum pump (3 µm of porosity). Aliquots of 100 µL of extract

were mixed with 1 mL Folin–Ciocalteu (0.25 N) with vortex for 10 seconds. After 3 min, 1 mL of sodium carbonate 7.5% was added. After 7 min, 5 mL of distilled water were added and the mixture was kept under agitation for 30 min, at room temperature. The absorbance was read in a spectrophotometer (Thermo scientific, Evolution 60S, USA) at 765 nm. The results were expressed in mg of gallic acid equivalents per gram of flour (mgGAE/g), using a standard curve obtained by different concentrations of gallic acid ($y = 30.763x + 0.0818$; $R^2 = 0.996$).

Condensed tannins

The content of total tannins was evaluated by Vanillin reaction (BURNS, 1971), with modifications of Maxson and Rooney (1972) and Price, Van Scoyoc and Butler (1978). Tannins were extracted from samples (200 mg) in 1% HCl in methanol, under agitation (80 rpm, 20 min, 30ºC). After extraction, solutions were centrifuged (3,000 rpm, 20 min). Supernatant (1 mL) was mixed with 2.5 mL of 1% solution of vanillin in methanol and 2.5 mL of 8% HCl in methanol. After 20 min under rest, absorbance was measured at 500 nm using a spectrophotometer (Multiskan 60 Themo Scientific®) compared to the blank. A standard curve of catechin was used ($y = 0.5002x + 0.0052$; $R^2 = 0.999$) and results were expressed as mg equivalent of catechin per 100 grams of sample. The standard curve was prepared using a stock solution of catechin in methanol (1 mg/mL) and aliquots of 2.5, 5, 10, 20, 25 and 50 mL were removed from the concentrated solution and volume adjusted to 100 mL with methanol in a volumetric flask. The reaction was performed under the same conditions described for the sample.

Phenolic compounds identification

The determination of the concentration of phenolic compounds in foods was performed using the Folin Ciocalteu reagent as described by Singleton et al. (1999). For

this analysis, samples (400 mg) were extracted with 10 mL of methanolic solution:water:cloridic acid (50:48;2). The material was sonicated for 1 h and the extract was centrifuged (8000 rpm). An aliquot (1.5 mL) was filtered in PTFE 0.22 µm and placed in vials to UPLC-QTOF analysis, using a Acquity UPLC (Waters) system, coupled to a quadrupole-time-of-flight (QTOF, Waters). Chromatography was performed in a Waters Acquity UPLC BEH column (150 x 2,1 mm, 1,7µm), fixed temperature of 40 ˚C, mobile phases were water with 0,1% formic acid (A) and acetonitrile with 0,1% formic acid (B), gradient of 0-15 min (2-95%) of B; 15.1-17 min (100%) of B; 17.1-19.1 min (2%) of B, flow of 0.4 mL/min and injection volume of 5 µl. The ESI mode was acquired at 110-1180 Da, temperature stationary source at 120 ˚C, desolvation temperature at 350 ˚C, gas desolvation flow of 350 L/h, extraction cone of 0.5 V, capillary voltage of 2.6 kV. The cone tension of 35 V, collision energy of 5eV was used as low scan. For high scan cone tension was 35 V with power ramp of collision of 20-40 eV. Encephalin leucine was used as lock mass. Acquisition mode was MS^E. The instrument was controlled by Masslynx 4.1 software (Waters corportation). Chromatograms were analyzed by MarkerLynx software for discrimination of potential chemical markers. Method conditions were: retention time of 1.2 to 7.0 min, 120 to 1200 Da, and level 5 of noise elimination. Markers were identified by correlation between retention time (rT) and mass/charge ratio (*m/z*) resultant from the elution order and normalized based on each ion intensity. Centered on the average was the scale used to form the principal component analysis (PCA). Through the comparison between each sample was generated the orthogonal projections to latent structures-discriminant analysis OPLS-DA for construction of S-plot and distinction of potential markers. S-plot graphic shows the ions of variation in each sample or time of storage of cultivar.

2.2.4. Antioxidant capacity

Antioxidant activity was evaluated by oxygen radical absorbance capacity (ORAC) according to Prior et al. (2003), using 20 µL 6-hydroxy-2,5,7,8-tetramethylchroman 2-carboxylic acid, 1–8 µM final concentration (Trolox standard; y =

0.9905x - 8.172 $R^2 = 0.989$) and whole bean flour (1 mg). ORAC results were expressed in mmol TE/g of dry flour.

2.3. Statistical Analysis

Results were presented as the mean ± SD. Samples from a unique source of beans, at time zero and six months of storage, were suitably homogenized, and each experiment was performed in triplicate. Data were subjected to one-way analysis of variance (ANOVA) with two sources of variation (bean and time) and bean*time interaction. Significant interactions ($P < 0.05$) were fragmented and the effect of time was tested by models of linear and quadratic equation in function of time of storage for each bean. Differences between bean cultivars were tested by t-Student test at 5% of probability. The software Statistical Analysis System (SAS-Institute Inc., North Carolina, USA, 1989), version 9.3 was used. For characterization of phenolic compounds principal component analysis (PCA) was used and orthogonal projections to conduct latent structures-discriminant analysis (OPLS-DA) for S-plot graphics construction and distinction of potential markers.

3. Results and discussion

3.1. Macronutrients and dietary fiber

The evaluated carioca beans, BRS Pontal and BRSMG Madreperola, showed no statistical differences for time of storage or bean cultivar ($p>0.05$) for moisture, proteins, total dietary fiber, soluble and insoluble fiber and carbohydrates (**Figure 1**). Regardless of the Carioca bean cultivar, only lipids decreased with storage time (from zero to six months) ($p = 0.007$) (**Figure 1C**). However, this reduction was an inexpressive nutrition loss (PO: 1.4±0.03 to 1.2±0.4; MP: 1.3±0.1 to 1.2±0.2 g/100 g), mainly considering that

common bean present lower lipid content. In agreement with others studies, the main effect of storage seems to be on the lipid content, in which a reduction may be observed, even in controlled conditions (SAWAZAKI *et al.*, 1985; RIBEIRO; PRUDENCIO-FERREIRA; MIYAGUI, 2005). Lipids are an important parameter of bean quality during storage, since they are the most reactive components under adverse conditions (ANWAR; CHATHA; HUSSAIN, 2007). Degradation of lipids results in changes of free fatty acid content, increasing the sensitivity to fatty acid oxidation (ANWAR; CHATHA; HUSSAIN, 2007).

The initial moisture content (**Figure 1A**) was similar to the values observed for carioca beans stored under normal and modified conditions (VANIER *et al.*, 2014). For Pinto bean, similar to carioca, the lower moisture content (12 and 14%) was maintained throughout the storage period (360 days), while samples with higher initial moisture content (16-20%) decreased during initial weeks of storage (RANI *et al.*, 2013). Moisture content around 14% may increase the microbial stability of common beans during storage (UEBERSAX; SIDDIQ, 2012). In addition, low moisture content (< 16%) is important to reduce the hardness during storage.

As aforementioned, no effect on protein was observed for PO and MP beans submitted to short storage (six months) (**Figure 1B**). The same was verified by Rani *et al* (2013) for beans stored by four months. However, Coelho *et al.* (2013) observed strong decrease in the percentage of soluble proteins along period of storage under natural environmental conditions (3, 6, 9 and 12 months). Vanier *et al.* (2014) observed decrease in the protein content after 12 months, but under high relative humidity (75 ± 5%). As suggested by Vanier *et al.* (2014) the reduction of proteins may be associated with the decrease in the tissue pH, which is inversely proportional to time of storage, and may partially contribute to the development of the HTC defect.

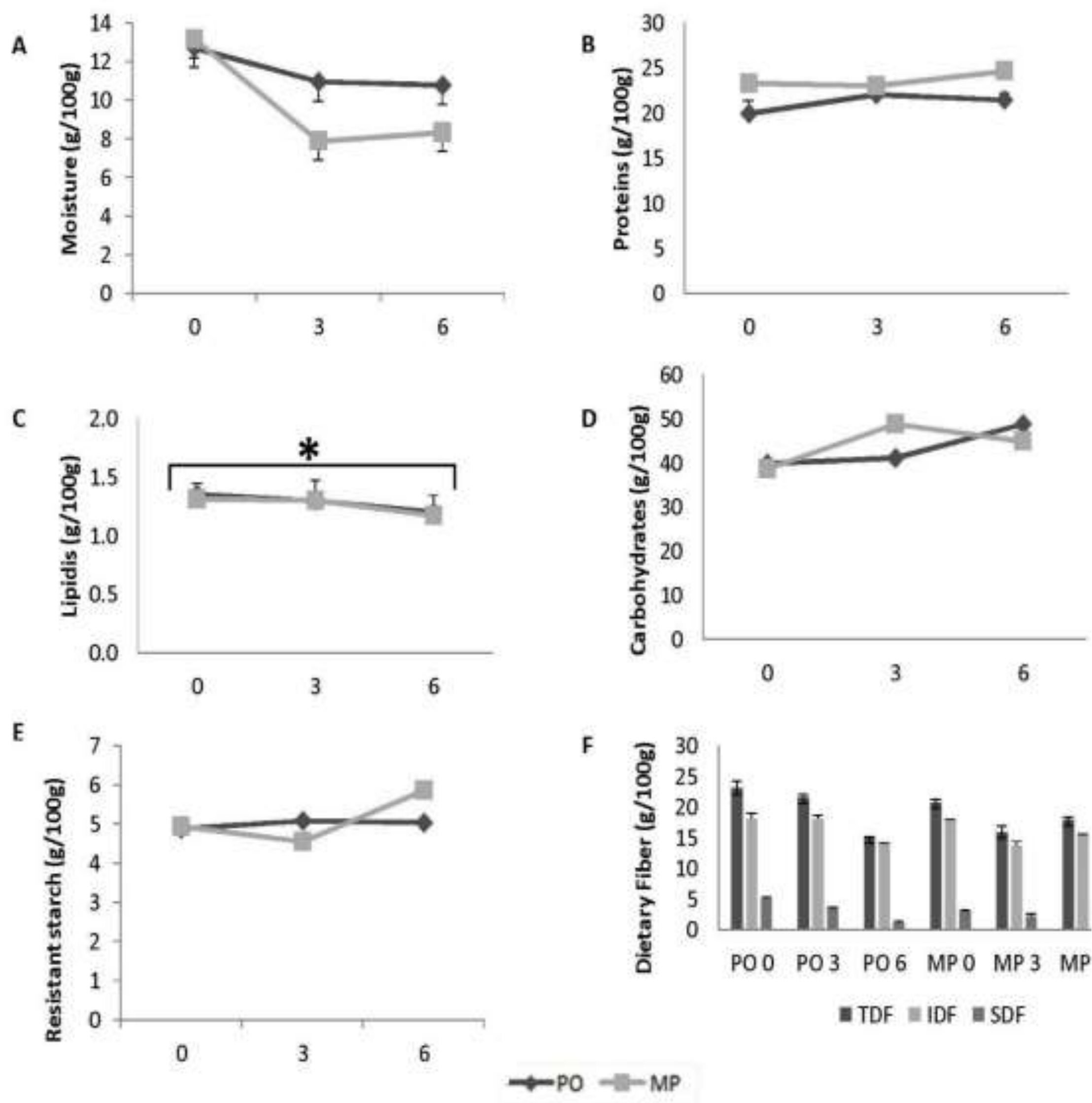

Figure 1. Macronutrients composition of Carioca beans stored up to six months. **PO**: BRS Pontal; **MP**: BRSMG Madreperola; TDF: total dietary fiber; IDF: insoluble dietary fiber; SDF: soluble dietary fiber. Numbers 0, 3 and 6 indicate months of storage. Results are present by g/100g of dry sample. *Indicate significant difference along storage time, by ANOVA with bean*time interaction of variation ($p < 0.05$). No difference was observed between bean cultivars, tested by t-Student test at 5% of probability.

Even though chemical or structural alteration of starch is independent of the environmental conditions (HOHLBERG; STANLEY, 1987; SIQUEIRA *et al.*, 2014), the carbohydrate content (**Figure 1D**) of PO and MP remained unchanged, which is a positive point to prevent hardness. Changes of this macronutrient due to starch retrogradation contributes to hardness during storage (SHEHATA, 1992; FERREIRA *et al.*, 2016) and loss of capacity to form a thick broth, which is an important quality aspect for American Latin consumers (GARCIA; LAJOLO, 1994; SÁNCHEZ-ARTEAGA *et al.*, 2015). Regarding the resistant starch, no effect due to storage time or differences among beans were observed ($p > 0.05$) (**Figure 1E**). The resistant starch content of PO and MP varied from 4.95 ± 0.14 to 5.85 ± 0.22 and 4.89 ± 0.09 to 5.03 ± 0.06 g/100 g, respectively, from 0 to 6 months of storage. These results were higher than the values observed by Costa *et al.* (2006), of 2.33 ± 1.23 mg/100g for cooked carioca beans. These differences can be attributed to methods of analysis and cooking process, since Costa *et al.* cooked the beans after soaking for a period of 16 h (1:2 w/v). Other factors may be related to these differences such as genetic, crop management or environmental aspects.

No change on dietary fiber content was observed in the present study (**Figure 1F**), and PO and MP beans presented lower soluble/insoluble dietary fiber mean rate ($P < 0.75$), indicating low propensity to hardening, since this rate when higher than 0.75 can contribute to hardness during storage (REYES-MORENO; PAREDEZ-LÓPEZ, 1993). In addition, storage period and temperature seem to be crucial to observe alterations on fiber content, as verified in beans stored for prolonged time and higher temperature (36 ºC; 24 months) (REYES-MORENO; PAREDEZ-LÓPEZ, 1993).

3.2. Mineral content

The time of storage did not affect the ash content (**Figure 2A**), as also observed by Vanier *et al*. (2014) for beans stored under nitrogen-modified atmosphere or normal atmosphere (oxygen), even for longer storage conditions (up to 360 days, at 25º C). The storage conditions did not affect mineral content, which are shown in **Figures 2B to 2I**. Some variations are expected for minerals when environmental conditions are able to

promote membranes damage (SHEHATA, 1992; PARMAR *et al.*, 2017). Thus, the storage conditions used in the present study was suitable to preserve mineral content of the carioca beans.

However, some differences between cultivar were observed. At periods 0, 3 and 6 months, PO presented higher concentrations of manganese (30.3, 23.03 and 31.04% higher, respectively) (**Figure 2D**) ($p = 0.01$) while MP presented higher concentrations of phosphorus (14.6, 12.2 and 19.1% higher, respectively) ($p = 0.03$) (**Figure 2G**).

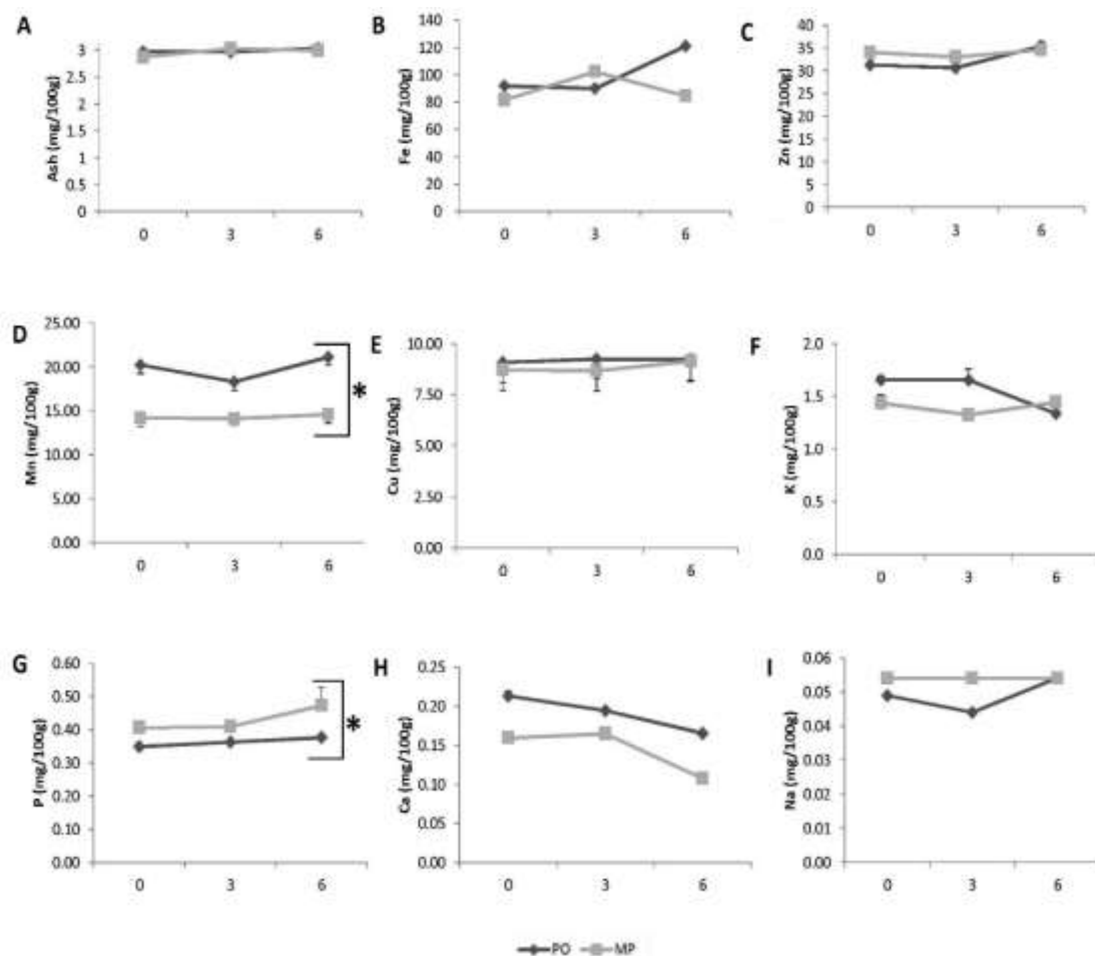

Figure 2. Ash (A) Mineral content (B to I) of Carioca beans stored up to six months.

PO: BRS Pontal; **MP**: BRSMG Madreperola; Fe: iron; Zn: zinc; Mn: manganese; Cu: cupper; K: potassium; P: phosphorus; Ca: calcium; Na: sodium. Numbers 0, 3 and 6 indicate months of storage. *Indicate significant difference between beans ($p < 0.05$) tested by t-Student test at 5% of probability. No effect of time of storage was observed, by ANOVA with bean*time interaction of variation ($p < 0.05$).

3.3. Phytochemicals and antioxidant capacity

No statistical difference among beans was observed for total phenolic content (P = 0.18) (**Figure 3A**). MP presented higher tannin content (38.3, 24.12 and 25.8%; at 0, 3 and 6 months respectively; P = 0.042) (**Figure 3B**), while PO presented higher antioxidant capacity (9.8, 8.9 and 6.58%; at 0, 3 and 6 months respectively; P = 0.01). For phytate no difference was observed (P > 0.05) between cultivar throughout storage (**Figure 3C**). Overall the few differences between beans, the commercial storage did not affect phytochemicals content, and consequently antioxidant capacity (P > 0.05).

PO and MP at time zero presented total phenolic content (1.32±0.2 and 1.25±0.04, respectively) similar to that observed for Pontal bean (without storage) (1.33±0.15 mg EqGA/g) (DIAS *et al.*, 2015). Usually a reduction of the total phenolic content of stored beans is observed where oxidation is associated with darkening and hardening during storage (VANIER *et al.*, 2014). According to Vanier *et al.* (2014), the total phenolic is probably the same content, but these compounds may form complexes with proteins and other macromolecules during cell stress making them less extractable and, thus, a lower amount of phenolics by Folin-Ciocalteau method is detected, as analyzed in the present study. In addition, monomeric phenolics, such as tannins, are relatively polar and soluble in water, exhibiting mobility in environments with high water activity (AW; SWANSON, 1985), which did not happen in the present study, since relative humidity was lower than 65%.

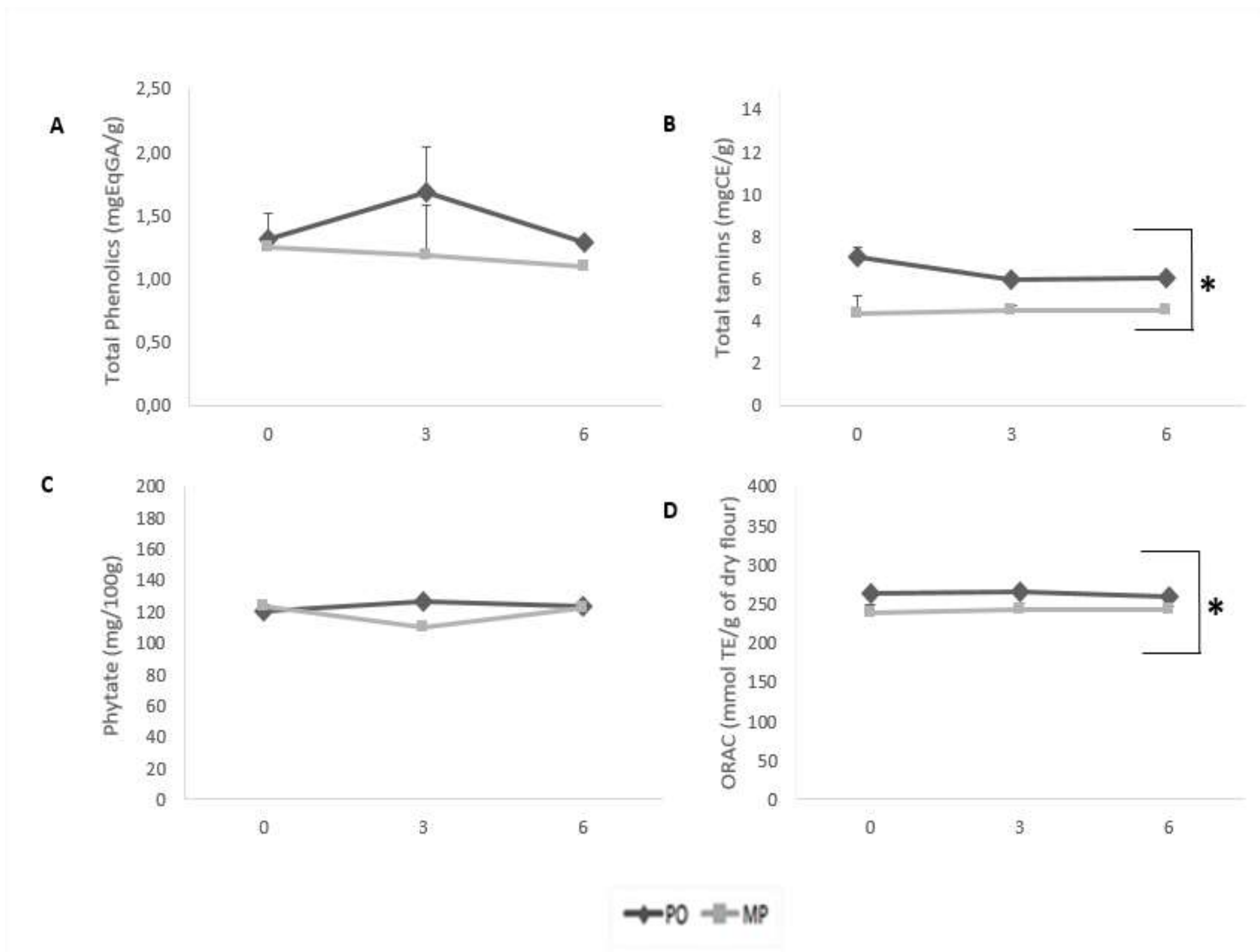

Figure 3. Phytochemical and antioxidant capacity of Carioca beans stored up to six months.

PO: BRS Pontal; **MP**: BRSMG Madreperola; **ORAC**: oxygen radical absorbance capacity; **GAE**: gallic acid equivalents; EC: equivalent of catechin. Numbers 0, 3 and 6 indicate months of storage. *Indicate significant difference between beans ($p < 0.05$) tested by t-Student test at 5% of probability. No effect of time of storage was observed by ANOVA with bean*time interaction of variation ($p < 0.05$).

According to PCA the separation between MP and PO indicates that these cultivars differ in phytochemical composition, which can be due to intensity or absence of characteristic ions, including primary metabolites (**Figure 4A** and **4B**). In addition, phytochemical composition of Pontal bean seems to be affected by storage time, since there is a separation at PC2 (12%) related to 0, 3 and 6 months of storage (**Figure 4A**). The slight difference among time of storage for MP at PC2 and the almost absence of variation at PC1 (57%) shows a constant phytochemical composition during the period of storage analyzed (**Figure 2**). The OPLS-DA comparison shows through S-plot graphic that citric acid, catechin and glycosylated kaempferol were the representative chemical markers for chemical differentiation between the varieties (**Figure 4C**).

The concentration of glycosylated kaempferol increased throughout storage (0 to 6 months) for both bean cultivars, constituting a chemical marker for differentiation along time storage (**Figures 5A** and **5B**). **Figures 6A** and **6B** show the extract's chromatograms and mass spectrums, respectively.

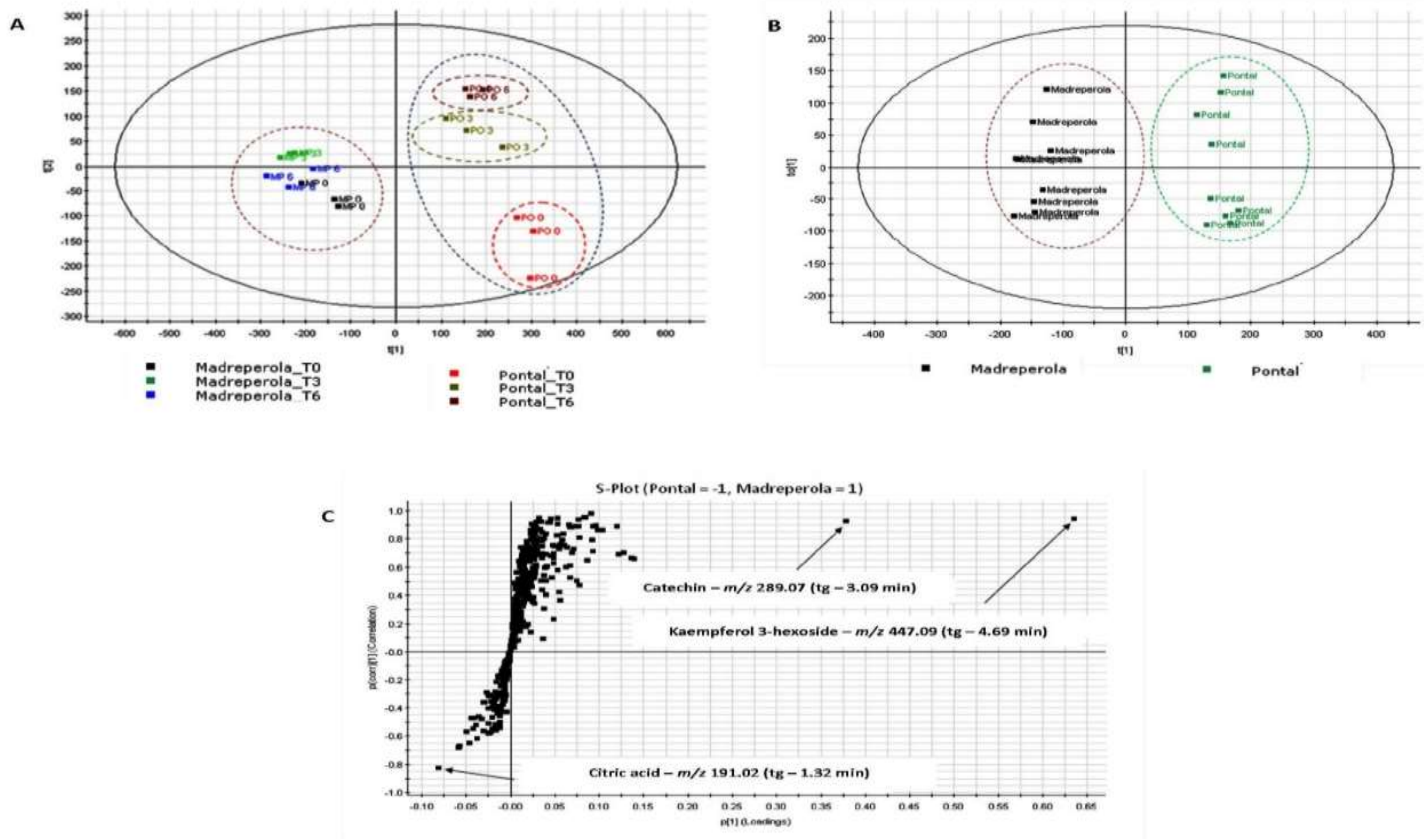

Figure 4. Principal component analysis (PCA) showing phytochemical composition of Carioca beans stored up to six months.

(A) Storage time effect. (B) Beans comparison. (C) Representative chemical markers. T0, T3 and T6 means months of storage zero, three and six months, respectively.

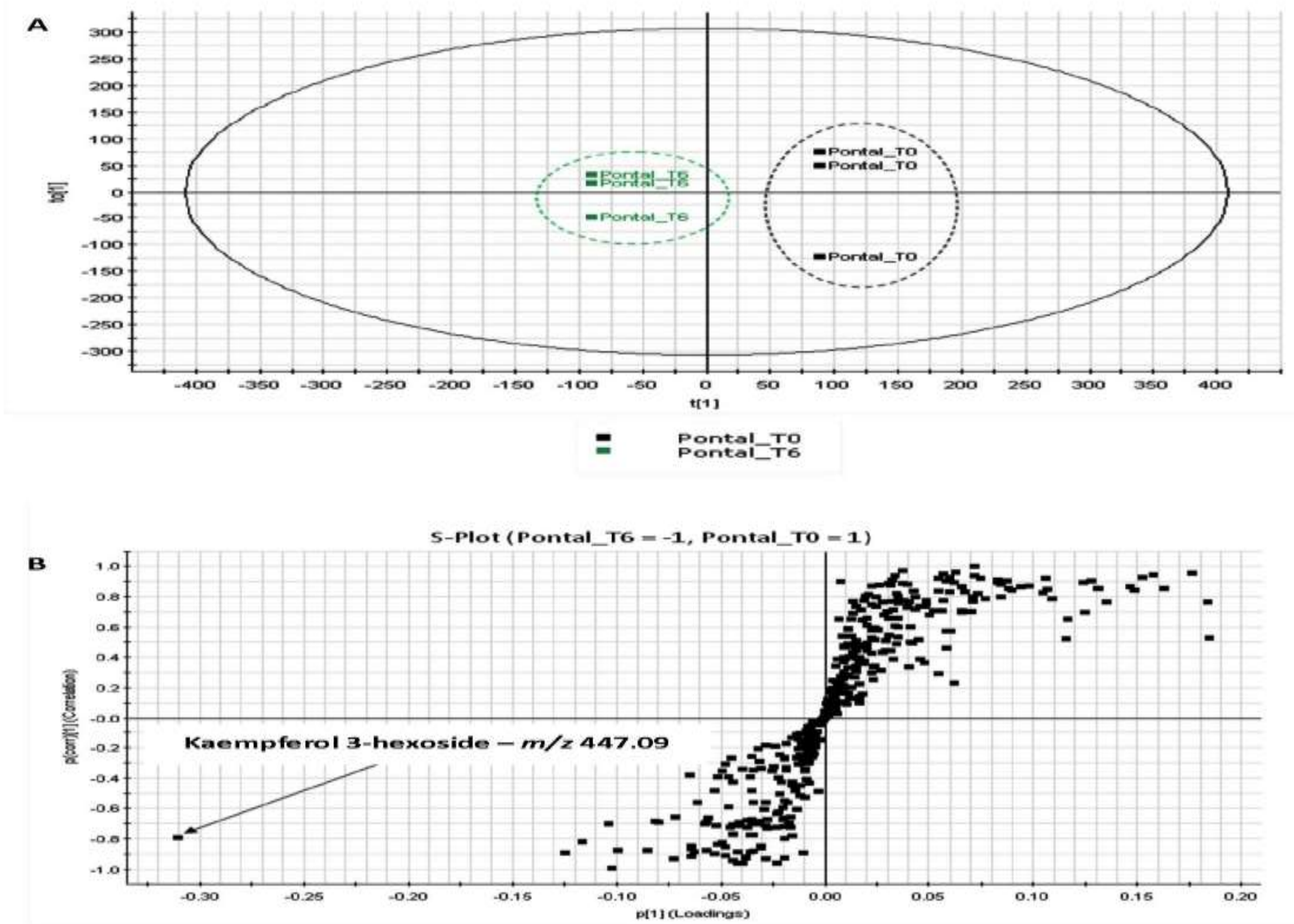

Figure 5. Principal component analysis (PCA) showing effect of six months of storage on BRS Pontal bean phytochemicals (A) and representative chemical marker (B). 0, T3 and T6 means months of storage zero, three and six months, respectively.

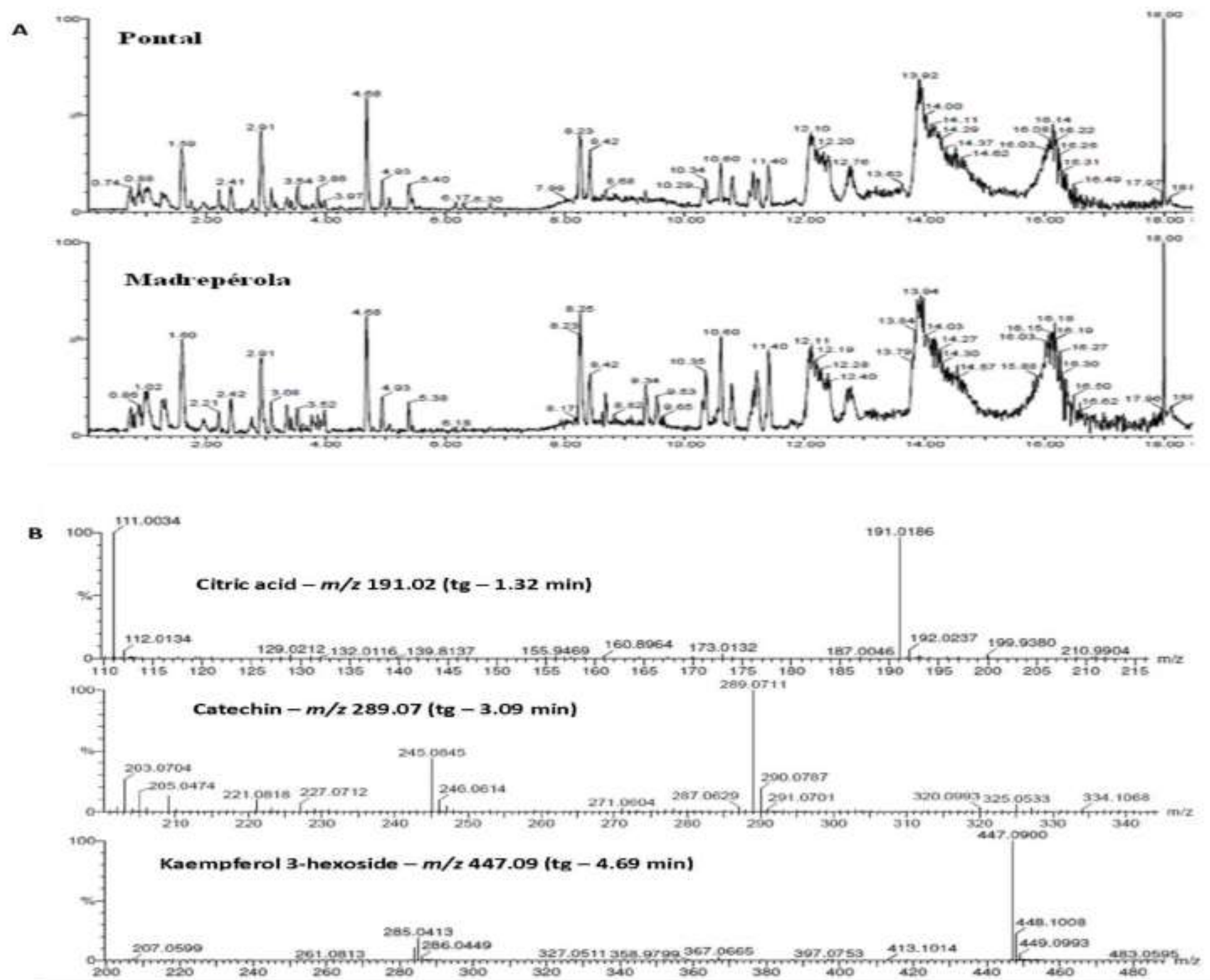

Figure 6. Extracts chromatograms and mass spectrums for Pontal (A) and Madreperola (B).

In the present study the phytochemical analysis were performed in the whole grain after storage and cooking process, based on consumer's preparation habits. However, most of the available studies perform these determinations in the coat or cotyledon. Ranilla, Genovese and Lajolo (2007), for example, evaluated 25 Brazilian cultivars of raw *Phaseolus Vulgaris* L, including BRS Pontal, stored up to one year at 4 °C and relative humidity of 85%. Unfortunately kaempferol data is not yet available for BRS Pontal and phenolic content was analyzed in the seed coat and cotyledon, separately. Mojica *et al.* (2015) also evaluated coat of raw carioca beans, including BRS Pontal, regarding the phenolic composition. These previous studies demonstrated that carioca beans present high levels of kaempferol glycosides (RANILLA; GENOVESE; LAJOLO, 2007), in addition to other flavonol glycosides, such as quercetin 3-O-glucoside and myricetin 3-O-glucoside (MOJICA *et al.*, 2015).

In the present study, stored and fresh cooked whole carioca beans presented higher antioxidant capacity (251.7±11.4 mmol TE/g of dried flour, on average) than seed coats of raw and fresh BRS Pontal (209.9±28.2 mmol TE/g), evaluated by the same method (MOJICA *et al.*, 2015).

Some market class of dry beans, such as pinto, reds and carioca present postharvest seed coat darkening, which results in considerable economic loss due undesirable decline in the visual quality that consumers associate with prolonged cooking time (SIQUEIRA *et al.*, 2014). It is suggested that the hardening and/or darkening phenomena is initiated by complex reactions activated inside the grains, involving different cell components such as cell wall polymers, phenolics, starch, protein and enzymes (BERRIOS; SWANSON; CHEONGH, 1988). In addition, environmental (climate, crop season and soil) conditions and intrinsic characteristics contribute to the degree and speed at which grains develop these phenomena (RIBEIRO; PRUDENCIO-FERREIRA; MIYAGUI, 2005). The postharvest darkening includes a combination of environmental, genetics and chemical changes. Darkening is accelerated by exposure to light, high temperature and humidity during postharvest carioca bean storage. Therefore, assessment of commercial storage conditions is important to ensure nutritional and sensory quality. As observed from this study, commercial storage time (six months), even under uncontrolled relative humidity and ambient temperature (22 ± 3 ºC) was able to preserve important nutrients and phytochemicals, as well as the antioxidant capacity of PO and MP carioca beans. We

emphasize that under these conditions, even PO which is less resistant to darkening and hardening during storage than MP, no significant changes were observed in the evaluated parameters.

4. Conclusion

This is the first study showing that the commercial storage time did not impair the nutritional and phytochemical composition of carioca beans with contrast to darkening resistance and hardening during storage. Glycosylated kaempferol was identified as a potential chemical marker to differentiate throughout storage time between PO and MP beans. Storage for up to six month under room temperature without relative humidity control is suitable to retain nutritional quality of BRSMG Madreperola and BRS Pontal carioca beans.

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6.2. ARTIGO 2

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Postharvest storage of Carioca bean (*Phaseolus vulgaris* L.) did not impair inhibition of inflammation in lipopolysaccharide-induced human THP-1 macrophage-like cells

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Storage can affect the nutritional quality of common bean (*Phaseolus vulgaris*), but little is known about the postharvest effect of storage on inflammation. The aim of this work was to evaluate, *in vitro*, the effect of postharvest storage-time on inflammation by Carioca beans, Madreperola (MP) and Pontal (PO), stored (0, 3 and 6 months), cooked and subjected to pepsin-pancreatin simulated gastrointestinal digestion. Anti-inflammatory potential was investigated in human THP-1 macrophage-like cells. Commercial storage time did not affect protein concentration, degree of hydrolysis, hydropathicity or antioxidant capacity. All PO hydrolysates reduced tumour necrosis factor-α (TNF- α) (31% on average). MP hydrolysates decreased interleukin 1β (IL-1β) (PO 0: 0.1 mg/mL, 20.7%; PO 0 and 6: 5 mg/mL, 24.8 and 48.2%, respectively) and prostaglandin E-2 (PGE-2) (PO 0: all concentrations, 18% on average; PO 6: 0.1 mg/mL, 18.6%). Carioca beans inhibited inflammation and commercial storage time did not impair physicochemical or biological properties.

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1. Introduction

Common bean (*Phaseolus vulgaris* L.) is widely consumed and culturally important to human nutrition (FAO, 2015). Globally, postharvest losses of bean quality are extensive and impact the bioavailability of nutrients and consumer acceptability (Uebersax & Siddiq, 2012). Postharvest changes due to prolonged and adverse storage conditions, such as high temperature (>25 °C) and relative humidity (RH) (>65%), can promote darkening and hardening of common beans affecting their agronomical, sensory and nutritional aspects (Siqueira, Bassinello, Malgaresi, Pereira, & Fernandes, 2016).

The suitable period of storage depends on environmental conditions (mainly temperature and humidity) and initial moisture of beans (Rani, Chelladurai, Jayas, White, & Kavitha-Abirami, 2013). Long time storage increases hydration defects (Rios, Abreu, & Corrêa, 2003), losses of phytates and lignification due to changes in phenolic metabolism (Nasar-Abbas et al., 2008), resulting in hardening and darkening. In addition, large proteins such as phaseolin are vulnerable to enzymatic and non-enzymatic hydrolysis, which release small polypeptides and aromatic amino acids that contribute to hardening of beans due to the formation of insoluble polymers in the middle lamella of the bean cells (Hohlberg & Stanley, 1987).

Carioca bean is a variety cultivated in Brazil that undergoes fast darkening after harvesting (Faria et al., 2004). This phenomenon prevents its storage over long periods, since consumers consider darkening a characteristic of aging and hardening (Siqueira et al., 2016). This variety has been modified by selective breeding to delay darkening (Faria et al., 2004) and hardening upon storage (Siqueira et al., 2016). However, it has been demonstrated that after six months of storage under ambient conditions, common beans may present hard-to-cook defects, increase cooking time, and reduce *in vitro* protein digestibility (Nyakuni et al., 2008). For Carioca beans, only 2 months of storage is enough to observe hardness, even under control conditions (5 °C) (Coelho, Prudencio, Christ, Sampaio, & Schoeninger, 2013).

Carioca beans present high antioxidant capacity (Mojica, Meyer, Berhow, & González de Mejía, 2015) and may reduce the pro-inflammatory state linked to Non Communicable Chronic Diseases (NCCD) (Carrasco-Castilla et al., 2012; Luna-Vital, Mojica, González de Mejía, Mendoza, & Loarca-Piña, 2014; Oseguera-Toledo, Gonzalez de Mejía, Dia, & Amaya-Llano, 2011; Rui, Boye, Simpson, & Prasher, 2012). It has been found that polyphenolic compounds provide health benefits, and others bioactive compounds, such as bioactive peptides, may contribute to decreasing the risk of obesity, hypercholesterolemia, diabetes mellitus, cardiovascular disease and cancer (Hayat, Ahmad, Masud, Ahmed, & Bashir, 2014; Luna-Vital et al., 2014). Bean enzymatic hydrolysis produces hydrolysates and peptides with anti-inflammatory (Oseguera-Toledo et al., 2011) and antioxidant activities (Carrasco-Castilla et al., 2012; Oseguera-Toledo et al., 2011) that could inhibit the chronic inflammatory process initiated by human macrophages. Macrophages are the major source of pro-inflammatory cytokines, and several markers can be used to measure chronic inflammation, such as tumour necrosis factor-α (TNF-α), interleukins (IL), prostaglandin E-2 (PGE-2), and others. TNF-α has a pivotal role in the progression of inflammation since it induces the production of IL-1β, among other pro-inflammatory cytokines (Lefkowitz, Lefkowitz, & Lefkowitz, 2001) and increases PGE-2 (Williams, Mann, & DuBois, 1999).

Although Carioca beans are widely consumed by the Brazilian population, no studies have been reported on the effect of commercial storage time (up to six months) on their functional properties. Most of the studies have focused on the hard-to-cook-beans and beans stored for long periods, aiming to produce functional ingredients mainly for use by the food industry, but not for direct human consumption, due to the physicochemical and nutritional changes. Therefore, we highlighted the importance to evaluate the effect of commercial storage time (up to six months) on functional properties of bioactive peptides, since Carioca beans with extended storage time are poorly consumed by humans. The aim of this investigation was to compare the effect of fresh and commercially stored (up to six months) Carioca bean varieties on the physicochemical and biological properties of their peptides produced after gastrointestinal digestion; also, their potential to inhibit markers of inflammation in lipopolysaccharide (LPS)-induced human THP-1 macrophage-like cells. This is a novel scientific contribution, and it is also important since people consume Carioca beans stored for up to six months; longer storage times provide beans not suggested for direct human consumption.

2. Materials and methods

2.1. Materials

Two varieties of "Carioca" beans, BRS Pontal (PO) and BRSMG Madreperola (MP) (*Phaseolus vulgaris* L.), were cultivated and harvested in Brazil in the Fall of 2013 (kindly provided by EMBRAPA Rice and Bean, Santo Antônio de Goiás, GO, Brazil). PO and MP were chosen based on their different responses to resist changes during storage conditions. Both of them were selectively bred by EMBRAPA Rice and Bean by crossing 2 lineages (MP: AN 512666-0 and AN 730031, and for PO: BZ3836//FEB 166/NA 910523). The best lineages were selected after six generations based on productivity potential and resistance to diseases. In addition, the grain aspects were also considered for MP beans resulting in a cultivar with both late darkening and late hardening during storage. Porcine pepsin (EC 3.4.23.1) and pancreatin (8xUSP, a mixture of digestive enzymes produced by the exocrine cells of the porcine pancreas, EC 232-468-9), were purchased from Sigma Aldrich (St. Louis, MO, USA). Human acute monocytic leukaemia cell line (THP-1) (TIB-2002™) from American Type Culture Collection (ATCC®, Manassas, VA, USA). Fetal bovine serum from Invitrogen (Grand Island, NY, USA). DC™ (detergent compatible) protein assay (500-0112), Precision Plus Protein™ Dual Colour Standards (1610374) and Polypeptide SDS-PAGE Standard (1610326) were purchased from Bio-Rad (Hercules, CA, USA). Streptomycin/penicillin and sodium pyruvate were purchased from Cellgro (Manassas, VA, USA). Sodium nitrite, sulfanilamide, N-1-(naphthyl) ethylenediamine-diHCl, lipopolysaccharide (LPS from *Escherichia coli* O55:B5), phorbol 12-myristate 13-acetate (PMA) and cellulose acetate membrane 500 Da molecular weight cut-off (Z367982) were

purchased from Sigma-Aldrich®. Human EIA kits from Cayman Chemical® (Ann Arbor, MI, USA) to analyse IL-1 β (#583311), and PGE-2 (#514010), and from Abcam (Cambridge, MA, USA) for TNF-α (#ab46087); dipeptidyl peptidase IV (DPPIV)-Glo protease assay kit (#G8350) from Promega (Madison, WI, USA) and COX-2 inhibitor screening kit (#K547-100) from BioVision Incorporated (Milpitas, CA, USA).

2.2. *Common bean storage and processing*

After harvesting, the beans were stored at room temperature (22 ± 3 °C), without humidity control (<65%) and evaluated at three and six months; time zero was used as control. After each period the beans were cooked under pressure (1:2 bean: water; 120 °C, 50 min), dried (air-circulating oven for 8 h/60 °C) and ground (sieve of 600 μm, size 30 mesh; Grinder Vertical Rotor MA 090 CFT, Marconi Equipment, Piracicaba, SP, BR). The resulting flour was packed under vacuum and stored at −20 °C until analysis.

2.3. *Simulated gastrointestinal digestion*

In order to simulate gastrointestinal digestion, pepsin-pancreatin hydrolysis was used as described by Megías et al. (2004). Briefly, a sequential enzyme digestion was carried out for two hours with pepsin (pepsin/bean flour 1:20, pH 2.0) plus two hours with pancreatin (pancreatin/bean flour 1:20, pH 7.5) at 35 °C, in triplicate. The hydrolysis was stopped by heating at 75 °C for 20 min. The hydrolysates were centrifuged at 20,000 *g* for 15 min at 4 °C, dialysed to remove salts (cellulose acetate membrane 500 Da molecular weight cut-off, Sigma-Aldrich) and freeze dried in a LabConco FreeZone Freeze dry system (Kansas City, MO, USA). Samples were stored at −20 °C until analysis.

2.4. *Determination of degree of hydrolysis (DH) and soluble protein*

DH was determined, in triplicate, as described by Cabra, Arreguin, Vazquez-Duhalt, and Farres (2007). The percentage of the dissolved protein after precipitation with 0.2 M trichloroacetic acid (TCA) was compared to the total dissolved common bean flour protein (100%). This was obtained after complete hydrolysis with 2 M sulphuric acid at 100 °C for 4 h. Detergent compatible (DC™)-protein assay (#500-0112; Bio-Rad®) was used to determine protein concentration, according to the manufacturer's instructions at 630 nm using a bovine serum albumin (BSA) standard curve: $y = 0.0002x - 0.0048$; $R^2 = 0.999$. Soluble protein concentration was expressed in percent related to the total amount of complete hydrolysate.

2.5. *Gel electrophoresis analysis SDS-PAGE of common bean flour and peptide fractions*

PO and MP proteins were analysed, in duplicate, by sodium dodecyl sulphate–polyacrylamide gel electrophoresis analysis (SDS-PAGE). Samples were diluted (1:1, v/v) in Laemmli buffer 5% of β-mercaptoethanol, pH 6.8 (Bio-Rad, 161–0737), boiled for 5 min, and 20 μg of protein was loaded in pre-cast (4–20%) gradient polyacrylamide Tris–HCl gels. Standard dual colour protein markers (10–250 kDa) were used to calculate molecular mass of separated proteins. Constant voltage of 200 V for 35 min was applied; the gels were stained with Simply Blue Safe Stain overnight and de-stained with water (three times-5 min each).

Peptide size present in PO and MP hydrolysates were analysed, in duplicate, as described by Rocha, Hernandez, Chang, and González de Mejía (2014) with modifications. Samples were diluted in tricine sample buffer (5% BME; pH 6.8; 1:2 sample: buffer) and the polypeptide SDS-PAGE standard (1.4–26.6 kDa) diluted 1:1 (v/v) in the same buffer. The gels were run using 16.5% Tris-Tricine polypeptide ready gels (Bio-Rad®) and a 1X tris-tricine SDS running buffer, for 90 min at 125 V constant. The gels were washed with water (three times-5 min each), stained overnight and de-stained for 2 h with water, changing the water each 30 min. All gels were visualized using the GL 4000 Pro Imaging System.

2.6. *Peptides separation*

The hydrolysates were passed through size exclusion chromatography (SEC), in duplicate, and the peptides were separated based on molecular mass, using a gel filtration column (30 cm × 10 mm, 13 μm average particle size) packed with Superdex 30 prep grade resin with a size exclusion limit of 10 kDa (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The hydrolysates (20 mg/mL) were eluted using 20 mM Tris–HCl buffer pH 7.5 and flow rate was set in 0.5 mL/min. Peaks were detected using GE AKTA prime plus system (GE Healthcare Life Sciences) at 280 nm. The fractions were collected every 3 min using an automated fraction collector attached to the GE AKTA System. The protein content of fractions was determined by DC™ protein assay.

2.7. *Biochemical properties of peptide fractions and complete hydrolysates*

To confirm the biochemical properties of the peptide fractions, dipeptidyl peptidase-IV (DPP-IV) activity, cyclooxygenase-2 (COX-2) inhibitor screening assay and antioxidant activity were performed in triplicate, using the fractions at concentration of 1 mg of protein/mL. DPP-IV activity was evaluated by DPP-IV Glo Protease assay (G8351, Promega), according to Mojica, Chen, and González de Mejía (2015). COX-2 activity was measured using a COX-2 inhibitor screening assay, following the manufacturer's instructions (BioVision, K547-100). Per cent inhibition was calculated from the blank and enzyme control for each sample. Antioxidant activity was evaluated by oxygen radical absorbance capacity (ORAC) according to Prior et al. (2003), using 20 μL 6-hydroxy-2,5,7,8-tetramethylchroman 2-carboxylic acid, 1–8 μM final concentration (Trolox standard; $y = 0.9905x - 8.172$ $R^2 = 0.989$) and peptides fraction (at 1 mg of protein/mL). The same method was used to evaluate antioxidant capacity of complete hydrolysates at concentration of 1 mg/mL (freeze-dried hydrolysates diluted in 75 mM phosphate buffer, pH 7.4). ORAC results were expressed in mmol TE/mg of protein for peptides fractions and mmol TE/g for complete dry hydrolysates.

Nitric oxide (NO) radical scavenging capacity of complete hydrolysates was determined using sodium nitroprusside (SNP) as the NO donor (Green et al., 1982). Briefly, 10 mM SNP solution was incubated with hydrolysates (1 mg of dry hydrolysate/mL) in PBS at 25 °C. After 150 min, 0.5 mL of incubated solution

was mixed with 0.5 mL of Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dichloride and 3% phosphoric acid). The absorbance was immediately measured at 546 nm. Results were expressed as % inhibition in relation to PBS, used as control.

Hydropathicity of complete hydrolysates (50 mg dry hydrolysate/mL) was determined in duplicate according to Dia, Bringe, and González de Mejía (2014), using a hydrophobic interaction chromatography column and 1 mL phenyl Sepharose high performance column (0.7 × 2.5 cm; 34 µm average particle size). The peptides angiotensin (H = 0.36, RT = 3.82 min), Bowman Birk Inhibitor (H = –0.159; RT = 3.41 min) and a synthetic peptide from common bean (KKSSG; H = –1.96; RT = 2.95 min) were used to construct a standard curve of hydropathicity ($y = 2.6919x - 9.72$; $R^2 = 0.926$). ProtParam Expasy® toll (http://web.expasy.org/protparam/, accessed 13.02.15) was used to calculate the Grand Average of Hydropathicity (GRAVY), which indicates the solubility of the proteins according to the sum of hydropathicity values of all amino acids in the sequence divided by the protein length. Positive scores indicate more hydrophobic amino acids, and negative scores more hydrophilic amino acids (Kyte & Doolittle, 1982).

2.8. *Characterization of the peptide fractions*

The peptide fractions from pepsin-pancreatin hydrolysates obtained after gel filtration were analysed by high-performance liquid chromatography–electrospray-ionization-mass spectrometry (HPLC–ESI–MS) using a Q-tof Ultima mass spectrometer (Waters, Milford, USA), equipped with an Alliance 2795 HPLC system, according to Mojica et al. (2015). Only peaks with intensity higher than 50%, and the sequences with more than 70% of probability of identity were analysed using the MassLynx 4.1V software (Waters). The presence of bioactive sequences in the main common bean proteins was confirmed using UniProt database from BLAST® tool using the keyword "*Phaseolus vulgaris* L" to filter by organism (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi, accessed 12.03.15). Query covers below 60% were not included. The potential biological activity of the peptides was predicted by using BIOPEP database (http://www.uwm.edu.pl/biochemia, accessed 12.03.15). Peptide structures were predicted using PepDraw tool (http://www.tulane.edu/~biochem/WW/PepDraw/, accessed 12.03.15). The amino acids were presented in one letter nomenclature.

2.9. *Anti-inflammatory potential of common bean hydrolysates*

THP-1 cells, a human monocytic-derived cell line, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% penicillin/streptomycin, 1% sodium pyruvate and 10% foetal bovine serum at 37 °C in 5% CO_2/95% air using a CO_2 Jacketed Incubator (NuAIRE DH Autoflow, Plymouth, MN, USA). Phorbol 12-myristate 13-acetate (PMA) was added at a concentration of 162 nM to promote differentiation of THP-1 cells into macrophages (Takashiba et al., 1999). Macrophage differentiation was allowed to occur for 24 h and confirmed by cell morphology and total adhesion to the plate. Human THP-1 macrophages-like were seeded at a density of 1,000,000 cells per 2 mL in a 6-well plate. The efficiency of THP-1 cells growing in the presence of sterile-filtered hydrolysates (0.1 to 5 mg/mL for 24 h) was assessed by Aqueous Solution CellTiter® 96 One Proliferation Assay Kit. THP-1 cells were treated with LPS (1 µg/mL) to induce inflammation and the hydrolysates (0.1 mg/mL, 1 mg/mL, 2.5 mg/mL and 5 mg/mL) for 24 h. LPS plus PBS, and PBS were used as positive and negative controls, respectively. The treatments were performed in duplicate. After 24 h of treatment, the growth medium were collected and frozen at –80 °C until analysis.

Prostaglandin-2 (PGE-2) was measured using a PGE-2 EIA monoclonal kit following the manufacturer's instructions (Cayman Chemical). Briefly, 50 µL of diluted (1:500, v/v) cell supernatant was plated in a 96-well goat anti-mouse IgG coated plate and incubated for 18 h at 4 °C. After incubation, the plate was washed using the provided washing buffer, the colour was developed by adding 200 µL of Ellman's reagent and shaking the plate for 60–90 min in the dark. The amount of PGE-2 was calculated using the generated PGE-2 standard curve ($y = -1.1 \ln(x) + 4.2078$, $R^2 = 0.998$). TNF-α and IL-1β were measured in the cell culture supernatant using commercially available assays from Cayman Chemical® and Abcam, respectively, following the manufacturer's instructions. The cell culture supernatant was diluted 1:100 (v/v) for TNF-α and 1:50 (v/v) for IL-1β (sample: buffer). The amount of TNF-α ($y = 0.7991x - 2.0792$, $R^2 = 0.998$), and IL-1β ($y = 0.0066x + 0.0249$, $R^2 = 0.999$) were calculated using their respective standard curves run at the same time as the treatments. Results were expressed in ng/mL.

2.10. *Statistical analysis*

Results are presented as the mean ± SD of at least two independent experiments, and each experiment was performed in triplicate. Data were subjected to one-way analysis of variance (ANOVA) with two sources of variation (bean and time) and bean*time interaction. Significant interactions ($P < 0.05$) were fragmented and the effect of time was tested by models of linear and quadratic equation in function of time of storage for each bean. The differences between types of bean were tested by t-Student test at 5% of probability. For effect of common bean hydrolysates on markers of inflammation, different doses were tested and compared to a negative (PBS) and a positive control (PBS and LPS) by contrast at 5% of probability. Principal component analysis was performed to obtain a graphic representation of hydrolysates and dose effect on inflammatory markers. The software Statistical Analysis System (SAS-Institute Inc., Cary, NC, USA, 1989), version 9.3 was used.

3. Results

3.1. *Electrophoretic profile and soluble protein concentrations from common bean after simulated gastrointestinal digestion*

Fig. 1A depicts the images of raw and cooked Carioca beans selected for this study. Fig. 1B shows the main proteins from both varieties before simulated gastrointestinal digestion for fresh and stored beans. PO and MP beans did not show different protein profiles at three or six months of storage, with

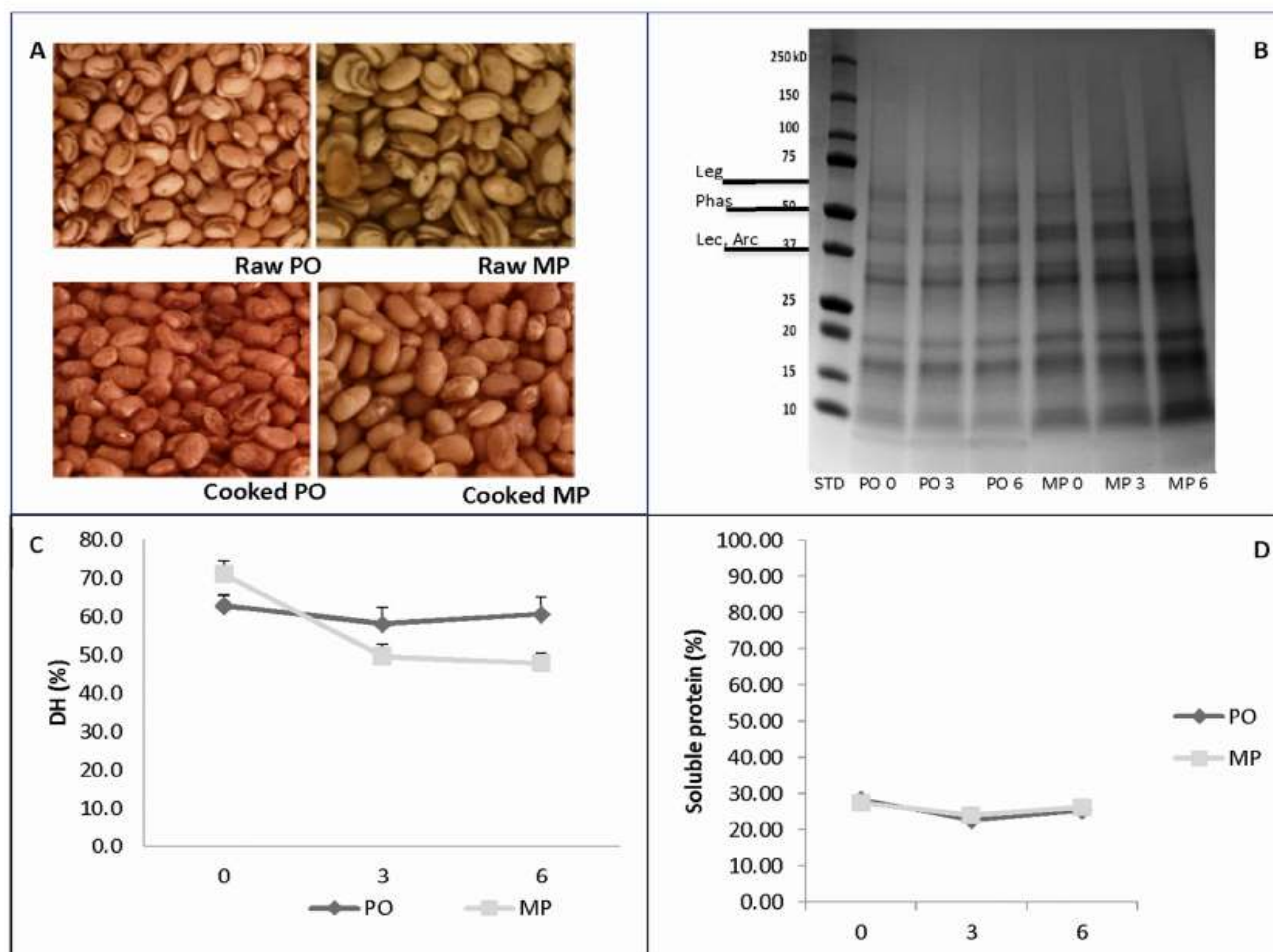

Fig. 1 – Image of raw and cooked Carioca beans (A). SDS–PAGE for whole bean flour (B). Degree of hydrolysis (C) and soluble protein concentration (D) of pepsin-pancreatin hydrolysates obtained from PO and MP at 0, 3 and 6 months of storage. STD, indicate molecular weights of proteins in the Dual Colour Standards BioRad (Hercules, CA, USA). PO, BRS Pontal and MP, BRSMG Madreperola. Numbers 0, 3 and 6 refer to time of storage. Lines show the major proteins from common beans: Leg, legumin; Phas, phaseolin; Lec, lectin; Arc, arcelin. Results are expressed as mean ± SD of three independent experiments. Significant differences between beans were evaluated by t-Student test at 5% of probability ($P < 0.05$). Time effect was evaluated by models of linear and quadratic equation in function of time of storage for each bean ($P < 0.05$).

the most intense bands between 18 and 30 kDa. The protein bands observed by SDS-PAGE between 50–75 kDa appeared to be legumin (Leg) (UniProtKB, 2015; Yin et al., 2011). Phaseolin (Phas), the main storage protein (40–50%) of common bean, was indicated by bands at 37–50 kDa, and lectin (Lec) and arcelin (Arc) at 25–37 kDa (UniProtKB, 2015). After pepsin–pancreatin hydrolysis of large PO and MP bean proteins, in all times of storage, were converted into smaller molecular peptides (<10 kDa) (Supplementary Fig. S1A). This was confirmed by polypeptide SDS-PAGE gel that showed peptides with molecular weight lower than 1.4 kDa (Supplementary Fig. S1B).

The mean yield after enzymatic hydrolysis, dialysis and freeze-dried process was 51.2 ± 4%. The storage time did not affect ($P > 0.05$) the degree of hydrolysis (DH) (PO: 60.4 ± 14.0%; MP: 56.1 ± 14.1%, in average) (Fig. 1C) or soluble protein concentration (PO: 26 ± 2%; MP: 25 ± 1%, in average) (Fig. 1D). No significant differences were observed among hydrolysates for these parameters ($P > 0.05$).

3.2. *Biochemical properties of hydrolysates and peptide fractions*

Fig. 2 shows the size exclusion chromatography (SEC) performed by gel filtration. A.1, A.2 and A.3 shows the peptide sequencing from HPLC–ESI–MSMS analysis. Antioxidant capacity (ORAC) of main peptide fraction (A.2) did not differ among bean samples ($P = 0.30$) (Table 1). MP peptide fractions were more powerful to inhibit DPP-IV (66.7 ± 12.0%, in average) than PO fractions (49.3 ± 8.1%, in average) ($P < 0.01$). Peptide fractions were less potent to inhibit COX-2 activity, wherein PO fractions (11.1 ± 1.6%, in average) presented higher inhibition than MP (0.9 ± 1.6, in average) ($P = 0.01$). No time effect was observed

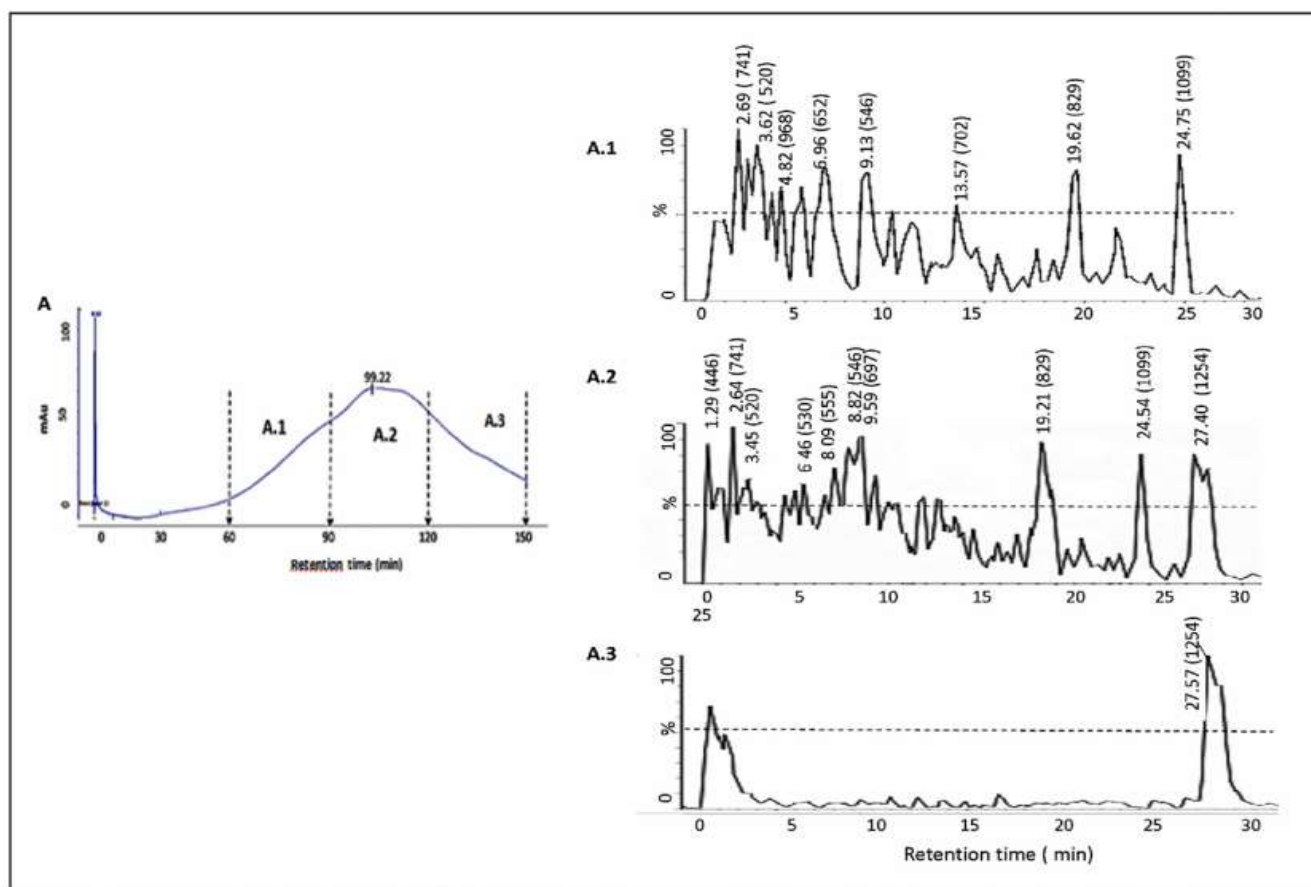

Fig. 2 – Schematic diagram showing the identification of peptides from PO and MP after simulated gastrointestinal digestion with pepsin-pancreatin (A). Hydrolysates were subjected in duplicate to size exclusion chromatography (SEC at 280 nm) (<10 kDa) and the major fractions were subjected to HPLC-ESI-MSMS analysis for sequence identification. Peak selected from each fraction (A.1, A.2 and A.3) for MS/MS analysis corresponded to at least 50% of the total net intensity and are represented by retention time followed by molecular weight.

($P > 0.05$) (Table 1). Complete hydrolysates did not affect DPP-IV and COX-2 activity (data not shown) ($P > 0.05$).

Antioxidant capacity evaluated by ORAC and NO inhibition of complete hydrolysates was not affected by storage-time (Table 2). However, MP presented higher ORAC than PO hydrolysates in all times of storage (88.9 ± 4.0 and 82.7 ± 3.3 mmol TE/g dry hydrolysate, respectively) ($P = 0.04$). No differences were observed for NO inhibition among bean samples ($P = 0.62$). Negative values were observed for total hydropathicity in the complete hydrolysate (ranging from

Table 1 – Biochemical properties of hydrolysates fractions of PO and MP beans stored for 3 and 6 months.

Hydrolysates	Fraction (RT[1])	ORAC (mmol TE/mg of protein)	DPP-IV inhibition (%)	COX-2 inhibition (%)
PO 0	A2 (90–120)	39.25 ± 0.12	41.25 ± 6.09	12.8 ± 0.5*
PO 3	A2 (90–120)	39.28 ± 0.03	57.54 ± 5.54	9.61 ± 1.1*
PO 6	A2 (90–120)	36.35 ± 0.28	19.07 ± 6.09	11.0 ± 2.1*
MP 0	A2 (90–120)	39.80 ± 0.11	55.82 ± 7.75*	0.36 ± 1.5
MP 3	A2 (90–120)	39.59 ± 0.10	79.58 ± 0.74*	−0.36 ± 1.0
MP 6	A2 (90–120)	39.10 ± 0.17	64.65 ± 1.11*	2.80 ± 1.4

RT[1], retention time of gel filtration for the main fraction, A2 (Fig. 2). PO, Pontal hydrolysates and MP, Madreperola hydrolysates before (0) and after 3 and 6 months of storage (3 and 6, respectively). ORAC, oxygen radical absorbance capacity; DPP-IV, dipeptidyl peptidase IV; COX-2, cyclooxygenase-2. A2: *Indicate significant differences between beans by t-Student test at 5% of probability ($P < 0.05$). No difference by time was observed ($P > 0.05$; ANOVA followed by models of linear and quadratic equation in function of time of storage for each bean).

Table 2 – Biochemical properties of complete hydrolysates of PO and MP beans stored for 3 and 6 months.

Hydrolysates	ORAC (mmol TE/g dry hydrolysates)	NO inhibition (%)	Hydropathicity Index
PO 0	84.88 ± 3.38*	86.10 ± 0.38	−0.80 ± 0.25
PO 3	78.10 ± 4.93*	81.24 ± 2.97	−1.25 ± 0.06
PO 6	85.24 ± 5.39*	83.22 ± 0.56	−0.49 ± 0.53
MP 0	92.31 ± 8.50	86.19 ± 1.65	−0.64 ± 0.51
MP 3	85.67 ± 3.05	88.28 ± 1.98	−1.00 ± 0.19
MP 6	88.99 ± 12.28	80.95 ± 0.41	−0.89 ± 0.08

PO, Pontal hydrolysates and MP, Madreperola hydrolysates before (0) and after 3 and 6 months of storage (3 and 6, respectively). ORAC, oxygen radical absorbance capacity; NO, nitric oxide inhibition.

* Indicate significant differences between beans by t-Student test at 5% of probability ($P < 0.05$). No difference by time was observed ($P > 0.05$; ANOVA followed by models of linear and quadratic equation in function of time of storage for each bean).

−1.25 ± 0.06 to −0.49 ± 0.53), without differences among beans and time of storage ($P > 0.05$) (Table 2).

3.3. *Peptide sequence and biological potential*

Table 3 presents the resulting peptides after hydrolysis, their biological potential and physicochemical characteristics. MP presented higher number of bioactive sequences (11 peptides) than PO hydrolysates (6 peptides). All bioactive sequences presented positive values of hydrophobicity and few sequences presented negative values (KLLALQKMRK and QTSTPLFS from MP 3 and MP 6, respectively). Antioxidative peptides were found in all hydrolysates, except in PO 6. Only PO 6 did not present the antioxidative peptide VELVGPK. Besides VELVGPK, PO 0 also presented the antioxidative sequence ALVPVGSKPK. Anti-inflammatory peptides (ubiquitin-mediated proteolysis activating peptide – UMPA) were found in PO 0 (LAMPV) and MP 3 (KLLALQKMRK). The most prevalent bioactivity was related to inhibition of angiotensin converting enzyme (ACE) and DDP-IV and stimulating of glucose uptake (glucose uptake stimulating peptide – GUSP) (Fig. 3).

3.4. *Effect of common bean hydrolysates on markers of inflammation*

Viability of human THP-1 macrophages-like cells was not affected by PO or MP hydrolysates at concentrations from 0.1 to 5 mg/mL ($P > 0.05$), with values of viability higher than 90% (data not shown). Fig. 4 presents the effect of PO and MP hydrolysates on TNF-α, IL-1β and PGE-2 produced by LPS-stimulated THP-1 human macrophages. The *in vitro* model showed that the anti-inflammatory properties of PO and MP hydrolysates were not affected by storage-time ($P > 0.05$). Comparing to positive control (C+), all concentrations of PO hydrolysates reduced TNF-α (around 31% on average) ($P < 0.05$) (Fig. 4A) and MP hydrolysates decreased IL-1β at 0.1 mg/mL (20.7%, time 0) and at 5 mg/mL (24.8% and 48.2%, time 0 and 6, respectively) ($P < 0.05$) (Fig. 4D). No significant changes were observed for PGE-2 after treatment with PO hydrolysates, but it decreased at all concentrations (18% on average) for MP 0, and at the lowest concentration for MP 6 (18.6%, 0.1 mg/mL) ($P < 0.05$) (Fig. 4F).

Principal component analysis and cluster analysis can be observed in Fig. 5. MP hydrolysates at times 0, 3 and 6 and doses 0.1, 2.5 and 5 were more effective to reduce IL-1 β, while PO hydrolysates from fresh (time 0) and stored beans (time 6) were more related to inhibit TNF-α production at doses of 0.1, 1.0 and 5 mg/mL.

4. Discussion

Brazil is the largest producer and consumer of common bean in the world. The favourite type is Carioca bean, which represents around 70% of the total consumption and 52% of the cultivated area in the country (Martino et al., 2012). Although beans have long shelf life, storage time accentuates the difference between quick darkening and darkening-resistant Carioca bean genotypes (Silva, Ramalho, Abreu, & Silva, 2008). It has been demonstrated that after six months of storage under ambient conditions, common beans may present hard-to-cook defects (Nyakuni et al., 2008). The development of hard-to-cook bean defects prevents its consumption due to the inability of cotyledons to soften sufficiently within a reasonable time during cooking, resulting in flatulence, low digestibility of macronutrients and negative changes in texture and palatability (Njoroge et al., 2015). Carioca bean type presents fast darkening of the tegument, impeding the storage over long periods (Siqueira et al., 2014). Consumers associate darkening to aging of grains and hard-to-cook beans. Due to the consumers preferences, some producers anticipate the harvest of Carioca bean and most of them avoid storage of the product for long periods (Faria et al., 2004). Postharvest storage time of common beans (*P. vulgaris* L.) can affect their sensory and nutritional quality. Common bean with prolonged storage is not usually consumed due to hard-to-cook defects and poor digestibility. In summary, Carioca beans are cultivated and harvested twice per year, and up to six months is the usual commercial self-life. In addition, little was known about the effect of commercial storage time (up to six months) on physicochemical and biological properties of peptides from Carioca bean. We have evaluated, for the first time, the effect of 3 and 6 months of commercial storage on physicochemical and biological properties of two Carioca beans: BRS-Pontal (PO) and BRSMG-Madreperola (MP) cooked and after simulated gastrointestinal digestion with pepsin-pancreatin.

Storage for 3 and 6 months did not alter the natural enzymatic hydrolysis as showed by degree of hydrolysis. DH

Table 3 – Bioactive peptides identified by HPLC-ESI-MS/MS in PO and MP hydrolysates fractions.

Beans	Fraction (RT¹)	RT²	Prob (%)	Molecular mass Da	Peptide sequence*	Biological activity	Parental protein	QC	IP	Net charge	Hydrophobicity kcal mol⁻¹	Hydropathicity Index
PO TIME 0	1 (60–90)	2.6	99.9	741.4	VELVGPK	ACE inhibitor, anti-amnesic, antioxidative, antithrombotic, DPP-IV inhibitor, GUSP, PRSM	Alpha and beta phaseolin	100	6.5	0	13.45	0.4
		6.9	96.6	530.3	LAMPV	ACE inhibitor, DPP IV inhibitor, UMPA,	Alpha and beta phaseolin	100	5.6	0	6.16	2.0
	2 (90–120)	2.6	99.5	995.6	ALVPVGSKPK	ACE inhibitor, DPP-IV inhibitor, antioxidative, GUSP	Arcelin and phytohemagglutinin	100	10.6	2	13.72	0.2
PO TIME 3	2 (60–90)	2.4	99.9	741.4	VELVGPK	ACE inhibitor, antioxidative, DPP-IV inhibitor, GUSP, PRSM, PEI;	Alpha and beta phaseolin	100	6.5	0	13.45	0.4
		11.7	62.5	627.3	SLPVLV	GUSP; DPP-IV inhibitor	Alpha and beta phaseolin	100	5.4	0	5.08	2.2
PO TIME 6	2 (90–120)	3.4	99.9	644.3	TRGVLV	ACE inhibitor; DPP-IV inhibitor; GUSP	Alpha-amylase inhibitor 2	83	11.1	1	8.94	1.1
MP TIME 0	1 (60–90)	4.6	100	679.4	LSSLEM	DPP-IV inhibitor	Alpha and beta type phaseolin	83	3.2	−1	9.2	0.7
		6.5	99.9	861.5	LVSCLVDL	DPP-IV inhibitor, GUSP	Phytohemagglutinin	100	3.1	−1	7.3	2.2
	2 (90–120)	2.8	100	741.4	VELVGPK	ACE inhibitor, antioxidative, DPP-IV, GUSP, PRSM, PEI	Alpha and beta type phaseolin	100	6.5	0	13.4	0.4
MP TIME 3	1 (60–90)	2.5	100	741.4	VELVGPK	ACE inhibitor, antioxidative, DPP-IV, GUSP, PRSM, PEI	Alpha and beta phaseolin	100	6.5	0	13.4	0.4
		4.5	100	679.3	LSSLEM	DPP-IV inhibitor	Alpha and beta phaseolin	83	3.1	−1	9.2	0.7
	2 (90–120)	2.7	100	741.4	VELVGPK	ACE inhibitor, antioxidative, DPP-IV, GUSP, PRSM, PEI	Alpha and beta phaseolin	100	6.5	0	13.4	0.4
		32.2	93.0	1228.7	KLLALQKMRK	ACE inhibitor, UMPA, DPP-IV, GUSP, PRSM, PEI	Alpha and beta phaseolin	50	11.8	4	14.9	−0.4
MP TIME 6	1 (60–90)	6.2	81.6	861.4	LVTTTVDL	GUSP, DPP-IV inhibitor	Phytohemagglutinin	87	3.1	−1	8.8	1.3
		6.2	93.4	880.4	QTSTPLFS	ACE inhibitor, DPP-IV inhibitor	Alpha-amylase inhibitor 1	75	5.3	0	7.2	−0.1
	2 (90–120)	2.6	99.9	741.4	VELVGPK	ACE inhibitor, antioxidative, DPP-IV, GUSP, PRSM, PEI	Alpha and beta phaseolin	100	6.5	0	13.4	0.4
		3.4	99.9	644.3	TRGVLV	ACE inhibitor, DPP-IV inhibitor GUSP	Alpha-amylase inhibitor 2	83	11.1	1	8.9	1.1

PO, BRS Pontal hydrolysates; MP, BRSMG Madreperola hydrolysates; RT¹, retention time of gel filtration (Fig. 2A); RT², retention time of HPLC-ESI-MSMS; Prob (%), probability of the peptide sequence; QC, query cover from BLAST® toll; IP, isoelectric point; Hydropathicity, by grand average of hydropathicity (GRAVY); ACE inhibitor, angiotensin-converting-enzyme inhibitor; DPP-IV inhibitor, dipeptidyl peptidase IV inhibitor; GUSP, glucose uptake stimulating peptide; PRSM, peptide regulating the stomach mucosal membrane activity; PEI, prolyl endopeptidase inhibitor; UMPA, ubiquitin-mediated proteolysis activating peptide; *Peptides sequenced by HPLC-ESI-MS/MS with intensity at least 50% and 70% of probability. Biological activities were obtained from the BIOPEP database; Highlighted and underlined portion of the sequence refer to part of the peptide with reported antioxidant and anti-inflammatory activity, respectively (BIOPEP database). Only sequences of main proteins of *Phaseolus vulgaris* L. are presented in the table and were confirmed with BLAST® tool (QC >60%). Physicochemical properties were determined using PepDraw and GRAVY using ExPASy – ProtParam. The amino acids are presented in one letter nomenclature.

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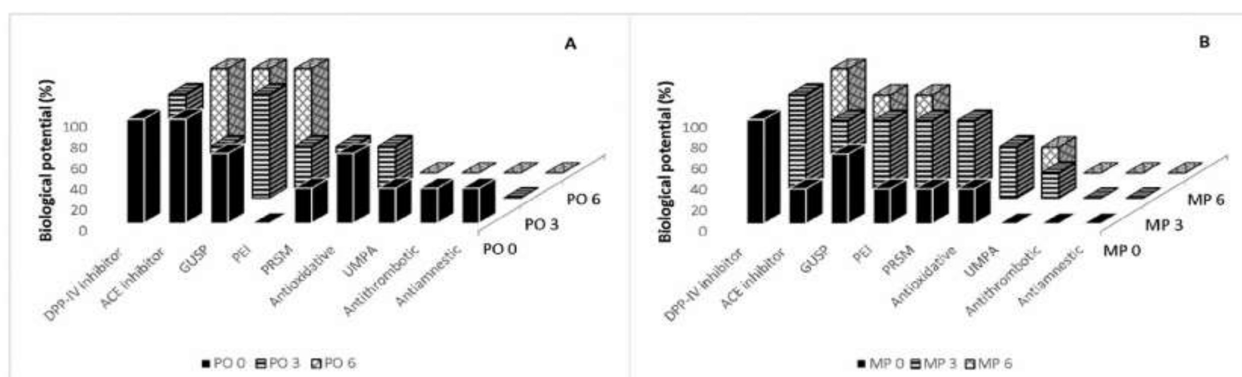

Fig. 3 – Percentage of potential biological activity of peptides from BRS Pontal (A) and BRSMG Madreperola (B) after simulated gastrointestinal digestion with pepsin-pancreatin. PO, BRS Pontal; MP, BRSMG Madreperola. Numbers 0, 3 and 6 refers to time of storage. Percentage is related to total number of peptides present in each hydrolysate. DPP-IV, dipeptidyl peptidase-IV inhibitor; ACE, angiotensin-converting-enzyme inhibitor; GUSP, glucose uptake stimulating peptide; PEI, prolyl endopeptidase inhibitor; PRSM, peptide regulating the stomach mucosal membrane activity; UMPA, ubiquitin-mediated proteolysis activating peptide.

(58.3 ± 8.6%, in average) was higher than that reported for raw (20%) and pre-cooked common beans (boiling for 15 min) (30%) (Mojica et al., 2015). Heat treatment may increase DH due to partial denaturation and sequential exposure of hydrophobic residues of globular structure proteins, increasing the susceptibility to proteolysis (Montoya et al., 2008). Our results for cooked beans confirmed that cooking time can be an additional factor to increase DH. Others factors include method of analysis, cultivars, time of hydrolysis and enzymes used in the *in vitro* digestion.

Storage-time did not affect antioxidant capacity or ability of peptide fractions to inhibit DPP-IV, although a minor effect was observed for COX-2 activity inhibition. Due to the higher protein content, peptide fractions presented higher antioxidant capacity than complete hydrolysate (38.89 ± 1.27 mmol TE/mg of protein vs. 0.321 ± 0.017 mmol TE/mg of protein, in average). ORAC values for PO and MP hydrolysates (321.59 ± 17.8 mmol TE/g of protein, in average) were lower than those observed for hydrolysates from pre-cooked pinto beans (*P. vulgaris* L), similar to Carioca beans, which presented around 450 mmol TE/g of protein (Mojica et al., 2015; Rui et al., 2012). Comparing to the original food matrix, hydrolysates presented higher ORAC (85.9 ± 4.7 mmol TE/100 g of dry hydrolysate, in average) than those observed for raw (5.06 to 12.90 mmol TE/100 g) and boiled (0.418 to 1.5 mmol TE/100 g) pinto beans, similar to Carioca beans (USDA, 2010). A high antioxidant capacity has been reported for hydrolysates due to the production of small peptides (Luna-Vital et al., 2014), and the presence of aromatic (Trp, Tyr, Phe) and basic amino acids (His, Arg) associated with proton donors (phenolic, indole and imidazole groups) (Sarmadi & Ismail, 2010).

The negative values of hydropathicity for PO and MP hydrolysates indicated the presence of peptides with more hydrophilic amino acids (Kyte & Doolittle, 1982). The production of peptides with a higher or less hydrophobicity, as well as the DH, depends on hydrolysis time, food matrix (Guzmán-Méndez, Jaramillo-Flores, Chel-Guerrero, & Betancur-Ancona, 2014), enzyme/substrate ratio, heat treatment (Luna-Vital et al., 2014) and number of aromatic acid residues (Guo, Kouzuma, & Yonekura, 2009). In our study, the sequencing of bioactive peptides showed sequences with hydrophobic character. It is important to increase interactions with cell membranes as well as the antioxidant capacity of peptides (Guzmán-Méndez et al., 2014). In fact, the hydrophilic character of complete hydrolysates did not affect antioxidant capacity, evaluated by ORAC and NO inhibition. In agreement with other findings, the antioxidant capacity was also independent of the hydrophilic character for African yam bean hydrolysates (Ajibola, Fashakin, Fagbemi, & Aluko, 2013), and pea hydrolysates (Pownall, Udenigwe, & Aluko, 2010).

Bioactive peptide sequences confirmed the antioxidant and anti-inflammatory potential of PO and MP hydrolysates. The antioxidative sequences in PO and MP hydrolysates were EL and KP, also described for other protein sources (Montoya-Rodríguez, Gómez-Favela, Reyes-Moreno, Milán-Carrillo, & González de Mejía, 2015). Bioactive sequences related to ubiquitin-mediated proteolysis activating peptide (UMPA) were LA, RA and WA (Li & Aluko, 2010; Montoya-Rodríguez et al., 2015; Turner, Du, & Varshavsky, 2000), wherein LA was found in PO and MP hydrolysates.

The mechanism of antioxidant action of peptides to decrease inflammation was related to the reduction of reactive oxygen species (ROS) (Hernández-Ledesma, Hsieh, & de Lumen, 2009). ROS stimulate the release of inflammatory mediators and recruitment of additional macrophages, contributing to chronic inflammation (Hernández-Ledesma et al., 2009). In this respect, the antioxidative peptides can be useful to improve the antioxidant defence and decrease the inflammatory process. UMPA peptide, found in PO and MP hydrolysates (time 0 and 3 months, respectively), accelerates its own absorption increasing the import capacity of cells (Turner et al., 2000). These properties of small compounds to stimulate its own uptake by ubiquitin system suggest a potential to regulate other ubiquitin-dependent pathways (Turner et al., 2000), such as in the control

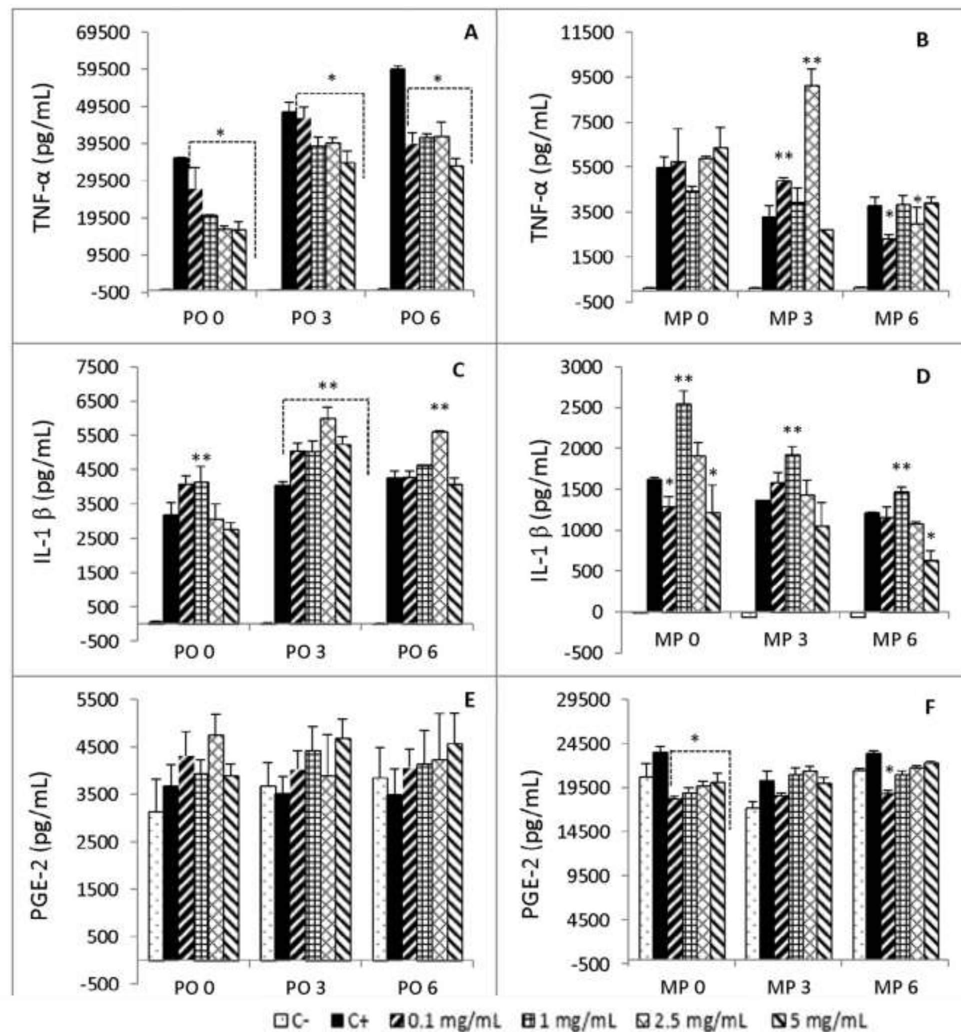

Fig. 4 – Effect of BRS Pontal and BRSMG Madreperola pepsin–pancreatin hydrolysates (0.1 mg/mL to 5 mg/mL) on tumour necrosis factor-α (TNF-α) secretion (A, B), interleukin-1β (IL-1β) (C, D) and prostaglandin E-2 (PGE-2) (E, F) secretion in LPS-stimulated THP-1 macrophages. PO, BRS Pontal; MP, BRSMG Madreperola. Numbers 0, 3 and 6 refer to storage time of storage. Treatments: C–, negative control (PBS); C+, positive control (PBS + LPS) and doses of hydrolysates: 0.1, 1.0, 2.5 and 5.0 mg/mL. *Indicate significant decrease and **indicate significant increase related to C+ ($P < 0.05$).

of inflammatory process by degradation of intracellular key proteins (Elliott, Zollner, & Boehncke, 2003).

We have demonstrated that bean storage time, up to six months, did not affect the efficacy of pepsin-pancreatin hydrolysates to decrease markers of inflammation in lipopolysaccharide (LPS)-induced human THP-1 macrophage-like cells. For some hydrolysates, even the lowest concentrations (0.1 mg/mL) decreased production of TNF-α (PO 0, 3 and 6, and MP 6), IL-1 β (MP 0) and PGE-2 (MP 0 and 6). THP-1 macrophages constitutively secrete high levels of PGE-2 as a consequence of the differentiation process by PMA, as observed by Schwende, Fitzke, Ambs, and Dieter (1996), which can explain the no statistical difference of PGE-2 observed for the negative and positive controls. Although antioxidative and anti-inflammatory peptides were not found in PO 6, its complete hydrolysate was able to inhibit TNF-α production. This effect can be related to other peptides not still recognized in the data bank and scientific literature, or also due to the fact that these antioxidative peptides were not considered in the analysis due to their low intensity in the HPLC–ESI–MSMS analysis (<50%). In addition, phenolic compounds interact non-covalently with storage proteins in the hydrolysates (Garcia-Mora et al., 2015) and may contribute to decrease ROS production and inflammation, since ROS increase TNF-α production by activation and translocation of NF-κB (Marín-García, 2014).

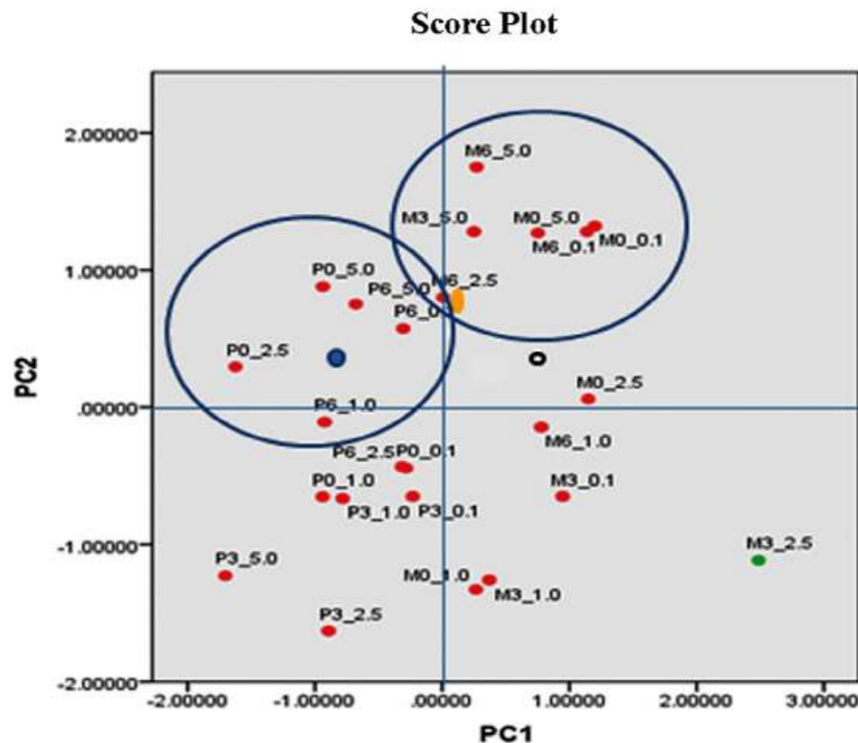

Fig. 5 – Principal component analysis score plot and clusters of PO and MP hydrolysates at 0.1 to 5 mg/mL. Score plots for the first two principal components PC1 (37.0%) and PC2 (24.6%). P, BRS Pontal; M, BRSMG Madreperola; TNF-alpha (●), tumour necrosis factor-alpha; IL-1β (●), interleukin-1β, PGE-2 (○), prostaglandin E-2. Numbers 0, 3 and 6 indicate the time of storage, followed by concentration of the hydrolysate.

Peptides are inactive within the protein sequence, but are released during protein hydrolysis (Di Bernardini et al., 2011). Furthermore, peptides produced depend on the site of the protein cleaved during enzymatic hydrolysis. Despite the differences in the cell model (human THP-1 macrophages vs. RAW-264.7 –mouse macrophages) and enzymatic hydrolysis (pepsin-pancreatin vs. alcalase), the anti-inflammatory effects also varied between beans for hydrolysates obtained after protein extraction of *P. vulgaris* L. (Pinto Durango and Negro 8025), which decreased PGE-2 production (Oseguera-Toledo et al., 2011). Anti-inflammatory potential of pepsin-pancreatin hydrolysates was likewise observed for other food matrices, such as hydrolysates from amaranth (reduction of TNF-α and PGE-2 THP-1 macrophages) (Montoya-Rodriguez, González de Mejía, Dia, Reyes-Moreno, & Milan-Carrillo, 2014) and hydrolysates from soy products (decrease of IL-1β and TNF-α in RAW-24.7 macrophages) (Dia et al., 2014).

Ours results suggest that bean hydrolysates can act in different pathways related to the inflammatory process. The common beans used in the present study were cooked fresh and after storage and the hydrolysates resulting after digestion were used to evaluate the anti-inflammatory effect in human THP-1 macrophage. We showed that common beans stored for six months, cooked and digested by gastrointestinal enzymes produced peptides with antioxidant and anti-inflammatory potential.

5. Conclusion

This is the first *in vitro* study to show that cooked Carioca beans BRS Pontal and BRSMG Madreperola inhibit markers of inflammation, and commercial storage time (6 months) of these beans did not alter the physicochemical and biological properties of pepsin-pancreatin hydrolysates. *In vivo* studies are needed to assure the bioavailability and bioefficacy of peptides from stored and cooked Carioca beans.

Conflict of interest

The authors declare no conflict of interest.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2016.02.029.

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6.3. ARTIGO 3

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Digested protein isolate from fresh and stored Carioca beans reduced markers of atherosclerosis in oxidized LDL-induced THP-1 macrophages

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ABSTRACT

Oxidized LDL (ox-LDL) and its interaction with lectin type ox-LDL receptor (LOX-1) determine atherosclerosis progression. The potential of peptides from digested protein isolate (DPI) derived from fresh and stored Carioca bean (*Phaseolus vulgaris* L.) to prevent ox-LDL induced inflammation in human THP-1 macrophage-like cells was examined here. BRS Pontal (PO) and BRSMG Madreperola (MP) fresh and stored for 6 months were cooked, and protein isolates were digested by pepsin–pancreatin. Peptides from stored Carioca beans decreased prostaglandin E-2 (PGE-2) (from 17.7 to 47%) and tumour necrosis factor-α (TNF-α) (12.3 to 82%). Stored MP (0.01 mg/mL) inhibited reactive oxygen species (ROS). LOX-1 and matrix metalloproteinase-9 (MMP-9) expression were reduced by peptides from all beans. Stored PO and MP digested protein isolates reduced expression of 10 cytokines related to atherosclerosis process. Peptides from Carioca beans presented anti-atherosclerotic properties comparable to simvastatin, which were related to inhibition of LOX-1, MMP-9 and ICAM-1.

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Abbreviations: CVDs, Cardiovascular diseases; DPI, digested protein isolate; GCSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage-stimulating factor; GRO-CXC, chemokine Growth-regulated Oncogene; GRO-α, GRO-alpha; ICAM-1, intracellular adhesion molecule-1; IL, interleukin; INF-γ, interferon gamma; LOX-1, ox-LDL receptor; MCP, monocyte-chemo attractant protein; MIG, monokine induced by IFN-γ; MMP-9, matrix metalloproteinase-9; MP, BRSMG Madreperola; ox-LDL, oxidized LDL; PGE-2, prostaglandin E-2; PO, BRS Pontal; RANTES, chemokine (C-C motif) ligand; TGF-β, transforming growth factor-beta; THP-1, human acute monocytic leukaemia cell line; TNF-α, tumour necrosis factor-alpha; TNF-β, tumour necrosis factor beta

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1. Introduction

Cardiovascular diseases (CVDs) are the main cause of death worldwide and due to increased ageing and obesity, prevalence is estimated for 2030 in 23.6 million of deaths (WHO, 2011). Atherosclerosis is responsible for a large proportion of CVDs, such as coronary artery disease (e.g. heart attack), cerebrovascular disease (e.g. stroke), and diseases of aorta and arteries (e.g. hypertension) (WHO, 2011). Atherosclerotic process is associated to pro-atherogenic and pro-inflammatory mediators that lead to formation of plaques and progressive stenosis (Cardilo-Reis et al., 2012; Khan, Spagnoli, Tardif, & L'Allier, 2015). Monocytes and macrophages present an underlying role in the atherosclerotic process and are involved in all stages of plaque development (Saha et al., 2009). *In vitro* studies with these type of human cells has been performed to simulate atherosclerosis process and to evaluate prevention mechanism by dietary bioactive compounds and peptides (Aluganti Narasimhulu, Selvarajan, Brown, & Parthasarathy, 2014; Chu, Lee, Chu, Yin, & Tseng, 2009; Del Bo et al., 2016).

The initial step of atherosclerosis involves high levels of low-density lipoprotein (LDL), LDL oxidation and recruitment of monocytes. These cells adhere to activated endothelium and migrate to the intima and differentiate into macrophages, whose scavenged modified lipid become foam cells (Neele, Van den Bossche, Hoeksema, & de Winther, 2015). The following steps are activation and cytokine secretion, cell foam apoptosis, plaque growth, smooth muscle cell migration and fibrous cap formation (Neele et al., 2015). Oxidized LDL (ox-LDL) induces expression of adhesion molecules on endothelium and facilitates monocyte adhesion to intima, determining the extension of lesion formation and progression (Mehta et al., 1995). Ox-LDLs are recognized and bonded by plasma membrane receptors, including lectin type ox-LDL receptor 1 (LOX-1) (Marín-García, 2014). LOX-1 is a scavenger receptor whose levels are elevated upon recognition of ox-LDL. Among the several molecular determinants of atherosclerosis, inflammation presents an underlying role, and its suppression represents a potential benefit to reduce CVD incidence.

Nevertheless, most of CVD treatments consist of targeted reductions in serum lipoprotein levels rather than combating the deleterious effects of acute and chronic inflammation (Khan et al., 2015). Statins (e.g. simvastatin, atorvastatin, pravastatin, fluvastatin, and lovastatin) are some of the drugs that are shown as effective not only due to their cholesterol-lowering prop-
et al., 2015). Statins (e.g. simvastatin, atorvastatin, pravastatin, fluvastatin, and lovastatin) are some of the drugs that are shown as effective not only due to their cholesterol-lowering prop-
erties, but also to lipid-independent pleiotropic effects (Hofnagel, Luechtenborg, Weissen-Plenz, & Robenek, 2007).

Dietary sources may provide bioactive peptides that can be useful for the prevention or treatment of chronic diseases and with properties relevant to cardiovascular health (Erdmann, Cheung, & Schröder, 2008; Garcia-Mora et al., 2015; McCartan, Powell, Green, McEneny, & McGinty, 2015). *In vitro* studies have evidenced that bioactive peptides from common beans (*Phaseolus vulgaris* L.) have anti-inflammatory and antihypertensive activities, by suppression of nuclear factor-kappaB (NF-κB) pathways (Oseguera-Toledo, Gonzalez de Mejía, Dia, & Amaya-Llano, 2011), and inhibition of angiotensin I-converting enzyme (Garcia-Mora et al., 2015; Rui, Boye, Simpson, & Prasher, 2012, 2013; Torruco-Uco, Chel-Guerrero, Martínez-Ayala, Dávila-Ortíz, & Betancur-Ancona, 2009), respectively. Bioactive peptides are released from proteins during gastrointestinal digestion, fermentation and enzymatic hydrolysis (Wang & Gonzalez de Mejía, 2005). In this sense, time of storage is important to preserve bean quality and can increase the natural enzymatic hydrolysis (Hohlberg & Stanley, 1987), affecting biological properties. Carioca bean is a cultivar of common bean (*P. vulgaris* L.), similar to pinto bean, susceptible to fast hardening during storage, which prevents its storage over long periods (Faria et al., 2004; Siqueira et al., 2014). Old beans, stored for long time, are not usually consumed due the hard-to-cook-defects (Njoroge et al., 2015). However, little is known about the effects of commercial time of storage of Carioca bean on functional properties of peptides, mainly bioactive peptides related to LDL metabolism and atherosclerotic process.

Therefore, the objective of this research was to evaluate the physicochemical properties of peptides from fresh and stored Carioca bean protein isolates after simulated gastrointestinal digestion and investigate their biological potential to prevent ox-LDL induced inflammation by pleiotropic effects in human THP-1 macrophage-like cells. The results support the functional food nature of bean consumption.

2. Materials and methods

2.1. *Materials*

Carioca beans BRS Pontal (PO) and BRSMG Madreperola (MP) (*P. vulgaris* L.) were kindly provided by EMBRAPA Rice and Bean (Santo Antônio de Goiás, GO, BR). Porcine pepsin (EC 3.4.23.1), pancreatin (8xUSP, a mixture of digestive enzymes produced by the exocrine cells of the porcine pancreas, EC 232-468-9), simvastatin (S6196), sodium nitrite, sulphanilamide, N-1-(naphthyl) ethylenediamine-diHCl, phorbol 12-myristate 13-acetate (PMA) and 500 Da molecular weight cut-off membrane (Z367982) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RC DC (reducing agent and detergent compatible) protein assay (500-0122) and DC (detergent compatible) protein assay (500-0112) were purchased from Bio-Rad (Hercules, CA, USA). Human acute monocytic leukaemia cell line (THP-1) (TIB-2002) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Fetal bovine serum was purchased from Invitrogen (Grand Island, NY, USA). Streptomycin/penicillin and sodium pyruvate were purchased from Cellgro
(ATCC, Manassas, VA, USA). Fetal bovine serum was purchased from Invitrogen (Grand Island, NY, USA). Streptomycin/penicillin and sodium pyruvate were purchased from Cellgro
(Manassas, VA, USA). Medium oxidized (LDL) (770202) was purchased from Kalen Biomedical. Human EIA kits were purchased from Cayman Chemical (Ann Arbor, MI, USA) to analyse prostaglandin E2 (PGE2, #514010) and from Abcam (Cambridge, MA, USA) for tumour necrosis factor alpha (TNF)-α analysis (ab46087). Other products from Abcam were: cellular reactive oxygen species (ROS) detection assay kit (ab113851), human cytokine antibody array (#ab133996), lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) rabbit polyclonal primary antibody (ab60178) and matrix metallopeptidase 9 (MMP-9) rabbit monoclonal primary antibody (ab76003). Intracellular adhesion molecule-1 (ICAM-1) rabbit oligoclonal primary antibody (710278) was purchased from Invitrogen (Carlsbad, CA, USA).

2.2. *Carioca beans storage and processing*

BRS Pontal (Brazilian Pontal bean) (PO) and BRSMG Madreperola (Brazilian-Minas Gerais Madreperola bean) (MP) are Carioca beans (*P. vulgaris* L.), similar to Pinto bean, and were selectively bred by EMBRAPA Rice and Bean (Santo Antônio de Goiás, GO, BR). MP beans presented late darkening and hardening through storage time. Post-harvest beans were stored at room temperature (22 ± 3 °C), without humidity control (<65% relative humidity) for six months. Time zero was used as a baseline control for all comparisons in the present study. Beans were cooked (1:2 bean/water; 120 °C, 50 min, under pressure) at time zero (samples will be referred as PO 0 and MP 0) and after six months (samples will be referred as PO 6 and MP 6) of storage, dried (air-circulating oven for 8 h/60 °C) and ground (Grinder Vertical Rotor MA 090 CFT; sieve of 600 μm, size 30 mesh; Marconi Equipment, Piracicaba, SP, BR). The resultant flour was stored using vacuum packaging and kept at −20 °C until analysis.

2.3. *Extraction of bean protein isolates*

Triplicates of PO and MP flours were diluted in water (1:10 w/v) and pH adjusted to 8.0 with 0.1 M sodium hydroxide before extraction for 1 h at 35 °C, with stirring. The mixture was centrifuged at 5000 *g* for 15 min at 4 °C and the resultant precipitate was re-extracted under identical conditions to maximize yield. Both extracts were combined and pH adjusted to 4.3 with HCl to precipitate proteins, followed by centrifugation at 10,000 *g* for 20 min at 4 °C. Supernatant was discarded and the pellet was freeze-dried in a LabConco Freeze Dryer 4.5 (Kansas, MO, USA) and stored at −20 °C until further analysis.

2.4. *Simulated gastrointestinal digestion*

Pepsin–pancreatin hydrolysis was used to simulate gastrointestinal digestion as described by Megías et al. (2004). Bean protein isolates were suspended in water (1:20 w/v), in triplicate, and a sequential enzyme digestion was carried out with pepsin/substrate 1:20 (w/w) (pH 2.0) followed by pancreatin/substrate 1:20 (w/w) (pH 7.5) at 37 °C, for 2 h each. Digestion was stopped by heating at 75 °C for 20 min. Samples were centrifuged at 20,000 *g* for 15 min at 4 °C. The digested protein isolates (DPI) was dialysed to eliminate salts using a 500 Da molecular weight cut-off membrane (Z367982, Sigma-Aldrich) and freeze dried (LabConco). Samples were stored at −20 °C until analysis and will be referred as PO DPI (0 and 6) and MP DPI (0 and 6).

2.5. *Protein profile, soluble protein concentration and degree of hydrolysis*

The protein profile of whole bean flour and DPI was evaluated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis analysis (SDS-PAGE). Samples were diluted (1:1, v/v) in Laemmli buffer 5% of β-mercaptoethanol, pH 6.8 (161–0737, Bio-Rad, Hercules, CA, USA), boiled for 5 min and loaded in pre-cast (4–20%) gradient polyacrylamide Tris–HCl gels. Standard dual colour (10–250 kDa) was used to calculate molecular mass of separated proteins. After running (constant voltage of 200 V for 35 min) the gel was stained with Simply Blue Safe Stain overnight, destained with water (3–times of 5 min) and visualized using the GL 4000 Pro Imaging System (Carestream Health Inc., Rochester, NY, USA). The protein profile of IPD was compared to the respective whole beans before digestion.

Total protein concentration of DPI was evaluated in triplicate by DC (detergent compatible) protein assay (#500-0112; Bio-Rad), according to the manufacturer's instructions. Absorbance was read at 630 nm and protein concentration was calculated using the generated bovine serum albumin (BSA) standard curve ($y = 0.0002x + 0.0116$; $R^2 = 0.995$).

Degree of hydrolysis (DH) was performed in triplicate as described by Cabra, Arreguin, Vazquez-Duhalt, and Farres (2007). The percentage of the dissolved protein after precipitation with 0.2 M trichloroacetic acid (TCA) was compared to the total dissolved Carioca bean flour protein (100%), which was obtained after complete hydrolysis with 2 M sulphuric acid at 100 °C for 4 h. The protein concentration was also analysed by DC (detergent compatible) protein assay (standard curve: $y = 0.0002x + 0.0047$; $R^2 = 0.995$).

2.6. *Peptide characterization and bioactive potential*

Peptides were analysed by high-performance liquid chromatography–electrospray ionization–mass spectrometry (HPLC–ESI–MS) using a Q-ToF Ultima mass spectrometer (Waters, Milford, MA, USA), equipped with an Alliance 2795 HPLC system, according Mojica et al. (Mojica, Chen, & Gonzalez de Mejía, 2015). Only peaks with intensity higher than 70%, and sequences with more than 80% of probability were analysed. The presence of bioactive sequences in the main Carioca bean proteins was confirmed using UniProt database from BLAST tool, using the keyword "*Phaseolus vulgaris* L." to filter by organism (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on March 12, 2015). Query covers below 60% were not included. Biological activity of peptides was predicted by using BIOPEP database (http://www.uwm.edu.pl/biochemia, accessed on March 12, 2015). Percentage of potential biological activity of peptides was related to total number of peptides present in each sample. Peptide structures were predicted by PepDraw tool (http://www.tulane.edu/~biochem/WW/PepDraw/, accessed on March 20, 2015). The amino acids were presented in one letter nomenclature.

2.7. *Antioxidant capacity*

Antioxidant capacity was measured by Oxygen Radical Absorbance Capacity assay using 20 μL of 6-hydroxy-2,5,7,8-tetramethylchroman 2-carboxylic acid, Trolox standard at 1–8 μM final concentration for standard curve construction, DPI (1.0 mg/mL of freeze-dried samples in 75 mM phosphate buffer, pH 7.4), or blank (75 mM phosphate buffer, pH 7.4), with 120 μL of 116.9 nM fluorescein (final concentration 70 nm/well), and 60 μL of 40 mM α,α′-azodiisobutyramidine dihydrochloride (AAPH) per well. A black walled 96-well plate was read at 485 and 582 nm every 2 min at sensitivity 60 at 37 °C using a Synergy 2 multiwell plate reader (BioTek, Winooski, VT, USA). The assay was performed in triplicate and results were expressed as millimoles Trolox equivalents per g of dried DPI (mM TE/g).

2.8. Anti-atherosclerotic potential

THP-1 cells, a human monocytic-derived cell line, were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% penicillin/streptomycin, 1% sodium pyruvate and 10% fetal bovine serum at 37 °C in 5% CO_2/95% air using a CO_2 Jacketed Incubator (NuAIRE DH Autoflow, Plymouth, MN, USA). PMA was added at a concentration of 162 nM to promote differentiation of THP-1 cells into macrophages (Takashiba et al., 1999). Macrophage differentiation was allowed to occur for 48 h and confirmed by cell morphology and total plate adhesion. Efficiency of THP-1 cells growing in the presence of all treatment was assessed by aqueous solution CellTiter 96 one proliferation assay kit (Promega Corporation, Madison, WI, USA). THP-1 macrophages-like were seeded at a density of 1,000,000 cells per 2 mL in a 6-well plate. Medium oxidized (ox)-LDL was used to induce atherosclerosis (10 µg/mL) for 48 h. Prior to incubation with ox-LDL, THP-1 cells were treated for 2 h with PO and MP digested protein isolates at concentrations of 0.01, 0.05 and 0.1 mg/mL. Cells treated with phosphate buffered saline (PBS) and ox-LDL were used as a positive control and cells with PBS were used as a negative control. Another set of cells were treated with simvastatin diluted in PBS (10 µM) and ox-LDL as a pharmacological control. After treatment for 48 h, the growth medium and cell lysates were collected and frozen at −80 °C until analysis. All cell treatments were performed in duplicate.

2.8.1. PGE-2, TNF-α and ROS measurement in supernatant of THP-1 macrophages

Secretion of PGE-2 (diluted 1:250 sample:buffer) and TNF-α (without dilution), was evaluated, in triplicate, by commercially available ELISA assays, following the manufacturer's instructions. Inflammatory markers concentrations were calculated using their respective standard curves run at the same time as the treatments (PGE-2: $y = -1.016 \ln(x) + 3.463$; $R^2 = 0.995$, and TNF-α: $y = 0.978x - 2.532$; $R^2 = 0.997$). Results were expressed in pg/mL.

An independent cell treatment was performed, in triplicate, in 96 well plate for reactive oxygen species (ROS) inhibition assay, using the cellular reactive oxygen species detection assay kit (Abcam, ab113851). THP-1 cells (2.5×10^4 cells/well) were differentiated (48 h with PMA at 162 nM), followed by 48 h of treatment as described the previous section. One hour prior to completion of the treatment, the 2′,7′-dichlorofluorescin diacetate (DCFDA) were loaded in all wells (50 µM/total volume). After this period the plate was transferred to the microplate reader without washing and read with excitation wavelength at 485 nm and emission wavelength at 535 nm. Results were expressed as fluorescence intensity.

2.8.2. Western blot analysis of LOX-1, MMP-9 and ICAM-1

Cell lysates were used for western blotting to measure expression of the pro-atherosclerosis markers: oxidized low-density lipoprotein receptor (LOX-1), intracellular adhesion molecule-1 (ICAM-1) and matrix metalloproteinase-9 (MMP-9). Briefly, after treatments the cells were washed once with ice cold DMEM (1 mL) and twice with ice cold PBS (1 mL) and lysed with 200 µL of Laemmli buffer (Bio-Rad) containing 5% β-mercaptoethanol. Cell lysates were sonicated for 30 s and boiled for 5 min. Protein concentration was quantified using RC-DC Assay (Bio-Rad) and 20 µg protein was loaded in 4–20% Tris–HCl gels (Bio-Rad) for protein separation. Resolved protein were transferred to a PVDF (polyvinylidene difluoride membrane, Millipore, Billerica, MA, USA) and blocked with 3% nonfat milk in 0.1% TBST for 1 h at 4 °C. Membranes were washed with 0.1% TBST (5 times 5 min each) and incubated with respective primary antibody (LOX-1, MMP-9 or ICAM-1) in 1% nonfat milk in 0.1% TBST (1:500) at 4 °C overnight. Membranes were washed again and incubated with anti-rabbit horseradish peroxidase conjugate secondary antibody in 1% nonfat milk in 0.1% TBST (1:2500) for 3 h at room temperature. After incubation and washes, proteins were visualized using a chemiluminescent reagent (GE Healthcare) following manufacturer's instructions. The membrane pictures were taken with a GL 4000 Pro Imaging system (Carestream Health Inc.). Band intensity was normalized using GAPDH and probed with mouse GAPDH primary antibody in 1% nonfat milk in 0.1% TBST (1:500), using anti-mouse horseradish peroxidase conjugate as secondary antibody in 0.1% TBST (1:2500). Western blot analysis was performed in duplicate.

2.8.3. Protein/cytokines array

The human cytokine antibody array (#ab133996, Abcam) was used, in duplicate, to analyse the expression of 23 atherogenic-related protein/cytokines in the cell lysates of negative control (PBS), positive control (PBS plus ox-LDL – 10 µg/mL), pharmacological inhibitor (simvastatin, 10 µM) and PO-DPI and MP-DPI. For this assay DPI from stored PO and MP (6 months) at 0.1 mg/mL were used. The assay was performed according to manufacturer's instructions. Briefly, cell lysates were collected using cell lysis buffer and the protein concentration was determined by DC protein assay (Bio-Rad) ($y = 0.0002x + 0.014$; $R^2 = 0.993$). Provided membranes were blocked with 1X blocking buffer (2 mL) at room temperature for 30 min. After aspiration of 1X blocking buffer, diluted lysates (150 µg of protein/mL:1 mL of 1X blocking buffer) were loaded into each well and incubated overnight at 4 °C. Membranes were washed in clean containers with a large volume of wash buffer I (20 mL/30 min, with gentle shaking), as recommended after overnight incubation. The next incubations included aspiration and 2 washing steps with wash buffer I and wash buffer II (2 mL of each; 3 and 2 washes of 5 min, respectively). The incubation with 1X biotin-conjugated anti-cytokines (1 mL/well) was performed overnight at 4 °C and the subsequent incubation with 1X HRP-conjugated streptavidin (2 mL/well) occurred for 2 h at room temperature. Membranes were washed again and chemiluminescence detection was performed using equal volumes (1:1, v/v) of detection buffer C and detection buffer D. Membrane pictures were taken with a GL 4000 Pro Imaging system (Carestream Health Inc.). The results were expressed by mean intensity values and fold changes related to positive control (PBS plus ox-LDL).

2.9. In silico analysis of LOX-1 inhibition

Structural mechanism by which peptides from PO and MP digested protein isolates inhibit LOX-1 was evaluated by *in silico* analysis, through molecular docking as described by Luna-Vital et al. (Luna-Vital, Gonzalez de Mejía, Mendoza, & Loarca-Pina, 2015), using DockingServer.17 program. Peptides

were designed using Instant MarvinSketch (ChemAxon Ltd), and the MMFF94 force field was used for energy minimization of ligand molecules, peptides and simvastatin using DockingServer. Gasteiger partial charges were added to the ligand atoms. Non-polar hydrogen atoms were merged, and rotatable bonds were defined. The crystal structure file of LOX-1 was obtained from the Protein Data Bank (PDB: 1YXK). Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools. Affinity maps of 20 × 20 × 20 Å grid points and 0.375 Å spacing were generated using the Autogrid program. AutoDock parameter set- and distance-dependent dielectric functions were used in the calculation of the van der Waals and electrostatic terms, respectively. Docking simulations were performed using Lamarckian genetic algorithm (LGA) and the Solis and Wets local search method (Solis & Wets, 1981). Initial position, orientation, and torsions of the ligand molecules were set randomly. Each docking experiment was derived from 100 different runs that were set to terminate after a maximum of 2,500,000 energy evaluations. Population size was set to 150. During the search, a translational step of 0.2 Å, and quaternions and torsion steps of 5 were applied.

2.10. *Statistical analysis*

All analyses were performed in three independent replicates and the specific assays were also performed in triplicate; for cell treatments, experiments were performed in at least two independent replicates for each treatment and assayed in triplicate. Data are expressed as the mean ± standard deviation. The data were analysed by one-way analysis of variance (ANOVA) with two sources of variation (bean and time) and bean × time interaction. Significant interactions ($P < 0.05$) were fragmented and the effect of time was tested by models of linear and quadratic equation in function of time of storage for each bean. ANOVA was conclusive for significant differences between beans ($P < 0.05$). The effect of Carioca bean on markers of inflammation was tested by comparison of DPI, positive control (PBS and LPS) and simvastatin, at 5% of probability. Principal component analysis was performed to obtain a graphic representation of DPI and dose effect on inflammatory markers.

3. Results

3.1. *Protein profile, soluble protein and degree of hydrolysis*

Simulated gastrointestinal digestion of bean protein isolate resulted in peptides lower than 10 kDa before and after six months of storage (Fig. 1A). The soluble protein concentration increased after six months of storage for both DPI ($P < 0.05$); however, MP DPI presented higher values than PO DPI ($P < 0.05$) (Fig. 1B). The DH was negatively affected by time of storage ($P < 0.05$), with higher values for MP (MO 0 = 54.9 ± 6.1%; MP 6 = 48.6 ± 1.57% versus PO 0 = 51.3 ± 2.7%; PO 6 = 38.8 ± 1.7%, $P < 0.05$) (Fig. 1C). Antioxidant capacity (ORAC) was not affected after six months of storage, without significant difference between beans ($P > 0.05$) (Fig. 1D).

3.2. *Peptides profile*

Table 1 shows the bioactive peptides identified in PO and MP digested protein isolates. Peptides with low molecular mass were found in all DPI (490.28 kDa on average), except MP time 6, which presented peptides with higher molecular mass (1485.86 and 1557.88 kDa). PO 6 and MP 0 presented peptides from the most abundant common bean proteins (α-amylase and phaseolin, respectively). The bioactive sequences were mainly related to inhibition of angiotensin-converting-enzyme (ACE) and dipeptidyl peptidase-IV (DPP-IV). Antioxidative, stimulating vasoactive substance release (SVSR) and ubiquitin-mediated proteolysis activating (UMPA) peptides were found with less frequency. However, DPIs presented few differences on the percentage of bioactive potential and bean type and time of storage (Fig. 1E).

3.3. *Inhibition of PGE-2 and TNF-α secretion and ROS production*

Fig. 2 shows the effect of peptides from PO and MP DPI on PGE-2, TNF-α and ROS production in the model used with ox-LDL-induced THP-1 macrophages-like human cells. Both stored Carioca bean digested protein isolates (time 6) reduced PGE-2 production in a dose dependent manner, ranging from 17.7–47.0% for MP 6 to 25.1–36.3% for PO 6.

All concentrations used from PO and MP DPIs, except PO 6, reduced TNF-α ($P < 0.05$) in comparison with the activated ox-LDL positive control. The lower concentrations used for DPI resulted in higher reduction of TNF-α; from the lowest to the highest dose (0.01 to 0.1 mg/mL), PO 0 and PO 6 resulted in reductions ranging from 82–65.6% (0.01 mg/mL) to 24.3–12.3% (0.1 mg/mL), respectively, and MP 0 and MP 6 decreased TNF-α from 47–71.6% (0.01 mg/mL) to 39.4–47.1% (0.1 mg/mL), respectively.

ROS production was inhibited (55.7%) by MP6 DPI at the lowest concentration (0.01 mg/mL), which was comparable to inhibition caused by simvastatin (68.8%) ($P > 0.05$). Most DPIs did not affect ROS production. A positive correlation between ROS and TNF-α was observed for PO 0 and PO 6 ($R = 0.95$ and $R = 0.99$, respectively).

3.4. *Effect of PO and MP digested protein isolates on expression of markers of atherosclerosis*

Fig. 3 shows the effect of PO and MP DPIs on the expression of LOX-1 (Fig. 3A), MMP-9 (Fig. 3B) and ICAM-1 (Fig. 3C). The expression of LOX-1 was reduced by all DPIs from fresh and stored beans, at all concentrations ($P < 0.05$). The reduction was comparable to simvastatin from MP 0 and 6 DPI (all concentration) and from PO 0 (0.05 and 0.5 mg/mL) ($P > 0.05$). All concentrations of all DPIs reduced MMP-9 expression ($P < 0.05$). Peptides from DPI PO 0, MP0 and MP6 were equivalent ($P > 0.05$) or more potent than simvastatin at all concentrations, and PO 6 at 0.05 and 0.1 mg/mL ($P < 0.05$). Only DPI from PO 0 did not affect ICAM-1 expression. The reduction of ICAM-1 expression was equivalent to ($P > 0.05$) or higher than simvastatin for peptides from PO 6 and MP 0 (all concentration), and MP 6 (at 0.05 and 0.1 mg/mL) ($P < 0.05$).

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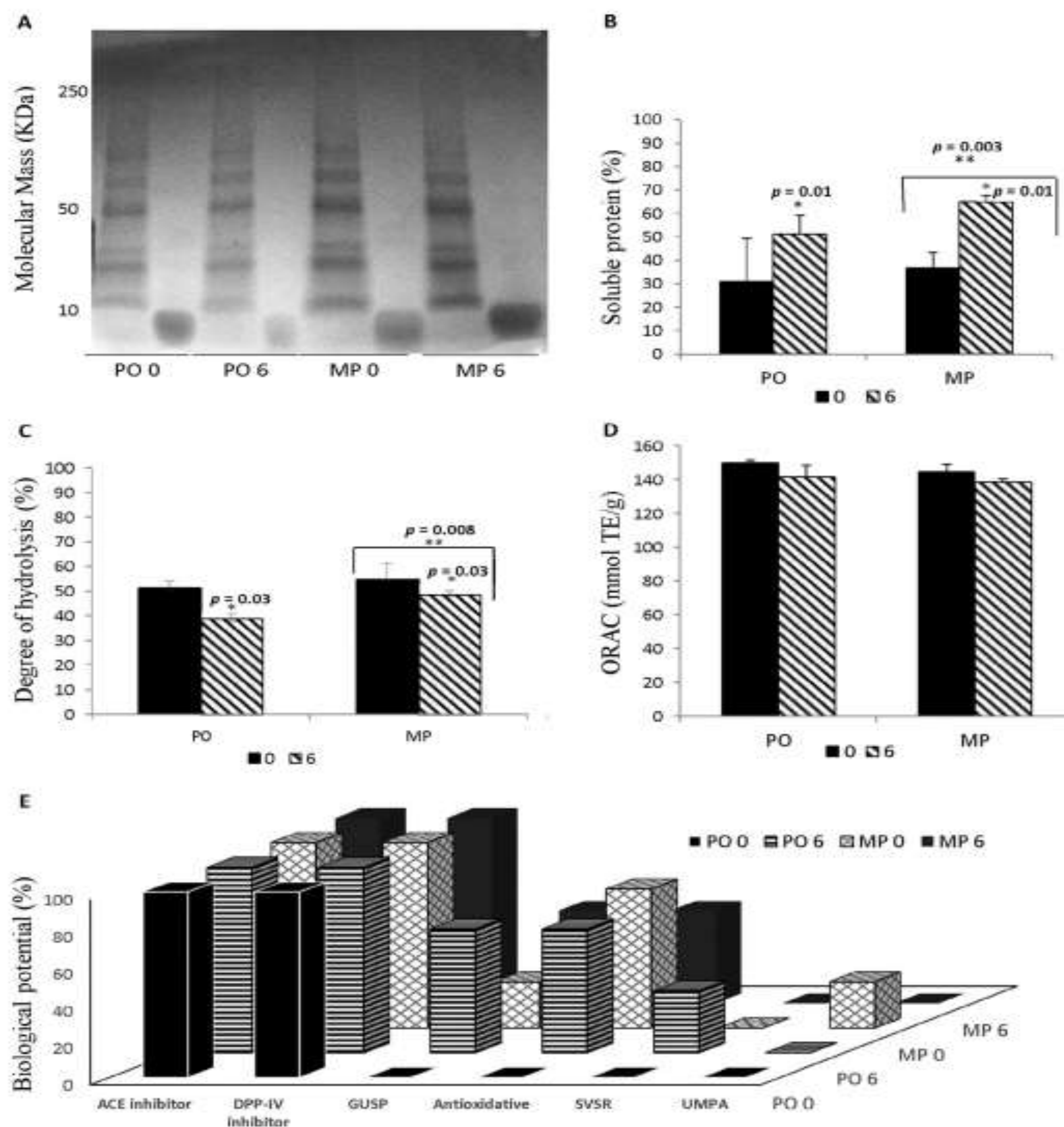

Fig. 1 – Electrophoretic profile (A), soluble protein concentration (B), degree of hydrolysis (C), antioxidant capacity of protein hydrolysates (D) and biological potential (%) (E) of digested protein isolates from PO and MP beans before and after 6 months of storage. PO, BRS Pontal; MP, BRSMG Madreperola; ORAC, oxygen radical absorbance capacity; ACE, angiotensin-converting-enzyme inhibitor; DPP-IV, dipeptidyl peptidase-IV inhibitor; GUSP, glucose uptake stimulating peptide; SVSR, stimulating vasoactive substance release peptide; UMPA, ubiquitin-mediated proteolysis activating peptide. *Significant difference with time of storage ($P < 0.05$; ANOVA followed by models of linear and quadratic equation in function of time of storage for each bean). **Significant difference between samples (by Student t-test). Percentage of biological potential is related to the total number of peptides present in each isolated digested protein.

Table 2 presents the percent reduction of protein markers involved in the inflammatory and atherogenic pathways in comparison to the positive control. Peptides from DPI PO 6 and MP 6 decreased interleukin expression (IL-1 α, IL-2, IL-3, IL-6, IL-10, IL-13 and IL-15), grow factors (GCSF), interferon gamma, monokine induced by gamma interferon (MIG), transforming growth factor-beta (TGF-β), TNF-α and TNF-β. IL-5 and IL-7 expression was reduced only by DPI MP. In general, most of the results were comparable ($P > 0.05$) to simvastatin, confirming that DPI MP and PO are able to decrease the expression of several anti-inflammatory and anti-atherosclerotic markers similarly to or even greater than simvastatin.

Together these results show that bioactive peptides from digested protein isolates from Carioca beans can act in the main steps of atherosclerosis mediated by LOX-1 signalling pathway (Fig. 4).

Table 1 – Bioactive peptides identified by HPLC-ESI-MS/MS in PO and MP digested protein isolates.

Digested protein isolates	RT (min)	Prob (%)	Molecular mass (Da)	Peptide sequences*	Biological activities	Parental proteins
PO TIME 0	56.4	88.6	479.24	FAAAT	ACE inhibitor DPP-IV inhibitor (FA, AT)	Eukaryotic translation initiation factor 5
PO TIME 6	53.81	71.7	1909.10	KELDERRLLLLLDRQ	ACE inhibitor (RL, RR, KE) SVSR (LLL) GUSP (LL) Antioxidative (EL) DPP-IV inhibitor (LL, DR, KE, RL, RR)	Alpha-amylase
	55.17	96.2	1359.79	KLLRRMMERK	ACE inhibitor (KL, RR, ME) GUSP (LL) Antioxidative (LLR) DPP-IV inhibitor (LL, ME, MM, RK, RM, RR)	Protein kinase PVPK-1
	56.82	70.8	496.38	YAAAT	ACE inhibitor (YA, AA, YAAAT), DPP-IV (AA, AT, YA)	Endoglucanase
MP TIME 0	46.7	68.84	1311.8512	RLLRAKMALLK	ACE inhibitor (RL, RA), GUSP (LL), Antioxidative (LK, LLR), UMPA (RA), DPP-IV inhibitor (MA, LL, RA, AL,RL)	Alpha and beta type phaseolin
	52.03	70.87	1551.97	KEAVRRLKLRRQ	ACE inhibitor (RL, LKL, EA, VR, KL, RR, KE), antioxidative (LK), DPP-IV inhibitor (VR, AV, KE, RL, RR)	Protein kinase PVPK-1
	54.68	68.53	495.23	FTAGT	ACE inhibitor (AG, GT), DPP-IV inhibitor (TA, AG)	DNA-directed RNA polymerase subunit beta
	57.55	87.85	1485.95	KKLNTKMRKLKV	ACE inhibitor (KL, LN), antioxidative (LK), DPP-4 inhibitor (KK, KV, LN, MR, NT, RK, TK)	Protein TIC 214
MP TIME 6	54.09	88.67	1557.88	RLRVDMLDTRRK	ACE inhibitor (RL, RR), DPP-4 inhibitor (ML, RK, RR, TR, VD)	Translation initiation factor IF-2, chloroplastic
	55.99	46.7	1485.86	RLLRARDAMTRK	ACE inhibitor (RL, RA, DA, AR), GUSP (LL), antioxidative (LLR), UMPA (RA), DPP-IV inhibitor (LL, RA, RK, RL, TR)	ATP-dependent Clp protease proteolytic subunit

PO, Pontal and MP, Madreperola digested protein isolates before (time 0) and after 6 months of storage (time 6). *Peptides sequenced by HPLC-ESI-MS/MS with intensity at least 70%. Potential bioactivities were obtained from the BIOPEP database. Only sequences of main proteins of *Phaseolus vulgaris* L. are presented in the table and were confirmed with BLAST tool. The amino acids are presented in one letter nomenclature. ACE, angiotensin-converting-enzyme inhibitor; DPP-IV, dipeptidyl peptidase-IV inhibitor; GUSP, glucose uptake stimulating peptide; SVSR, stimulating vasoactive substance release peptide; UMPA, ubiquitin-mediated proteolysis activating peptide.

3.5. *Molecular docking study of peptides inhibiting LOX-1*

The minimum estimated free energies of the interactions of the peptides with LOX-1 are shown in Table 3. The estimated free energies indicated that compounds with a more negative value are more likely to inhibit LOX-1. Peptides studied had free energy values ranging from −1.41 to $+8.02 \times 10^5$ kcal mol^{-1}, while simvastatin had a free energy value of −5.5 kcal mol^{-1}. Atom distances were shorter than 4 Å. The most stabilized poses of the peptide bonds with LOX-1 were obtained and an example can be observed in Fig. 5A,C, and simvastatin in Fig. 5B,D. The peptide FAAAT and simvastatin were able to inhibit LOX-1 mainly through contributions of hydrogen bonds, hydrophobic, polar and cation π interactions.

4. Discussion

The effect of fresh and stored beans on the functional properties of peptides obtained after simulated gastrointestinal digestion of the isolated proteins was evaluated. It has been reported that Carioca beans develop hardness after only 2 months of storage even under control conditions (5 °C) (Coelho, Prudêncio, Christ, Sampaio, & Schoeninger, 2013); therefore, this type of bean is commercially stored for up to 6 months for human consumption.

The simulated gastrointestinal digestion of the protein isolates from fresh and stored Carioca beans resulted in peptides with low molecular mass and their biological potential was mainly related to antioxidant and anti-inflammatory activities. Generally, stored beans present higher protein concentration than fresh beans, which can be due to physiological post-harvest changes. Natural enzymatic hydrolysis can happen during storage, facilitating protein cleavage. However, DH was not affected after six months of storage and was comparable to DH of pre-cooked beans (boiled for 15 min), without storage (35 to 60%) (Mojica et al., 2015). These DH values are considerably high and are in agreement with the heat treatment, since it promoted changes in the tridimensional structure of proteins, increasing accessibility of peptide bonds to enzymes (Schmidt & Markwijk, 1993).

The commercial time of storage (6 months) did not affect the antioxidant capacity evaluated by ORAC of DPI PO and MP. ORAC values did not differ between DPI from fresh and stored PO (149.7 ± 1.5 and 141.4 ± 4.3 mM TE/g of DPI, respectively) and

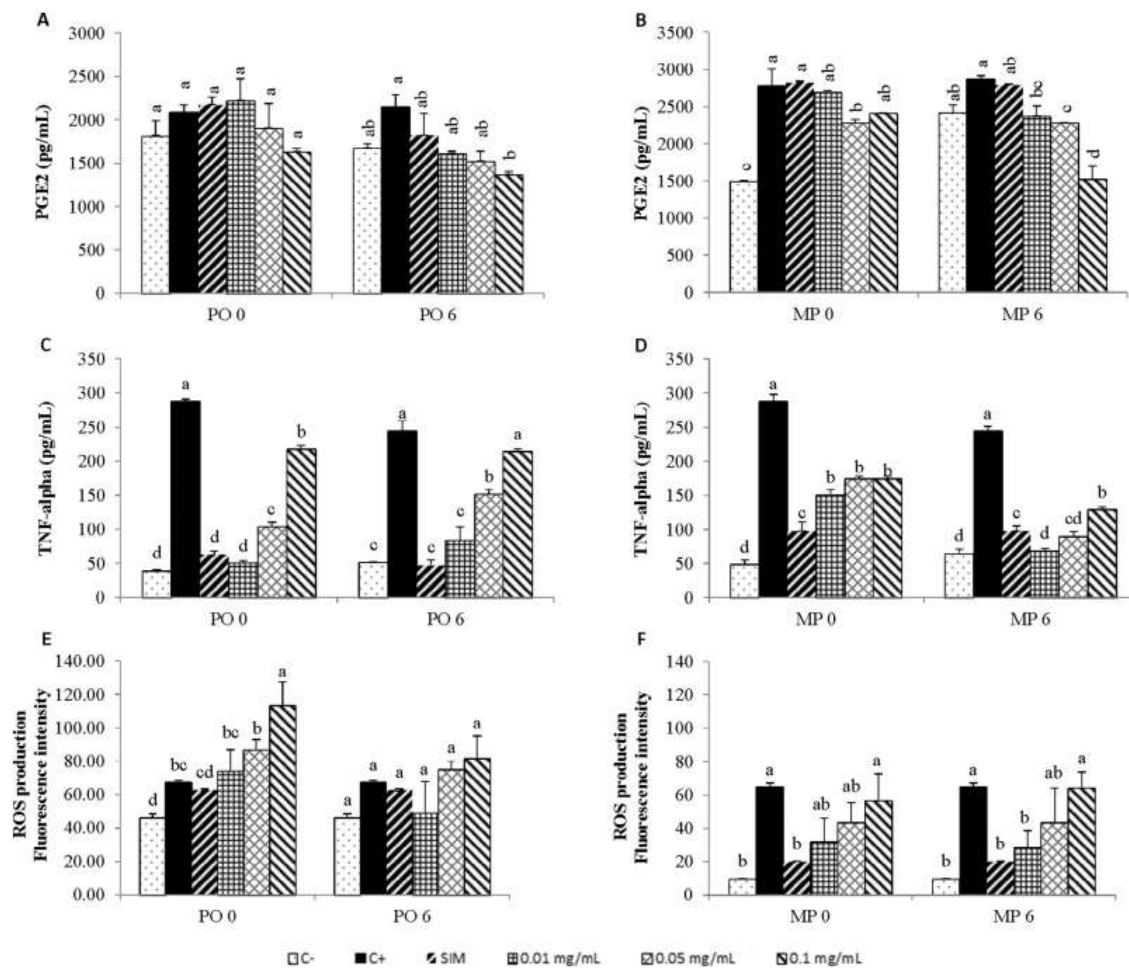

Fig. 2 – Effect of peptides from digested protein isolate of fresh and stored PO and MP beans on secretion of prostaglandin E (PGE-2) (A,B), tumour necrosis factor-alpha (TNF-α) (C,D) and reactive oxygen species (E,F) of oxLDL-stimulated THP-1 macrophages. All experiments were performed in at least two independent replicates. Different letter per column means statistically different relative to the positive control (C+). All treatments contain oxidized LDL (oxLDL, 10 μg/mL) except the negative control (C–) treated with PBS-alone. PO, BRS Pontal; MP, BRSMG Madreperola. Numbers 0 and 6 refer to storage time of storage; C–, negative control (PBS); C+ , positive control (oxLDL and PBS); SIM, simvastatin (10 μM/mL) and doses of digested protein isolates: 0.1, 1.0, 2.5 and 5.0 mg/mL. Means with different letters are significantly different (by Student t-test at 5% of probability). No difference by time was observed ($P > 0.05$; ANOVA followed by models of linear and quadratic equation in function of time of storage for each bean).

MP (144.7 ± 7.1 and 138.7 ± 1.5 mM TE/g of DPI, respectively). The time of storage did not affect peptides profile or their biological potential; DPI PO and MP presented some antioxidant and anti-inflammatory peptides, besides antihypertensive and anti-diabetic peptides, with potential to inhibit ACE and DPP-IV enzymes, respectively.

THP-1 is an excellent model to estimate immune-modulating effects of compounds in both activated and resting conditions of the cells, and it can suggest potential responses that may be reproduced *in vivo* (Chanput, Mes, & Wichers, 2014).

Anti-inflammatory potential was confirmed by the reduction of PGE-2 and TNF-α production in ox-LDL-induced THP-1 macrophages-like human cells. THP-1 macrophages constitutively secrete high levels of PGE-2 as a consequence of the differentiation process by PMA (Schwende, Fitzke, Ambs, & Dieter, 1996), which can explain the high values for the negative control. The highest concentration of PO 6 and MP 6 resulted in higher reduction of PGE-2 than simvastatin ($P < 0.005$).

Statins, such as simvastatin, cause regression of vascular atherosclerosis and reduce cardiovascular related morbidity and mortality, due to their cholesterol-lowering properties and also pleiotropic effects, such as reduction of oxidative stress, and consequently, reduction of generation of ox-LDL (Hofnagel et al., 2007). Statins also partially inhibit effects of TNF-α on

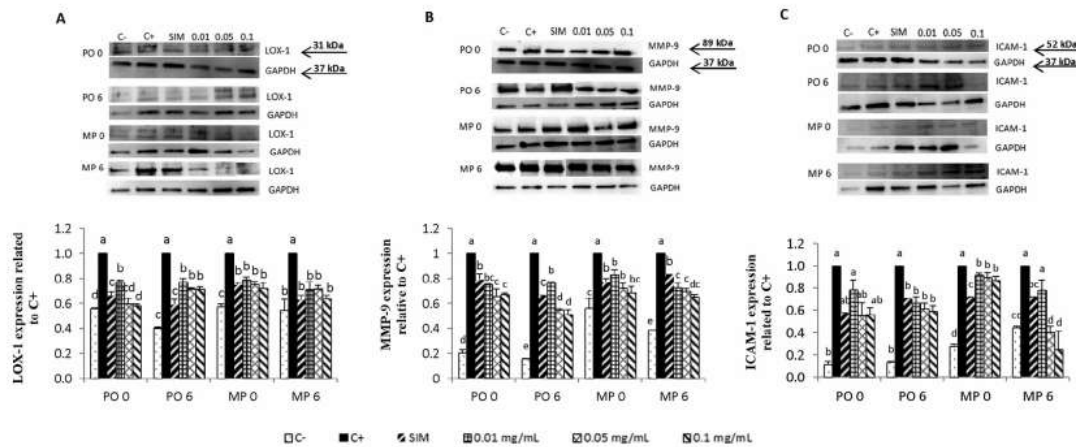

Fig. 3 – Effect of peptides from digested protein isolate of fresh and stored PO and MP beans on expression of lectin-like oxidized low-density lipoprotein receptor (LOX-1; 31 kDa) (A), matrix metalloproteinase-9 (MMP-9; 92 kDa) (B) and intracellular adhesion molecule-1 (ICAM-1; 58 kDa) (C). The net intensity of markers was normalized by net intensity of GAPDH (37 kDa), and the values were expressed in percentage of inhibition related to the positive control (C+). All treatments contain oxidized LDL (oxLDL, 10 µg/mL) except the negative control (C–) treated with PBS-alone. PO, BRS Pontal; MP, BRSMG Madreperola. Numbers 0 and 6 refer to time of storage; C–, negative control (PBS); C+, positive control (oxLDL and PBS); SIM, simvastatin (10 µM/mL) and doses of digested protein isolates: 0.1, 1.0, 2.5 and 5.0 mg/mL. Means with different letters are significantly different (by Student t-test at 5% of probability). No difference by time was observed ($P > 0.05$; ANOVA followed by models of linear and quadratic equation in function of time of storage for each bean).

ICAM-1, VCAM-1 and E-selectin expression (Wu, Tian, Zhou, & Wu, 2013).

TNF-α acts as a pro-inflammatory marker on the inflammation/atherosclerosis cascade (Frostegård et al., 1999), increasing expression and synthesis of pro-inflammatory cytokines such as MCP-1 and IL-6 (Norata, Cattaneo, Poletti, & Catapano, 2010). Inhibition of TNF-α impairs monocyte-endothelial cell interaction and reduces TNF-α mediated up-regulation of adhesion molecules and cytokines (Desai, Darland, Bland, Tripp, & Konda, 2012). Active macrophages accumulating at sites of the atherosclerotic lesions produce arachidonic acid, which is converted by cyclooxygenase 2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) to PGE-2, a main inflammatory mediator (Saha et al., 2009). PGE-2 suppresses chemokine production in human macrophages through the EP-4 receptor in early phases of atherosclerosis (Takayama et al., 2002), while contributing to the progression of plaque in later stages of the disease (Cipollone et al., 2001).

The effect on scavenging of ROS was not statistically different from the positive control. However, TNF-α values were lower than the positive control; its inhibition showed a trend to be inversely proportional to concentration, the lower concentrations of DPI were more effective. Cytokines, such as TNF-α, induces ROS generation, exacerbating the oxidative stress (Fernandez-Sanchez et al., 2011). Macrophages when stimulated produce large amounts of O^{2-} and H_2O_2, which exert cytotoxic activities against themselves and others cells (Albina, Cui, Mateo, & Reichner, 1993). Functions of macrophages in reverse cholesterol transport can be impaired by oxidative stress, favouring the atherosclerotic process (Korytowski, Wawak, Pabisz, Schmitt, & Girotti, 2014). In this sense, antioxidants from food may have protective effects on oxidative damage by increasing cellular antioxidant defence system. However, there is evidence that suggests that higher concentrations of antioxidants from dietary sources can act as pro-oxidants (Halliwell, 2013). In addition, ROS can stimulate a cytokine cascade effect through NF-κB-induced transcriptional events, inducing the expression of TNF-α (Martin, Hoeth, Hofer-Warbinek, & Schmid, 2000), which can explain the less effectiveness to reduce this marker at higher doses of DPI.

The potential mechanisms of action of anti-inflammatory bioactive peptides and peptide-rich hydrolysates are related to control of ROS production, increase of anti-inflammatory cytokines, decrease of inflammatory cytokines and inhibition of renin-angiotensin system (RAS), among others (Chakrabarti, Jahandideh, & Wu, 2014).

PO and MP DPI from fresh and stored Carioca beans were able to reduce LOX-1, MMP-9 and ICAM-1 expression, in a comparable or better way than simvastatin ($P < 0.05$). LOX-1 and ox-LDL present a crucial role in endothelial dysfunction and atherosclerosis. Their interaction activates NADPH oxidase, increasing ROS, which in turn promotes activation of a cascade of events that results in the development of inflammation, monocyte adhesion and platelet activation/aggregation. This may be caused mainly by activation and translocation of NF-κB that results in an increase of colony-stimulating factors (CSF), monocyte chemoattractant proteins (MCP-1), ICAMs, MMPs, among others (Marín-García, 2014). MCP-1 promotes monocyte migration to intima, and its expression is increased by activation of adhesion molecules, such as ICAM-1 (Dinarello,

Table 2 – Protein markers involved on inflammatory and atherogenic pathways, their action and percent of reduction by simvastatin, PO and MP digested protein isolates.

Protein name	Known acronym	Action	Reduction (%)[1]		
			SIM	PO	MP
Interleukin-alpha	IL-1 α	Induces the activation of nuclear factor-κB (NF-κB) (Libby, 2012)	100^{c}	100^{c}	100^{c}
Interleukin-2	IL-2	Promotes the expansion and activation of T cell subset and plaque development (Von der Thüsen et al., 2003)	100^{c}	77^{b}	100^{c}
Interleukin-3	IL-3	Stimulates cell adhesion (Von der Thüsen et al., 2003)	46^{b}	44^{b}	60^{c}
Interleukin-5	IL-5	Activates mast cells in the atherosclerotic plaque and plaque rupture (Von der Thüsen et al., 2003)	23^{ab}	3^{a}	34^{b}
Interleukin-6	IL-6	In chronic inflammation it is rather pro-inflammatory (Libby, 2012)	21^{ab}	0^{a}	42^{b}
Interleukin-7	IL-7	Similar to IL-2 actions (Von der Thüsen et al., 2003)	22^{bc}	0^{ab}	42^{c}
Interleukin-10	IL-10	Attenuates the production of pro-inflammatory cytokines by macrophages (Libby, 2012)	100^{a}	50^{b}	61^{c}
Interleukin-13	IL-13	Attenuates the expression of a wide range of inflammatory cytokines (Libby, 2012)	74^{b}	47^{b}	64^{c}
Interleukin-15	IL-15	Accelerates atherogenesis by modulation of monocyte levels, and activation of macrophages (Dadoo et al., 2014)	76^{c}	45^{c}	60^{d}
Granulocyte colony-stimulating factor	GCSF	Induces the production of TNF-α and GM-CSF (Xu et al., 2000)	100^{c}	42^{b}	56^{b}
Granulocyte macrophage-stimulating factor	GM-CSF	Its function is as a white blood cell growth factor (Xu et al., 2000)	29^{b}	21^{a}	36^{ab}
CXC Chemokines growth-regulated oncogene	GRO	It is part of the immune/inflammatory cascade (Ahuja & Murphy, 1996)	52^{b}	30^{ab}	0^{a}
GRO-alpha	GRO-α	It is a pro-inflammatory chemokine secreted by monocytes (Ahuja & Murphy, 1996)	69^{b}	69^{b}	34^{ab}
Interferon gamma	INF-γ	Pro-atherogenic (Libby, 2012)	77^{c}	43^{b}	58^{c}
Monocyte-chemo attractant protein-1	MCP-1	It has been linked with chronic inflammatory diseases and atherosclerosis (Von der Thüsen et al., 2003); involved in the recruitment of monocytes/macrophages and activated lymphocytes (McManus et al., 1998)	0	0	0
Monocyte-chemo attractant protein-2	MCP-2	Involved in the recruitment of monocytes/macrophages and activated lymphocytes (McManus et al., 1998)	100^{d}	41^{b}	64^{c}
Monocyte-chemo attractant protein-3	MCP-3	Involved in the recruitment of monocytes/macrophages and activated lymphocytes (McManus et al., 1998)	100^{c}	41^{b}	0^{a}
Monokine induced by IFN-γ	MIG	Related to coronary artery calcification in humans (Yu et al., 2015)	100^{d}	42^{b}	67^{c}
Chemokine (C-C motif) ligand	RANTES	Involved in several clinical inflammatory conditions, plays a crucial role in atherosclerosis (Virani et al., 2011)	2^{a}	0^{a}	0^{a}
Transforming growth factor-beta	TGF-β	Anti-atherogenic properties (Von der Thüsen et al., 2003)	58^{c}	21^{c}	38^{d}
Tumour necrosis factor alpha	TNF-α	It is expressed by the action of different stimulus; pro-inflammatory cytokine (Frostegård et al., 1999)	42^{b}	50^{c}	64^{d}
Tumour necrosis factor beta	TNF-β	Pro-inflammatory cytokine (Frostegård et al., 1999)	70^{b}	42^{b}	60^{b}

[1] Percent of reduction is relative to the positive control. PO, Pontal and MP, Madreperola digested protein isolates, prepared from PO and MP beans stored for 6 months at concentration of 0.1 mg/mL. Simvastatin was used at 10 μM). Means with different letters are significantly different (by Student t-test at 5% of probability).

2009). ICAM-1 is constitutively expressed by several cell types at low levels. This molecule is up-regulated by pro-inflammatory stimuli and involved in the progression of atherosclerotic lesions and its crosslinking results in signal transduction, and initiation of several pro-inflammatory signalling cascades and rearrangement of actin cytoskeleton (Wolf & Lawson, 2012). MMPs degrade collagen, which helps sustain plaque tension and stability. Therefore, MMPs increase plaque instability and are implicated in all phases of atherosclerosis, and the onset of symptoms. MMP-9 and MCP-1 have been suggested as useful biomarkers to distinguish stable and unstable plaques and predict future cardiovascular events (Ma, Yabluchanskiy, Hall, & Lindsey, 2014; McManus et al., 1998).

In addition, the potential of digested protein isolates from stored beans to reduce the expression of important markers of inflammation and atherosclerosis was confirmed by a cytokine array using the highest concentration tested (0.1 mg/mL). PO and MP DPIs reduced expression of ILs-1α, -2, -3, -5, -6, -7 and -15. All of them are related to atherosclerosis, by activation of NF-κB (Libby, 2012), plaque development, cell adhesion stimulation, plaque rupture (Von der Thüsen, Kuiper, Van Berkel, & Biessen, 2003) or activation of macrophages (Dadoo, Ashkar, Richards, & Trigatti, 2014). Also observed in this study are the reduction of the expression of granulocyte colony-stimulating factor, granulocyte macrophage-stimulating factor, CXC chemokines growth-regulated oncogene (GRO), GRO-α and interferon-γ, which are strong pro-atherogenic markers involved in the inflammatory cascade (Ahuja & Murphy, 1996; Libby, 2012; Xu, Hoglund, Hakansson, & Venge, 2000).

Expression of monokine induced by gamma interferon (MIG) and CC chemokine ligand-5 (RANTES) was reduced by DPI from stored PO and MP. MIG is a Th1-associated chemokines induced

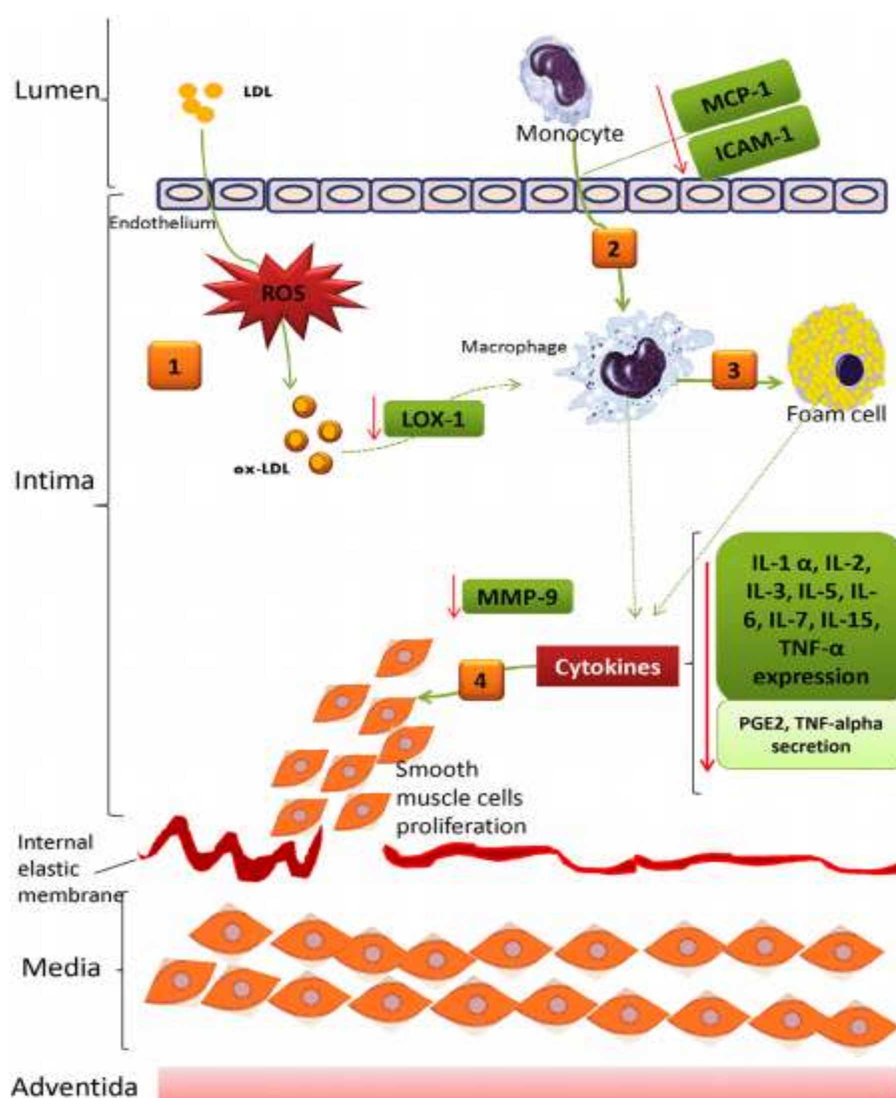

Fig. 4 – Potential mechanism of action of peptides from Carioca bean isolated protein after simulated gastrointestinal digestion related to protein expression of LOX-1 signalling pathway. Red arrows indicate in which steps Carioca bean peptides modulated the pathway in this study. Numbers 1, 2, 3 and 4 indicate the stages of atherosclerosis process. LDL, low-density lipoprotein; ROS, reactive oxygen species; ox-LDL, oxidized LDL; LOX-1, lectin type oxLDL receptor; IL, interleukins; TNF-α, tumour necrosis factor-α; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intracellular adhesion molecule-1; MMP-9, matrix metalloproteinase-9. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

by IFN-γ and suggested as a novel biomarker for T-cell inflammation and atherosclerotic plaque burden in humans (Yu et al., 2015). RANTES mediate transmigration of monocytes and T-cells into the intima, and induces the release of pro-inflammatory mediators. In addition, it is highly expressed in atheroma, and has been associated with the extent of carotid atherosclerosis and high-risk plaques (Virani et al., 2011). Regarding the complexity of atherosclerosis process, the therapies should target several inflammatory mediators and pro-inflammatory pathways, not only focusing in abnormal lipid levels treatment, but also in the anti-inflammatory alternative to reduce the progression and complications of atherosclerosis.

Only two peptides (FAAAT from PO 0 and YAAT from PO 6) had negative estimated free energy, meaning more potential to inhibit LOX-1. The higher free energy for simvastatin can be explained by the stereochemical configuration and smaller molecules, increasing the probability to access the catalytic sites (Luna-Vital et al., 2015). However, the anti-atherosclerotic effect found by the peptides from Carioca bean digested protein isolates in the present study can be due to peptides synergism

Table 3 – Estimated free energy binding and chemical interactions among the peptides present in Carioca beans (*Phaseolus vulgaris* L.) and the catalytic site of the LOX-1.

			Residues which showed interactions with the ligands and their atom distances (Å)			
Beans	Peptide sequences	EFE (kcal mol^{-1})	Hydrogen bonds	Polar	Hydrophobic	Cation-pi
PO TIME 0	FAAAT	−1.41	SER160(2.95)	GLN193(2.98), SER196(3.75), SER160(3.26)	LEU157(3.26), LEU158(3.67), LEU159(3.11), LEU160(3.54), LEU161(3.78)	–
PO TIME 6	YAAAT	−0.54	SER160(2.94)	GLN193(2.95), GLN193(3.10), SER160(3.04)	PHE261(3.76)	–
	KELDERRLLLLLDRQ	2.68E+05	GLN193(1.51), TYR197(2.91), ASP147(2.47), SER159(3.06), SER160(2.33), PHE190(1.97), ILE191(2.75), GLN192(2.99), ILE195(3.36), SER196(2.25), GLY249(3.17), ILE149(3.13), PHE158(3.39), ALA194(2.67),	ASP147(3.09), GLN193(2.67), SER196(2.33), TYR197(2.29), SER160(2.75), GLN193(0.72), ARG248(2.65)	ILE149(1.91), PHE158(2.55), ALA194(2.06), TYR197(2.42), PHE200(2.78), PHE261(3.01), PHE190(3.09), ILE191(2.87), ILE195(3.04)	TYR197(1.98), PHE261(2.89), PHE158(2.14), PHE190(1.92)
	KLLRRMMERK	4.47E+03	GLN193(2.65), ALA194(3.37), SER196(2.34), TYR197(2.74), ASP147(3.06), PHE158(3.32), SER159(2.86), SER160(2.63), GLY161(3.41), SER262(2.24), PHE261(3.54)	GLN192(3.54), GLN193(3.13), SER196(3.27), TYR197(3.01), ASP147(3.60), SER159(3.15),	ILE149(2.14), HIS151(3.12), TYR156(3.34), PHE158(2.74), PHE190(2.42), ILE191(3.18), ALA194(3.02), ILE195(3.11), TYR197(2.38), PHE202(3.35), ILE263(3.11), LEU157(3.11), PHE261(3.45)	PHE158(3.48), PHE190(1.72), TYR197(3.02), PHE202(3.69)
MP TIME 0	FTAGT	1.93	TYR197(2.74)	ASP147(3.51), TYR197(3.29)	ILE149(3.27), LEU157 (3.84), PHE158(2.77), ALA194(3.39) TYR197(3.47), PHE190(3.33)	PHE158(2.53), TYR197(3.16)
	KEAVRRLKLRRQ	2.95E+04	ASP147(1.84), TRP148(2.06), PHE190(2.72), GLN192(2.28), ALA194(2.81), SER196(2.46), TYR197(2.96), PHE158(3.35), SER159(3.22), SER160(2.36), ILE191(3.12), ILE149(3.72), ILE195(3.78), PHE261(3.77)	ASP147(3.23), SER160(2.92), GLN192(2.57), GLN193(2.52), SER196(3.28), TYR197(3.40)	ILE149(2.18), LEU157(2.52), PHE190(2.56), ALA194(2.63), TYR197(3.04), TRP148(3.04), HIS151(3.54), TYR156(3.18), PHE158(2.45), ILE195(3.73), PHE261(3.01)	PHE190(3.00), PHE158(3.60)
	KKLNTKMRKLKV	8.02E+05	ASP147(2.53), ILE149(1.63), ASN154(2.42), TYR156(2.76), PHE190(2.18), GLN193(2.28), SER196(2.53), TYR197(2.96), SER160(2.99)	HIS151(3.36), GLN193(1.80), TYR197(2.50), ASP147(2.17), SER159(3.29), SER160(2.30)	ILE149(2.30), HIS151(3.54), TYR156(2.94), LEU157(3.11), PHE158(2.90), PHE190(2.25), ALA194(2.74), PHE261(3.16)	TRP148(2.98), TRP150(3.51), HIS151(3.61), TYR156(3.59), PHE158(3.05), PHE190(1.07), TYR197(3.55), PHE202(3.21), PHE261(1.85)
MP TIME 6	RLLRAKMALLK	4.68E+03	SER160(3.07), GLN193(2.64), ALA194(2.40), SER196(2.51), TYR197(2.80), TYR156(3.07)	GLN193(2.07), SER196(2.77), TYR197(3.09), SER198(3.31), ASP147(3.36)	ILE149(2.61), LEU157(3.16), PHE158(2.95), PHE190(2.70), ALA194(3.82), ILE195(3.44), TYR197(2.72)	PHE158(2.99), PHE190(1.75), TYR197(2.91)
	RLLRARDAMTRK	1.01E+04	ASP147(1.99), SER159(3.03), SER160(2.87), GLN193(2.46), ALA194(1.72), TYR197(2.72), SER196(2.86), ILE149(3.80), PHE158(3.28)	ASP147(3.67), GLN193(3.05), TYR197(3.88), SER160(3.03), SER196(3.76)	ILE149(2.03), LEU157(3.06), PHE158(2.68), PHE190(2.89), ALA194(2.28), TYR197(3.19), PHE261(3.51)	PHE190(1.80), PHE200(2.95), PHE261(3.43), PHE158(1.62), TYR197(2.92)
	RLRVDMLDTRK	1.18E+05	ASP147(2.69), PHE158(2.73), SER159(1.85), SER160(2.45), GLN193(2.78), TYR197(2.69), SER262(2.41), GLN192(2.06), ALA194(2.91), SER196(2.27)	ASP147(3.72), SER159(2.95), SER160(3.73), TYR197(2.98), GLN193(2.51), SER196(2.42)	ILE149(1.78), LEU157(2.07), PHE158(1.78), TYR197(2.66), PHE202(2.91), PHE261(2.54), TRP148(3.77), TYR156(3.41), PHE190(2.98)	PHE158(2.46), PHE202(2.92), PHE261(3.00), TYR197(2.90)

PO, Pontal and MP, Madreperola digested protein isolates. EFE: estimated free energy, defined as the predicted Gibbs free energy between peptides and the catalytic site of LOX-1. Docking calculations were carried out using DockingServer. Negative values mean spontaneous reaction.

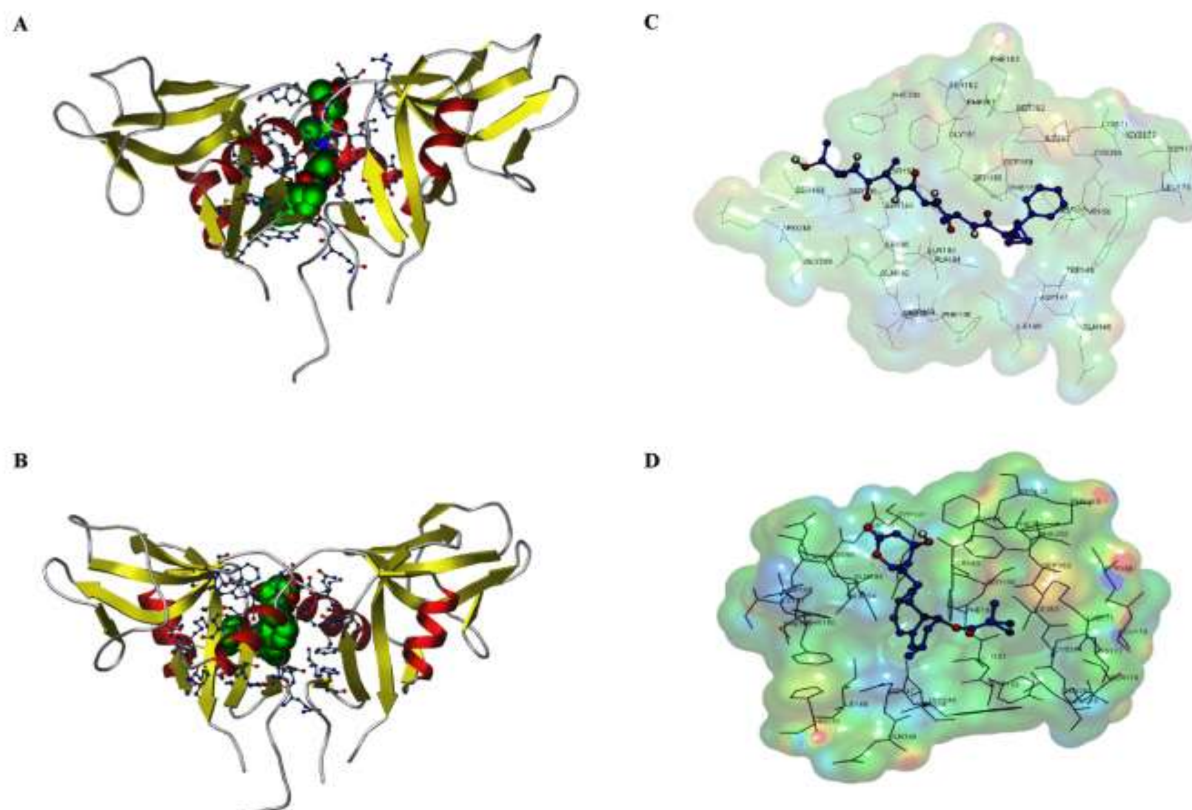

Fig. 5 – Molecular docking diagrams exemplifying the analysis using the FAAAT peptide (A) and simvastatin (B) showing the best pose of the peptide (spheres) inside the LOX-1 catalytic site, and the best pose of the peptide (C) and simvastatin (D) (sticks) with the interacting side chains of the catalytic site LOX-1, lectin type oxLDL receptor.

and pleiotropic effects. We demonstrated for the first time that gastrointestinal digestion of protein isolates from cooked Carioca bean can provide peptides capable to act in steps of the atherosclerotic process preventing events mainly mediated by LOX-1 signalling pathway.

4.1. *Potential mechanism*

A proposed mechanism of action of Carioca bean isolated protein, after simulated gastrointestinal digestion, was associated to protein expression of LOX-1 signalling pathway (Fig. 4). Therefore, the peptides may act on decreasing stimulus for internalization of LDL such as interleukins and oxidation of LDL by inhibiting its receptor (LOX-1) (1); decreasing migration and infiltration of monocytes by reducing expression of adhesion molecules (ICAM-1) and chemoattractant proteins (MCP-1) (2); decreasing foam cell formation mediated by LOX-1 and LDL interaction and monocytes migration (3); and potentially reducing the smooth muscle cells proliferation by decreasing in matrix-degrading proteases (MMP-9) and the inflammatory cytokines expression (4). This suggested mechanism of action needs to be tested in *in vivo* models.

5. Conclusion

Peptides from fresh and stored Carioca beans obtained by simulated gastrointestinal digestion of isolated proteins inhibited ox-LDL induced inflammation and also inhibited markers of atherosclerosis in human THP-1 macrophage-like cells. Storage for 6 months did not impair their biological properties to reduce the expression of LOX-1, MMP-9, ICAM-1 and cytokines related to initiation and progression of atherosclerosis, and some results were even better than for simvastatin.

Conflicts of interest

The authors declare no conflict of interest.

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7. CONCLUSÃO E CONSIDERAÇÕES FINAIS

Este estudo mostrou que as condições de armazenamento utilizadas foram adequadas para preservação da qualidade nutricional e do potencial anti-inflamatório e anti-aterósclerótico, *in vitro*, dos hidrolisados dos feijões carioca. Contudo, as características de resistência parecem determinar efeito diferenciado sobre determinados marcadores, visto que diferenças entre os cultivares foram observadas (0.1 mg/mL: redução de TNF-alfa pelos hidrolisados PO 0, 3 e 6, e por MP 6; redução de IL-1 β por MP 0 e PGE-2 por MP 0 e 6). Ressalta-se a importância desses achados quanto à redução de marcadores de aterosclerose (receptor de LDL-ox, de metaloproteína-9, molécula de adesão intracelular-1, e citocinas ligadas ao processo aterosclerótico). Considerando, que essas propriedades funcionais foram observadas após digestão gastrointestinal simulada, sugere-se que o consumo humano de feijões carioca pode resultar na liberação de peptídeos anti-inflamatórios, anti-oxidantes e anti-aterosclerótico, de tamanhos moleculares passíveis de abosorção. Nesse sentido, estudos com ratos Wistar estão sendo realizados por parte do grupo de pesquisa deste trabalho para avaliar se os efeitos observados *in vitro* acerca do potencial anti-inflamatório e anti-aterosclerótico se manterão *in vivo* após os processos digestivos e absortivos dos hidrolisados, e também da farinha integral dos feijões. Os resultados encontrados em animais poderão fornecer subsídios para a continuidade da investigação em ensaio clínico sobre a redução de risco cardiovascular em humanos.

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