VINÍCIUS PARZANINI BRILHANTE DE SÃO JOSÉ

EFEITO DA PROTEÍNA TOTAL DIGERIDA DA SEMENTE DE CHIA (*Salvia hispânica* L.) NA INFLAMAÇÃO, ESTRESSE OXIDATIVO E SAÚDE INTESTINAL DE CAMUNDONGOS ALIMENTADOS COM DIETA HIPERLIPÍDICA

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

Orientadora: Hercia Stampini Duarte Martino

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Hercia Stampini Duarte Martino
Orientadora

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RESUMO

SÃO JOSÉ, Vinícius Parzanini Brilhante de, M.Sc., Universidade Federal de Viçosa, julho de 2023. **Efeito da proteína total digerida da semente de chia (*Salvia hispânica* L.) na inflamação, estresse oxidativo e saúde intestinal de camundongos alimentados com dieta hiperlipídica.** Orientadora: Hercia Stampini Duarte Martino. Coorientadoras: Mariana Grancieri, Bárbara Pereira da Silva e Izabela Maria Montezano de Carvalho.

O consumo de dietas hiperlipídicas causa aumento da inflamação, do estresse oxidativo e piora da saúde intestinal, resultando em alterações metabólicas. Sendo assim, é de grande interesse o estudo de compostos bioativos que podem modular de forma positiva essas alterações. A chia é uma semente rica em proteína, sendo uma promissora fonte de peptídeos bioativos capazes de reduzir a inflamação e estresse oxidativo e melhorar a capacidade antioxidante e saúde intestinal. Diante disso, o presente estudo avaliou o efeito do consumo da proteína total digerida da semente de chia sobre a inflamação, estresse oxidativo e microbiota intestinal de camundongos C57BL/6 alimentados com uma dieta hiperlipídica. Para isso, 44 camundongos machos foram divididos em 4 grupos experimentais (n=11): AIN: Grupo dieta padrão (AIN-93M); HF: Grupo dieta hiperlipídica; AIN+DTP: Grupo dieta padrão AIN-93M adicionado de 400 mg/kg de proteína total digerida da semente de chia; HF+DTP: Grupo dieta hiperlipídica adicionada de 400 mg/kg de proteína total digerida da semente de chia. Ao final de 8 semanas, os animais foram eutanasiados para coleta do fígado, sangue, cólon e conteúdo do cólon. No artigo 1, foram utilizados 8 animais por grupo, selecionados de forma aleatória. Foram avaliadas a expressão gênica hepática de proteínas relacionadas ao metabolismo lipídico: PPAR-α, AMPK, SREBP-1, CPT-1 e AMPK; estresse oxidativo: NRF2, SOD; inflamação: IL-1β e NFκB; e a quantificação hepática de IL-1β e TNF-α. Também foi avaliada a morfologia hepática, para avaliação do acúmulo de gordura no tecido. O grupo HF+DTP apresentou menor acúmulo de gordura hepática comparado ao HF, com consequente redução da inflamação por meio da redução de NFκB e IL-1β e redução da peroxidação lipídica e atividade da catalase, comparado ao grupo HF. No artigo 2, foram utilizados os dados de 11 animais por grupo. Foi avaliada a expressão gênica de proteínas relacionadas a permeabilidade intestinal: claudina, ocludina e *zonula occludens*; a morfologia do cólon; e análise da microbiota intestinal através do rRNA 16S. Os grupos AIN+DTP e HF+DTP apresentaram a espessura de criptas e da camada muscular circular aumentadas, aumento na abundância de *Alistipes* e *Olsenella* e redução na abudância de *Akkermansia*, quando comparadas aos grupos AIN e HF respectivamente. Além disso, o grupo HF+DTP teve

redução da dominância bacteriana através do indice de Simpson, comparado ao HF. Ademais, todos os grupos se diferiram com relação a β-diversidade. Dessa forma, o consumo da proteína digerida de chia atua no fígado reduzindo o acúmulo lipídico, inflamação e estresse oxidativo, além de melhorar a morfologia com aumento da espessura das criptas e aumento da camada muscular circurlar do cólon e modificar a microbiota intestinal.

Palavras-chave: Peptídeos bioativos. Gordura saturada. Obesidade. Tecido hepático. Compostos bioativos de plantas. Microbiota.

ABSTRACT

SÃO JOSÉ, Vinícius Parzanini Brilhante de, M.Sc., Universidade Federal de Viçosa, July, 2023. **Effect of the digested total protein from chia seeds (*Salvia hispânica* L.) on inflammation, oxidative stress and intestinal health of mice fed a high-fat diet.** Advisor: Hercia Stampini Duarte Martino. Co-Advisers: Mariana Grancieri, Bárbara Pereira da Silva and Izabela Maria Montezano de Carvalho.

The consumption of high-fat diets causes an increase in inflammation, oxidative stress, and worsens intestinal health, resulting in metabolic changes. Therefore, the study of bioactive compounds that can positively modulate these changes is of great interest. Chia seeds are rich in protein, making them a promising source of bioactive peptides capable of reducing inflammation and oxidative stress while improving antioxidant capacity and intestinal health. In light of this, the present study evaluated the effect of consuming the total digested protein from chia seeds on inflammation, oxidative stress, and intestinal microbiota in C57BL/6 mice fed a high-fat diet. For this, 44 male mice were divided into 4 experimental groups (n=11): AIN: Standard diet group (AIN-93M); HF: High-fat diet group; AIN+DTP: AIN-93M standard diet group supplemented with 400 mg/kg of total digested protein from chia seeds; HF+DTP: High-fat diet group supplemented with 400 mg/kg of total digested protein from chia seeds. After 8 weeks, the animals were euthanized to collect liver, blood, colon, and colon content. In article 1, 8 animals per group were randomly selected. Hepatic gene expression of proteins related to lipid metabolism was evaluated: PPAR-α, AMPK, SREBP-1, CPT-1, and AMPK; oxidative stress: NRF2, SOD; inflammation: IL-1β and NFκB; and hepatic quantification of IL-1β and TNF-α. Hepatic morphology was also assessed for fat accumulation in the tissue. The HF+DTP group showed less hepatic fat accumulation compared to HF, with a consequent reduction in inflammation through the reduction of NFκB and IL-1β, and a reduction in lipid peroxidation and catalase activity compared to the HF group. In article 2, data from 11 animals per group were used. Gene expression of proteins related to intestinal permeability was evaluated: claudin, occludin, and zonula occludens; colon morphology; and analysis of intestinal microbiota through 16S rRNA. The AIN+DTP and HF+DTP groups showed increased crypt and circular muscle layer thickness, an increase in the abundance of Alistipes and Olsenella, and a reduction in Akkermansia abundance compared to the AIN and HF groups, respectively. Additionally, the HF+DTP group had reduced bacterial dominance through the Simpson index compared to HF. Moreover, all groups differed in terms of β-diversity. Thus, the consumption of digested chia protein acts on

the liver by reducing lipid accumulation, inflammation, and oxidative stress, while improving morphology with increased crypt thickness and circular muscle layer of the colon, and modifying intestinal microbiota.

Keywords: Bioactive peptides. Saturated fat. Obesity. Liver tissue. Bioactive plant compounds. Microbiota.

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LISTA DE ABREVIAÇÕES

°C: Graus Celsius

µL: Microlitros

ABTS: Ácido 2,2'-azino-bis(ácido 3-etilbenzotiazolina-6-sulfônico)

ACC: Acetil-CoA carboxilase

AIN-93M: Dieta para roedores AIN-93M

ALA: Alanina

ALT: Alanina aminotransferase

AMPK: Proteína quinase ativada por AMP

AP-1:

ARG: Arginina

AST: Aspartato aminotransferase

bp: Pares de base

CAT: Catalase

cDNA: DNA complementar

CEUA: Comissão de Ética no Uso de Animais

COX-2: Cicloxigenase 2

CPT: Carnitina palmitoil transferase

CYS: Cisteina

Da: Dalton

DEPC: Dietil-pirocarbonato

DNA: Ácido desoxirribonucleico

DNS: Departamento de Nutrição e Saúde

DPF: Frações proteicas digeridas

DPP IV: Dipeptidil peptidase 4

DPPH: 2,2-difenil-1-picrilhidrazil

DTP: Proteína total digerida

EDTA: Ácido etilenodiamino tetra-acético

EFE: Energia livre estimada

EUA: Estados Unidos da América

FAS: Ácido graxo sintase

FER: Coeficiente de eficiência alimentar

Fig: Figura

G: Grama

GLN: Glutamina

GLP-1: Peptídeo semelhante ao glucagon

GLU: Ácido glutâmico

GPx: Glutationa peroxidase

H: Hora

H_2O_2: Peróxido de hidrogênio

HF: Hiperlipídica

HFD: Dieta hiperlipídica

HFDP: Dieta hiperlipídica adicionada de proteína digerida

HIS: Histidina

HMG-Coa: 3-hidroxi-3-methyl-glutaril-CoA

IKKβ: Inibidor do fator nuclear kappa-B quinase subunidade beta

IL-10: Interleucina 10

IL-1β: Interleucina 1 beta

IL-6: Interleucina 6

ILE: Isoleucina

iNOS: Óxido nítrico sintase induzível

IκB: Inibidor do fator nuclear kappa B

JSH23: 4-Methyl-1-N-(3-phenylpropyl) benzene-1,2- diamine

Kcal: Quilocalorias

Kg: Quilograma

LEU: Leucina

LNE: Laboratório de nutrição experimental

LOX-1: Lipoxigenase 1

LPL: Lipase Lipoproteica

LPS: Lipopolissacarídeos

LYS: Lisina

MCP-1: Proteína de quimioatração de monócitos

MDA: Malondialdeído

MET: Metionina

Mg: Miligrama

Min: Minutos

mL: Mililitros

MTT: Brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio

NCBI: National Center for Biotechnology Information

ND: Dieta padrão

NDDP: Dieta padrão adicionada de proteína digerida

NFκB: Fator nuclear kappa B

nm: Nanometros

NRF2: Fator nuclear relacionado ao fator eritroide 2

OTU: Unidades taxonômicas operacionais

ox-LDL: Lipoproteína de baixa densidade oxidada

PCoA: Análise das coordenadas principais

PCR: Reação em cadeia da polimerase

PHE: Fenilalanina

p-JNK:

PPAR-α: Receptor alfa ativado por proliferadores de peroxissomos

PPARγ: Receptor gama ativado por proliferadores de peroxissomos

PRO: Prolina

PTN: Proteína

RNA: Ácido ribonucleico

ROS: Especies reativas de oxigenio

RT-qPCR: Transcrição reversa-reação em cadeia da polimerase quantitativa

SCFA: Ácidos graxos de cadeia curta

SD: Desvio padrão

SER: Serina

SOD: Superóxido dismutase

SRA: Arquivo de leitura de sequência

SREBP1c: Proteína 1c de ligação ao elemento regulador de esterol

TAC: Capacidade antioxidante total

TBARS: Ácido tiobarbitúrico

THR: Treonina

TLR4: Receptor do tipo toll 4

TNF-α: Fator de necrose tumoral alfa

TYR: Tirosina

UFV: Universidade Federal de Viçosa

VAL: Valina

ZO-1: Zonula ocludens 1

SUMÁRIO

1. INTRODUÇÃO

A alimentação ocidental apresenta elevado conteúdo de gordura saturada. O uso crônico de dietas hiperlipídicas leva a alterações na cadeia transportadora de elétrons causando redução da β-oxidação hepática e aumento de espécies reativas de oxigênio (ROS) (SOUZA et al., 2022; ZOU et al., 2022). Com a redução da β-oxidação, há acúmulo de substrato energético, o qual será armazenado como triglicerídeos nos hepatócitos (FERREIRA et al., 2018). Assim, há formação de hepatócitos balonizados, levando à inflamação local e aumento da produção de citocinas inflamatórias como interleucina 1 beta (IL-1β) e fator de necrose tumoral alfa (TNF-α), causando alterações morfológicas como inflamação lobular, fibrose e necrose celular (LEE et al., 2018; HAN et al., 2023). Tais alterações causam desajustes na homeostase e funcionamento de diversos órgãos, como fígado e tecido adiposo, propiciando o desenvolvimento de doenças crônicas não transmissíveis (BAUER et al., 2023; DUAN et al., 2022).

Além disso, o consumo de dietas ricas em gordura saturada está diretamente relacionado à piora da saúde intestinal, com redução da riqueza e diversidade e aumento da dominância de algumas espécies bacterianas, como o filo Firmicutes, e também ao desenvolvimento e agravamento do processo inflamatório, devido ao aumento da permeabilidade intestinal com translocação de moléculas inflamatórias como os lipopolissacarídeos (LPS) (ZHENG et al., 2016; HUANG et al., 2021; WANG et al., 2021).

Ademais, as dietas hiperlipídicas também podem alterar a morfologia intestinal. Diversos estudos mostram aumento da permeabilidade intestinal devido ao consumo crônico deste tipo de dieta (LI et al., 2021; NAKANISHI et al., 2021; YANG et al., 2022). Isso se deve à ruptura das proteínas transmembranas das *tigh-junctions* (NASCIMENTO et al., 2021). Assim, há aumento da permeação intestinal, com passagem de microrganismos e antígenos como o LPS, do lúmen intestinal para a matriz extracelular e circulação sanguínea, que levam à inflamação local e sistêmica (PAIK et al., 2013).

Sendo assim, o estudo de alimentos e compostos bioativos com potencial para redução da inflamação e estresse oxidativo causado por dietas hiperlipídicas, é de grande interesse para a comunidade acadêmica e saúde pública. Nesse contexto, a chia (*Salvia hispânica* L.) é uma semente que apresenta elevado conteúdo de proteínas (19 a 23%) as quais apresentam todos os aminoácidos essenciais (GRANCIERI; MARTINO; GONZALEZ DE MEJIA,

2019). As proteínas são moléculas grandes, as quais devem ser digeridas para que sejam absorvidas pelo organismo. Durante esse processo as proteínas dão origem a peptídeos bioativos de baixo peso molecular que apresentam potenciais benefícios para a saúde (HERNÁNDEZ-LEDESMA; MARTÍNEZ-VILLALUENGA, 2022).

Estudos *in vitro* demonstraram que a proteína total digerida de chia (DTP), é capaz de prevenir a adipogênese e inflamação em adipócitos por meio da redução da expressão do receptor gama ativado por proliferadores de peroxissomos (PPARγ), ácido graxo sintase (FAS), fator nuclear kappa B (NFκB) e TNF-α (GRANCIERI; MARTINO; MEJIA, 2020). Também, esse composto apresentou melhora em indicadores de estresse oxidativo em ensaios químicos e bioquímicos (GRANCIERI; MARTINO; GONZALEZ DE MEJIA, 2019). Resultados semelhantes, foram encontrados em estudos com camundongos C57BL/6, no qual a DTP de chia reduziu o percentual de gordura dos animais, área dos adipócitos e focos de inflamação do tecido adiposo. Ainda, houve redução dos niveis de NFκB e PPARγ, e redução da expressão da Proteína de ligação ao elemento regulador de esterol (SREBP1) e TNF-α (GRANCIERI et al, 2022) . Em estudo *in ovo*, a proteína hidrolisada de chia, apresentou melhora na inflamação e na permeabilidade intestinal, além de estímulo para a proliferação de bactérias benéficas (MISHIMA et al., 2023b).

No entanto, ainda são inexistentes estudos que avaliaram os efeitos da proteína digerida de chia na inflamação, estresse oxidativo, acúmulo de gordura e morfologia hepática e nas alterações da riqueza, diversidade e adundância da microbiota intestinal no contexto de uma dieta hiperlipídica. Assim, pretende-se, por meio desse estudo, investigar o efeito do consumo de proteína digerida da chia na inflamação, estresse oxidativo, microbiota intestinal e morfologia hepática e intestinal de camundongos alimentados com uma dieta hiperlipídica.

2. OBJETIVOS

2.1 Objetivo geral

Avaliar os efeitos da proteína total digerida da semente de chia na inflamação e estresse oxidativo hepático e na saúde intestinal de camundongos alimentados com dieta hiperlipídica.

2.2 Objetivos específicos

- Avaliar a expressão, quantificação e concentração de proteínas e enzimas relacionadas ao processo de inflamação, estresse oxidativo, peroxidação lipídica e atividade

antioxidante e morfologia hepática de camundongos alimentados com dieta hiperlipídica adicionada da proteína total digerida da semente de chia;

- Avaliar alterações nas *tight junctions*, morfologia e microbiota intestinal de camundongos alimentados com dieta hiperlipídica adicionada da proteína total digerida da semente de chia.

3. HIPÓTESE

A proteína digerida de chia irá fornecer peptídeos bioativos com capacidade anti-inflamatória e antioxidante, reduzindo a inflamação e o estresse oxidativo hepático causado pelo consumo de uma dieta hiperlipídica. Além disso, as propriedades antioxidantes, anti-inflamatórias e antimicrobianas dos peptídeos irão atuar na microbiota intestinal, melhorando sua permeabilidade e composição.

4. REVISÃO BIBLIOGRÁFICA

4.1 Dieta hiperlipídica e alterações metabólicas

O padrão de dieta ocidental é caracterizado por elevado conteúdo em açúcares e gorduras, principalmente as saturadas, sendo o padrão predominante em países como Brasil e EUA. Segundo o último *Dietary Guidelines for Americans* (2020-2025), o consumo de gorduras saturadas está acima da recomendação em todas as idades, sendo esse fator relacionado ao aumento da prevalência de diversas doenças, como câncer, obesidade e diabetes, e também está relacionado com o desenvolvimento de distúrbios metabólicos, como estresse oxidativo e inflamação hepática (USDA, 2020; CARDOSO et al., 2021).

Dentre os tecidos mais afetados pelas dietas hiperlipídicas estão o tecido adiposo e hepático, devido ao papel de ambos para a metabolização da gordura ingerida na dieta, as quais, são utilizadas para a síntese de hormônios, estruturas biológicas e como reserva energética (TURNER et al., 2014; VAN DER HEIJDEN et al., 2015; HACZEYNI; BELL-ANDERSON; FARRELL, 2018). No entanto, o consumo excessivo de gordura saturada, associado ao estilo de vida sedentário, promove o excesso de ácidos graxos livres circulantes, estimulando a sua deposição em órgãos não adiposos, como por exemplo o fígado (IPSEN; LYKKESFELDT; TVEDEN-NYBORG, 2018; LIAN et al., 2020).

Dentre os mecânimos responsáveis pelo aumento dos ácidos graxos livres na circulação, temos a absorção dos ácidos graxos advindos da dieta. Os lipídios ingeridos na

dieta, são absorvidos pelos enterócitos e incorporados aos quilomícrons na forma de triglicerídeos. Em seguida, são lançados para a circulação linfática e, após isso, seguem para a circulação sanguínea onde sofrem ação da lipase lipoproteica, liberando cerca de 30% do conteúdo de lipídios na forma de ácidos graxos livres, os quais são direcionados ao fígado para serem metabolizados (FIELDING, 2011). Além disso, o aumento de ácidos graxos livres pode ser devido ao aumento da lipólise e lipogênese de novo. Ambos os mecanismos são aumentados devido à dieta hiperlipídica, visto que, essa dieta causa inibição do receptor de insulina, levando à resistência insulínica. Assim, há estímulo para síntese de lipídios e liberação dos ácidos graxos armazenados no tecido adiposo (LIAN et al., 2020).

Dessa forma, a dieta hiperlipídica, causa aumento de ácidos graxos livres no fígado levando à redução da fluidez das membranas do retículo endoplasmático e alteração na cadeia transportadora de elétrons mitocondrial, causando aumento de proteínas mal dobradas, e geração de ROS como os radicais superóxido e hidroxila, resultando em aumento do estresse oxidativo (FU et al., 2011; NIVALA et al., 2013; ZOU et al., 2022). O aumento do estresse oxidativo, pode causar fosforilação e consequente degradação do inibidor do fator nuclear kappa-B quinase subunidade beta (IKKβ), permitindo a translocação de NFκB para o núcleo celular, aumentando a expressão de citocinas pró-inflamatórias como TNF-α e IL-1β, resultando em inflamação e dano hepático (NIVALA et al., 2013; AHMED; ROBINSON; O'FARRELLY, 2021; TILG et al., 2021).

Além disso, o excesso de ácidos graxos hepáticos estimula o receptor alfa ativado por proliferadores de peroxissomos (PPAR-α) a aumentar a oxidação lipídica, causando danos à função mitocondrial. Com isso, há menor oxidação mitocondrial e maior oxidação de ácidos graxos nos peroxissomos e citocromos, com produção de ROS (IPSEN; LYKKESFELDT; TVEDEN-NYBORG, 2018; JANA et al., 2019; SILVA et al., 2019). Esse aumento de ROS desencadeia a peroxidação lipídica, principalmente de lipídios insaturados, com formação de malondialdeído, que aumenta o dano mitocondrial e fibrose hepática (UCAR et al., 2013; MATSUMOTO et al., 2018).

Outra via importante na oxidação lipídica é a da proteína quinase ativada por AMP (AMPK), uma vez que é capaz de fosforilar e inativar a enzima acetil-CoA carboxilase (ACC), responsável pela síntese de ácidos graxos. Assim, não há uma inibição alostérica da carnitina palmitoil transferase (CPT) pela ACC, permitindo o transporte de ácidos graxos para dentro das mitocôndrias para serem oxidados (SCHINDLER et al., 2017; KE et al., 2018;

LINGESH et al., 2019). Apesar disso, o uso crônico de dietas hiperlipídicas pode atuar inibindo a AMPK, com consequente ativação da ACC, resultando em redução da oxidação lipídica e aumento da síntese de ácidos graxos, respectivamente. Dessa forma, há o acúmulo de lipídios intra-hepáticos, causando ativação de vias inflamatórias e dano celular (SHENG et al., 2019).

Além disso, o aumento da síntese de ácidos graxos está relacionado ao aumento de PPAR-γ, o qual é responsável pela diferenciação de adipócitos e pelo processo de lipogênese (GUO et al., 2022). O uso de dietas hiperlipídicas, causa maior ativação do PPAR-γ, o qual promove a regulação positiva da proteína de translocase de ácido graxo (CD36), responsável por facilitar o transporte de ácidos graxos de cadeia longa. Assim, há a maior internalização de lipídeos nos hepatócitos, podendo causar alterações metabólicas como resistência insulínica e esteatose hepática não alcoólica (FEBBRAIO, 2009; NIU et al., 2019; SILVERSTEIN).

O aumento de CD36 leva ao maior transporte da proteína 1c de ligação ao elemento regulador de esterol (SREBP1c) para o complexo de golgi, onde é processada e liberada na sua forma ativa, SREBP (MOON, 2017; SHIMANO; SATO, 2017; ZENG et al., 2022). O fator de transcrição SREBP é então translocado para o núcleo, estimulando a expressão do gene da ACC e FAS, aumentando assim a síntese de lipídeos intra-hepáticos (GONG et al., 2023; IPSEN; LYKKESFELDT; TVEDEN-NYBORG, 2018).

Este acúmulo de triglicerídeos hepáticos leva a alterações no metabolismo desse órgão (PENG et al., 2018; KOH et al., 2020), com aumento da geração de ROS, migração de células imunes, levando à ativação de vias inflamatórias com consequente produção de moléculas, TNF-α, NFκB e IL-1β (MORETTINI et al., 2015; AHMED; ROBINSON; O'FARRELLY, 2021).

Apesar disso, nosso organismo conta com mecanismos de defesa endógenos e exógenos que visam a neutralização de ROS, evitando assim o início do processo inflamatório exacerbado e possíveis danos celulares. Dentro da categoria dos endógenos, temos diversas enzimas antioxidantes atuam nesse processo, sendo as principais a catalase (CAT), superóxido oxidase (SOD) e a glutationa peroxidase (GPx) (VELLOSA et al., 2021). A enzima GPx atua na oxidação do H_2O_2, gerando água e a enzima CAT oxida H_2O_2 produzindo água mais oxigênio. A SOD atua na conversão do radical superóxido em oxigênio e H_2O_2, controlando

assim seu potencial citotóxico (WANG et al., 2018b ; SILVA et al., 2020; VELLOSA et al., 2021).

Dentro dos mecanismos de defesa exógenos temos a dieta, que pode atuar melhorando atividade das enzimas antioxidantes endógenas, ou fornecer vitaminas, minerais e compostos bioativos que possuem ação antioxidante, atuando na redução do estresse oxidativo e inflamação (BARBOSA et al., 2010; ALEKSANDROVA; KOELMAN; RODRIGUES, 2021). Dessa forma, diversos alimentos como o feijão, soja e chia vêm sendo pesquisados como alternativas para prevenir e reduzir o estresse oxidativo e inflamação (DA SILVA et al., 2019a; LIMA et al., 2019; PANG et al., 2020). Dentre esses, a chia na forma de semente, farinha e óleo apresenta grande potencial, devido ao seu conteúdo de antioxidantes, peptídeos bioativos, compostos fenólicos, ácido α-linolênico e fibra alimentar, os quais atuam na redução de mediadores inflamatórios, redução de tecido adiposo e melhora da atividade de enzimas antioxidantes (FERREIRA et al., 2018; DA SILVA et al., 2019b; ENES et al., 2020a).

4.2 Microbiota, morfologia e funcionalidade intestinal x Dieta hiperlipídica

Lederberg e McCray foram os primeiros cientistas a definir o conceito de microbiota, sendo essa “o conjunto de microrganismos presentes em um ambiente definido” (LEDERBERG; MCCRAY, 2001; MARCHESI; RAVEL, 2015). Desde a sua definição, cada vez mais estudos vêm sendo realizados para identificar a relação entre a microbiota humana ou animal, e sua influência no hospedeiro.

Dentro da saúde humana, a microbiota do trato gastrointestinal se destaca, por ser o ambiente corporal com maior densidade microbiana, sendo estimado que contenha aproximadamente 10^{14} microrganismos. A maior parte da população microbiana é composta por bactérias, as quais exercem funções comensais e simbióticas com o organismo hospedeiro (ARUMUGAM et al., 2011; DE MORAES et al., 2014). Análises taxonômicas da microbiota demonstraram que as bactérias mais predominantes são as pertencentes aos filos Firmicutes e Bacteroidetes (BOLAM; VAN DEN BERG, 2018). Apesar disso, a abundância tanto de filos, mas principalmente de espécies, é altamente influenciadas por diversos fatores como hábito alimentar, atividade física, uso de fármacos entre outros (BECATTINI; TAUR; PAMER, 2016; MALESZA et al., 2021a ; CAMPANIELLO et al., 2022).

Dentro dos hábitos alimentares, os estudos vêm demonstrando forte relação entre o consumo de dietas ricas em gorduras e alterações na composição da microbiota intestinal e desenvolvimento da obesidade (WAN et al., 2019; STANISLAWSKI et al., 2021; MISHIMA et al., 2023a). Estudos recentes demonstraram que o consumo de dietas ricas em gordura pode alterar a composição da microbiota intestinal, aumentando a proporção da razão *Firmicutes*/*Bacteroidetes*, a qual está relacionada com o desenvolvimento da obesidade (WANG et al., 2018a; JO et al., 2021). Além disso, o aumento dessa proporção tem sido postulado como um dos causadores da obesidade, uma vez que está relacionada ao maior aproveitamento energético alimentar, contribuindo assim, para o superávit calórico (KOLIADA et al., 2017). No entanto, tal proporção ainda apresenta resultados contraditórios, e por isso, alguns autores não aconselham o seu uso como marcador para doenças como a obesidade (MAGNE et al., 2020).

Uma metanálise, relacionando a microbiota intestinal e o consumo de dietas hiperlipídicas, revelou que esse padrão de dieta leva a uma redução modesta na riqueza e diversidade microbiana. Além disso, a avaliação da proporção *Firmicutes*/*Bacteroidetes* estava aumentada na maior parte dos estudos avaliados, apresentando forte correlação com o consumo de gordura (BISANZ et al., 2019).

Em outros estudos com animais a dieta hiperlipídica reduziu a abundância de *Bifidobactérias, Proteobacteria*, *Akkermansia* (HILDEBRANDT et al., 2009; JU et al., 2019; SHAO et al., 2020). Tais bactérias estão relacionadas produção de ácido butírico (RIVIÈRE et al., 2015), preservação da microbiota (REN et al., 2023), cognição e metabolismo energético cerebral (HIGARZA et al., 2021) e homeostase de glicose (YOON et al., 2021).

Além disso, o padrão de dieta ocidental está relacionado a alterações na permeabilidade intestinal, levando a hiperpermeabilidade intestinal (MUNKHOLM et al., 1994; HOU; ABRAHAM; EL-SERAG, 2011; MICHIELAN; D'INCÀ, 2015). Com isso, há maior translocação de microrganismos assim como antígenos alimentares ao longo do trato gastrointestinal, causando piora ou desenvolvimento de doenças intestinais como colite ulcerativa e doença intestinal inflamatória (PAIK et al., 2013; ANANTHAKRISHNAN, 2015; CHENG et al., 2016).

O aumento da permeabilidade se deve à capacidade das dietas hiperlipídicas de alterar a distribuição e expressão das *tigh-junctions* intestinais (ROHR et al., 2020). As *tigh-*

junctions são complexos multiméricos formado por proteínas transmembranas como a E-caderina, ocludina, claudina e molécula aderente juncional (SHEN et al., 2011; ROHR et al., 2020). Em estudos realizados com camundongos, o uso de dietas hiperlipídicas levou à redução da expressão de *tigh-junctions* aumentando a permeabilidade intestinal (CANI et al., 2008; SUZUKI; HARA, 2010; KIRPICH et al., 2012). Em estudos in vitro, foram observadas também alterações na distribuição das proteínas actina e *tigh-junctions*, mediado pela redução da proteína quinase C, a qual é capaz de aumentar a transcrição das proteínas ocludina e claudina (USAMI et al., 2003; KOIZUMI et al., 2008; TORELLI HIJO et al., 2019).

4.3 Efeitos da chia (*Salvia hispânica* L.) nas alterações metabólicas

A semente de chia (*Salvia hipanica* L.) é originaria de uma planta herbácea, nativa da Guatemala e sul do México (OLIVEIRA-ALVES et al., 2017). O gênero Salvia é o mais numeroso dentro da família Lamiaceae, sendo amplamente distribuída em regiões Sul da África, América e Sudeste da Ásia (TAKANO, 2017). Apesar do aumento do consumo da chia nos últimos anos, essa semente é consumida desde o século de 16 pelos povos pré-colombianos, com o objetivo de fornecer energia, resistência e força, e pelos soldados Astecas para suprir suas necessidades durante expedições e batalhas (AYERZA; COATES, 2009;).

A composição química e nutricional da chia pode variar de acordo com as condições climáticas e geográficas dos locais onde é cultivada (AYERZA, 2009). Mas, no geral, apresentam elevado conteúdo de fibras alimentares (35,3%), proteínas (18,9%) e lipídios (31,2%), com predomínio dos ácidos graxos poli-insaturados, principalmente o ômega 3 (ácido alfa linolênico) (20,37%), conhecido por efeitos benéficos em diversas doenças crônicas não transmissíveis (DA SILVA et al., 2017). Além disso, contém flavonoides, antocianinas, vitamina E, carotenoides e minerais (ferro, cálcio, fosforo e potássio) (AYERZA; COATES, 2011; SILVA et al., 2017). Tal composição química é uma das principais razões pela qual a chia vem sendo cada vez mais consumida, seja ela *in natura* ou adicionada a preparações, como bolos, pães, iogurtes, barras de cereal, dentre outros produtos. A adição dessa semente é uma alternativa para aumentar o conteúdo de fibra alimentar, lipídios poli-insaturados, compostos antioxidantes, proteínas e compostos fenólicos (predominantemente ácido rosmarínico e rosmarinil glicosídeo), gerando produtos com benefícios a saúde intestinal e em geral (BORNEO; AGUIRRE; LEÓN, 2010; HO et al., 2013; SILVA et al., 2017, 2019; ENES et al., 2020b; MOREIRA et al., 2022). Em estudo realizado em modelo animal, o extrato solúvel de chia se mostrou eficaz em melhorar

parâmetros histológicos, com aumento de vilosidades e células caliciformes (DA SILVA et al., 2019b)

Dentre os nutrientes da chia, destaca-se a proteína. Análises realizadas com sementes de chia brasileira mostrou que a mesma possui, aproximadamente, 19% de proteína (DA SILVA et al., 2017), percentual superior aos encontrados em outros cereais, como trigo (14%), cevada (9,2%), aveia (15,3%), milho (14%) e arroz (8,5%) (MONROY-TORRES et al., 2008). Além disso, as proteínas da chia apresentam boa estabilidade térmica entre temperaturas que variam de 70,4 a 125°C, boa retenção de água e óleo (4,06 e 4,04 g/g, respectivamente), além da capacidade de formar géis estáveis, principalmente pela presença de glutelinas (OLIVOS-LUGO; VALDIVIA-LÓPEZ; TECANTE, 2010).

Devido às suas características físico-químicas, concentrados proteicos de chia estão sendo utilizados como ingredientes em pães e suplementos proteicos veganos como alternativa para o fornecimento de peptídeos bioativos, melhora do perfil proteico e maior conteúdo antioxidante (MADRUGA, 2018; ATHLETICA NUTRITION, c2022).

Vinte proteínas da chia foram descritas usando como base sua sequência de aminoácidos (GRANCIERI; MARTINO; MEJIA, 2019a). Essas proteínas apresentam boa digestibilidade (78,9%), sendo capazes de fornecer todos os aminoácidos essenciais necessários para a nutrição humana (SANDOVAL-OLIVEROS; PAREDES-LÓPEZ, 2013). Diante dessa elevada concentração proteica e satisfatório perfil de aminoácidos, a chia é promissora fonte de peptídeos bioativos (GRANCIERI; MARTINO; MEJIA, 2019c).

Os peptídeos bioativos da chia se encontram inativos enquanto não houver a hidrólise das estruturas proteicas. Diferentes métodos de digestão proteica como a fermentação, hidrólise química, digestão gastrointestinal e enzimática podem levar à formação de diferentes peptídeos, visto que cada um irá exercer sua ação em aminoácidos específicos (UDENIGWE; ALUKO, 2012; ORONA-TAMAYO et al., 2015).

Assim, após sua absorção, esses peptídeos podem exercer diferentes funções biológicas como antimicrobianas (aumentando a permeabilidade de membrana de bactérias gram positivas, reduzindo assim seu crescimento), hipocolesterolemiantes (através da inibição da enzima HMG-Coa redutase), hipoglicemiantes (inibindo a degradação da dipeptidil peptidase IV), hipotensivas (inibindo a atividade da enzima conversora da angiotensina), antioxidantes e anti-inflamatórios (através da inibição da translocação de NFκB) (ORONA-TAMAYO et al., 2015; CICERO; FOGACCI; COLLETTI, 2017; COELHO et al., 2018;

GRANCIERI; MARTINO; MEJIA, 2019a; AGUILAR-TOALÁ; DEERING; LICEAGA, 2020; GRANCIERI; MARTINO; DE MEJIA, 2021).

4.4 Proteína digerida de chia, estresse oxidativo, inflamação e saúde intestinal

A proteína de chia, quando hidrolisada, gera diversos compostos bioativos, que podem variar de acordo com o método usado para hidrólise (GRANCIERI; MARTINO; MEJIA, 2019b). Quando realizada hidrólise sequencial da proteína de chia, utilizando alcalase e flavourenzima, são formados alguns di e tetras peptídeos que apresentam alta capacidade antioxidante. Esses peptídeos de baixo peso molecular se mostraram ricos em aminoácidos aromáticos, capazes de doar elétrons, estabilizando assim moléculas reativas, o que justifica o aumento da capacidade antioxidante provocada por esses peptídeos (SARMADI; ISMAIL, 2010; SILVEIRA COELHO et al., 2019; URBIZO-REYES et al., 2019). Além disso, alguns dos peptídeos gerados pela digestão da proteína de chia apresentam enxofre, o que torna esses peptídeos mais biodisponíveis para quelar metais livres, reduzindo o poder pró-oxidante desses metais (BARBOSA et al., 2010; URBIZO-REYES et al., 2019). Essa capacidade antioxidante dos peptídeos da chia é importante para diminuir os danos vasculares causados por dietas hiperlipídicas e/ou hiperglicídicas (SCHAAN; MARCOS VARGAS DA SILVA; CLÁUDIA IRIGOYEN, 2010).

Em uma revisão elaborada sobre as proteínas de chia, foram catalogadas um total de 20 proteínas na semente de chia, sendo 12 relacionadas ao processo de metabolismo das células das plantas, e 8 proteínas que atuam sobre a produção e armazenamento lipídico. Em seguida, foi avaliada a sequência de aminoácidos dessas proteínas e gerado o perfil de peptídeos, dos quais muitos apresentavam funções bioativas, como ação hipoglicemiante, hipotensiva, inibição da dipeptidil peptidase IV, inibição da enzima conversora de angiotensina e capacidade antioxidante. O efeito antioxidante foi o que apresentou maior frequência (GRANCIERI; MARTINO; MEJIA, 2019a).

A partir desses resultados, o mesmo grupo de pesquisa realizou um estudo *in silico* para determinar o potencial antioxidante, anti-inflamatório e anti-aterosclerótico dos peptídeos provenientes da proteína total digerida de chia e suas frações (albumina, globulina, prolamina e glutelina). Assim, foram identificados 596, 631, 612, 634, e 707 peptídeos da DTP e suas frações respectivamente. No entanto, apenas os peptídeos com ação antioxidante (54 peptídeos) foram analisados. Os efeitos dos peptídeos foram avaliados nos marcadores LOX-1, COX-2, p-65 NFκB e TLR4. O peptídeo TGPSPTAGPPAPGGGTH, proveniente da

fração albumina foi o que apresentou maior interação com o LOX-1 e TLR-4, sendo essa maior que o dos controles farmacológicos sinvastatina e atorvastatina. Além disso, a albumina também gerou o peptídeo SPKDLALPPGALPPVQ, que apresentou alta interação com o COX-2, sendo superior ao controle dexametasona. A DTP gerou o peptídeo HYGGPPGGCR, que apresentou maior interação que o controle farmacológico JSH23 (4-Methyl-1-N-(3-phenylpropyl) benzene-1,2- diamine), para o marcador p-65 NFκB (GRANCIERI; MARTINO; MEJIA, 2019b).

Ainda nesse mesmo estudo, foram realizadas análises bioquímicas para avaliar o potencial antioxidante, anti-inflamatório e anti-aterosclerótico da DTP e suas frações. Em relação à capacidade antioxidante, a fração glutelina apresentou a maior capacidade de eliminação do anion superóxido. Em relação à eliminação do radical óxido nítrico, a maior capacidade de eliminação foi para a fração albumina. Para a eliminação de peróxido de hidrogênio, tanto a DTP quanto suas frações não apresentaram diferença. Em relação à inibição de DPPH, a maior capacidade de inibição foi encontrada para a DTP e as frações albumina e glutelina. As análises bioquímicas anti-inflamatórias, as frações globulina, prolamina e glutelina, apresentaram maior inibição da 5-LOX, enquanto a DTP e albumina inibiram mais a COX 1 e 2 (GRANCIERI; MARTINO; MEJIA, 2019b).

A partir dos resultados positivos na redução da inflamação e estresse oxidativo, encontrados nas análises *in silico* e bioquímicas, nosso grupo de pesquisa realizou estudos *in vitro*. Assim, macrófagos RAW 264.4 foram estimulados a desenvolver inflamação com LPS e aterosclerose com ox-LDL, em seguida foram tratados com a DTP e suas frações. Tanto DTP quanto as frações foram capazes de reduzir p-NFκB, iNOS, p-JNK, e AP-1, e também reduziram a translocação de NFκB para o núcleo. A fração glutelina reduziu a secreção de óxido nítrico, espécies reativas de oxigênio, prostaglandinas, TNF-α, proteína de quimioatração de monócitos (MCP-1), interleucina 6 (IL-6) e interleucina 10 (IL-10). Além disso, a DTP foi capaz de reduzir a secreção de óxido nítrico, acúmulo lipídico, prostaglandinas, TNF-α, MCP-1 e IL-6. Dessa forma, a DTP e glutelina foram eficazes em reduzir as vias inflamatórias e aterogênicas *in vitro* (GRANCIERI; MARTINO; MEJIA, 2019e).

Em um novo experimento *in vitro* realizado com pré-adipocitos (3T3-L1) tratados durante a diferenciação com a DTP ou albumina ou glutelina ou peptídeos puros (NSPGPHDVALDQ e RMVLPEYELLYE) foi observada a prevenção da adipogênese em

todos os tratamentos, através da redução da expressão de PPARγ, LPL, FAS e SREBP1. Além disso, nesse experimento também foi feita a indução da inflamação, a qual foi inibida pela DTP, através da redução da expressão de NFκB e da secreção de TNF-α, óxido nítrico e COX-2 (GRANCIERI; MARTINO; DE MEJIA, 2021).

A partir dos resultados positivos encontrados nos estudos *in silico* e *in vitro*, um estudo *in vivo* com camundongos C57BL/6 alimentados com uma dieta rica em gordura saturada adicionada de 400mg/kg de DTP de chia, foi realizado por nosso grupo de pesquisa. Nesse experimento foi confirmado o potencial anti-adipogênico e anti-inflamatório da DTP, sendo observado redução do percentual de gordura e circunferência da cintura dos animais. Além disso, observou-se redução da área dos adipócitos, focos de inflamação no tecido adiposo e dos níveis de NF-κB p65, PPARγ, e SREBP1 (GRANCIERI et al., 2022).

Por fim, nosso grupo de pesquisa buscou avaliar os efeitos do hidrolisado proteico de chia nas populações bacterianas intestinais, na barreira intestinal, na resposta inflamatória e na funcionalidade da membrana da borda em escova *in ovo* (*Gallus gallus*). Assim, foi observado melhoria na inflamação pela redução de TNF-α, assim como da permeabilidade intestinal e funcionalidade da membrana da borda escova, com aumento de ocludina, mucina e aminopeptidase. Além disso, foi observado aumento de *Lactobacillus* e redução de *Bifidobacterium*, e melhoria da morfologia intestinal com aumento da altura e área superficial das vilosidades, aumento das profundidade das criptas e aumento no número de celulas de Paneth (MISHIMA et al., 2023b).

Assim, baseados em estudos prévios, a proteína da chia e seus peptídeos bioativos, podem atuar em diversas etapas de prevenção e redução da adipogênese, estresse oxidativo, inflamação e saúde intestinal. Apesar disso, apenas um estudo avaliou o efeito da proteína de chia em modelo *in vivo* com alterações metabólicas (GRANCIERI et al, 2022). Sendo assim, pesquisas que utilizem a proteína hidrolisada de chia (desengordurada e sem fibras) em modelo animal com alterações metabólicas são necessárias, para entender o papel dessa fração isolada nas vias de oxidação celular, tornando-a um ingrediente com potencial para geração de compostos farmacologicamente ativos na redução do risco de doenças crônicas não transmissíveis.

5. METODOLOGIA

5.1 Local de execução

A proteína digerida de chia foi produzida na *University of Illinois* e transportada para o Laboratório de Nutrição Experimental (LNE). O estudo *in vivo*, assim como as análises de estresse oxidativo e inflamação foram conduzidos no LNE do Departamento de Nutrição e Saúde (DNS/UFV). As análises de expressão gênica foram conduzidas no LNE em parceria com Laboratório de Parasitologia e Epidemiologia Molecular. As análises histológicas foram desenvolvidas em parceria com o Laboratório de Histopatologia do Departamento de Veterinária da UFV.

5.2 Matéria prima

5.2.1 Caracterização da amostra

As sementes de chia utilizadas durante o experimento foram cultivadas em janeiro de 2017 e colhidas em junho de 2017, no estado do Rio Grande do Sul, Brasil. A região de cultivo apresenta clima temperado com temperaturas médias de 26°C, chuvas regulares, umidade relativa entre 60% e 80% e solo argiloso. Após a colheita, as sementes foram enviadas para o LNE, onde foram armazenadas em freezer a -18°C.

5.2.2 Obtenção da proteína total digerida

As sementes de chia foram processadas na *University of Illinois*, EUA, para a produção da DTP. Para isso, as sementes foram imersas em água destilada (1:10 g/mL) durante 1 hora para formação da mucilagem. Em seguida foram congeladas *overnight* (-80°C), e no dia seguinte foram liofilizadas (Labconco Freeze Dryer 4,5; Kansas, MO, EUA). Após isso, a mucilagem foi removida manualmente com auxílio de peneira (500 µm/35 mesh) e as sementes livres foram trituradas em moedor de café (Mr. Coffee®) e peneiradas (500 µm/35 mesh) para uniformização da farinha (ORONA-TAMAYO et al., 2015). Em seguida, a farinha foi desengordurada adicionando hexano (1:10 g/mL) a 60°C durante 2 horas, sob agitação. Posteriormente a mistura foi centrifugada a 6000g, durante 15 minutos a 4°C. O sobrenadante gerado após a centrifugação foi descartado e a farinha precipitada foi mantida em capela de fluxo a 4°C *overnight*, para em seguida ser armazenada até sua utilização (ORONA-TAMAYO et al., 2015).

Para obtenção da proteína concentrada, a farinha obtida acima foi misturada com água deionizada (1:20 g/mL), ajustado o pH para 8, com uso de hidróxido de sódio 0,1M, e mantido por agitação constante durante 1 hora a 35°C. Em seguida, essa mistura foi centrifugada (5000g; 15 minutos; 25°C) e o sobrenadante (proteína concentrada) foi recolhido, liofilizado e armazenado a -20°C (ALVES et al., 2016; MONTOYA-RODRÍGUEZ et al., 2014). A digestão da proteína foi realizada usando técnica que simula a digestão gastrointestinal (MEGÍAS et al., 2004). Nesta, a proteína concentrada foi suspensa em água deionizada (1:20 g/mL), o pH foi ajustado para 2,0 e foi adicionada a enzima pepsina (1:20 g/g enzima/proteína). A reação foi mantida sob agitação durante 2 horas a 37°C. Em seguida, o pH foi reajustado para 7,5, e foi adicionada a enzima pancreatina (1:20 g/g enzima/proteína), e novamente a reação foi mantida por 2 horas, a 37°C sob agitação. Logo após, a digestão foi cessada colocando a mistura em banho maria a 75°C por 20 minutos. Após isso, a amostra foi centrifugada 2 vezes a 20.000g, durante 15 minutos, a 4°C. O sobrenadante (proteína total digerida) foi coletado e em seguida dialisado em membrana de exclusão de peso molecular de 100-500 Da (Spectra/Por®, Biotech CE Membrane), liofilizado e armazenado a -20°C até sua utilização.

5.3 Ensaio biológico

Para avaliar o efeito da DTP de chia foram utilizados 44 camundongos C57BL/6, machos, linhagem Inbred, com 8 semanas de vida. Os animais foram obtidos no Centro de Biologia da Reprodução da Universidade Federal de Juiz de Fora. Os animais foram transportados para o Biotério Setorial do LNE do DNS da UFV, onde foram distribuídos em gaiolas individuais de aço inoxidável, e permaneceram em ambiente com temperatura controlada a 21±1°C e fotoperíodo de 12 horas, controlado automaticamente. Os animais receberam água deionizada e dieta experimental *ad libitum*. Ao chegar no Laboratório de Nutrição Experimental, os animais foram distribuídos em 4 grupos experimentais, sendo eles: AIN (animais receberam dieta padrão AIN-93M), HF (animais receberam dieta hiperlipídica), AIN+DTP (animais receberam dieta AIN-93M adicionada de DTP) e HF+DTP (animais receberam dieta hiperlipídica adicionada de DTP) (Figura 1). O estudo foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Federal de Viçosa (CEUA/ UFV), com número de processo n°1/2019 (ANEXO I), e todos os procedimentos experimentais foram realizados em consonância com os princípios éticos na experimentação animal.

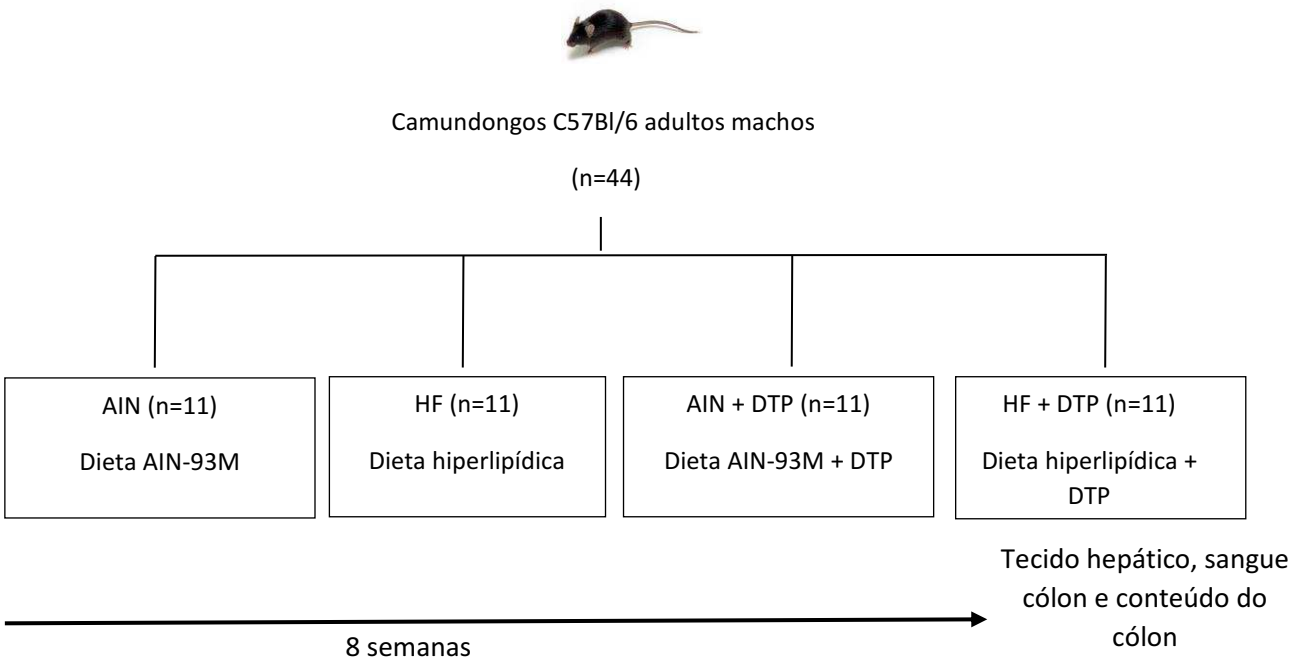

Figura 1: Desenho experimental. AIN: dieta padrão AIN-93M; HF: dieta hiperlipídica; AIN + DTP: dieta AIN-93M + DTP; HF + DTP: dieta hiperlipídica + DTP; DTP: proteína total. digerida.

5.4 Cálculo amostral

Para calcular o número de repetições estimado utilizou-se o cálculo do "n" amostral para estudos descritivos conforme detalhado por Fontelles *et al.* (2010), por meio da seguinte equação:

$$n = \frac{s^2}{(\bar{x} - \mu)^2} X (z_{\frac{\alpha}{2}})^2$$

Onde:

n: número de animais por grupo

s^2: variância dos dados de referência

$\bar{x} - \mu$: diferença máxima razoável, admitida entre a média obtida da amostra e a verdadeira média da população.

$z\frac{\alpha}{2}$: Erro alfa do tipo I: Determinado por meio da tabela de valores críticos da distribuição normal gaussiana.

Não foi realizado experimento prévio para cálculo do número de repetições ideal, pois seria necessário a utilização de animais. Portanto, utilizou-se dados de peso de um estudo que induziu a obesidade nos camundongos (SCHOEMAKER et al., 2017).

Considerou-se α=5%, e portanto um $z\frac{\alpha}{2}$ =1.96, conforme utilizado nos estudos de saúde (FONTELLES et al., 2010). O número de repetições calculado foi 9,42. Considerando-se possíveis perdas foi adicionado 10% ao n amostral calculado, portanto, foram solicitados 11 animais por grupo. Como trabalharemos com 4 grupos, foram necessários 44 animais.

5.5 Dietas experimentais

Os grupos AIN e AIN+DTP receberam diariamente dieta AIN-93M (REEVES; NIELSEN; FAHEY, 1993) em pellet contendo 15,2%, 9,7% e 75,1% do valor calórico proveniente, respectivamente, de proteína, lipídeos e carboidratos. Os grupos HF e HF+DTP receberam dieta hiperlipídica composta por 10,7% de proteínas, 60,3% de lipídeos e 29,0% de carboidratos (Tabela 1) (Research Diets, New Brunswick, NJ).

Todos os ingredientes foram pesados em balança semi-analítica (Gehaca, BG2000, Brasil), misturados manualmente, peneirados em peneira de plástico e homogeneizados em batedeira industrial (Leme®) durante 15 minutos. Após o preparo, as dietas foram peletizadas e acondicionadas em sacos de polietileno, devidamente rotulados e armazenados em freezer a -18 ± 1°C por no máximo 15 dias.

A DTP foi ofertada diariamente, incorporada ao pellet. A dose ofertada foi de 400 mg de DTP/kg de peso corpóreo do animal (DE LIMA et al., 2019; MOJICA et al., 2017). As perdas da dieta com a DTP foram quantificadas para cálculo final da ingestão de DTP.

No fim das 8 semanas de experimento, os animais foram mantidos em jejum por 12 horas e anestesiados com isoflurano (Isoforine, Cristália®) inalatório e eutanasiados por punção cardíaca. Parte do fígado e cólon foram coletados, lavados em solução salina, pesados e imediatamente congelados com nitrogênio líquido e armazenado a -80°C até o momento das análises. Um fragmento de fígado e cólon foi separado e armazenado em solução de

formaldeído 10%, para análises histológicas. O conteúdo do cólon, foi coletado e armazenado a -80°C, para realização de análises de microbiota. O sangue foi coletado em tubos contendo heparina, 16 x 100 mm (BD Vacutainer®) e centrifugados (Hermle®, modelo Z216MK, Alemanha) a 2865g por 10 minutos para separação do plasma e células sanguíneas.

Tabela 1. Composição das dietas experimentais.

Ingredientes (g/kg-1)	AIN93-M	HF	AIN+DTP	HF+DTP
Albumina*	179,71	179,71	179,71	179,71
Proteína total digerida de chia**	-	-	0,50	0,50
Amido de milho	425,99	113,99	425,99	113,99
Amido dextrinizado	155,00	155,00	155,00	155,00
Sacarose	100,00	100,00	100,00	100,00
Banha de porco	-	312,00	-	312,00
Óleo de soja	40,00	40,00	40,00	40,00
Celulose	50,00	50,00	50,00	50,00
Mix mineral	35,00	35,00	35,00	35,00
Mix vitamínico	10,00	10,00	10,00	10,00
L-cistina	1,80	1,80	1,80	1,80
Bitartarato de colina	2,50	2,50	2,50	2,50
Carboidrato (%)	75,10	29,00	75,10	29,00
Proteína (%)	15,20	10,70	15,20	10,70
Lipídeo (%)	9,70	60,30	9,70	60,30
Densidade calórica (Kcal/g)	3,81	5,37	3,81	5,37

Composição das dietas experimentais baseada na dieta padrão para roedores (AIN-93M) (REEVS et al., 1993). HF: dieta rica em gordura saturada. *Albumina baseada no teor de 77,9% de proteína.** Adicionada diariamente à dieta na proporção de 400mg/kg de peso corporal do animal.

5.6 Extração de mRNA do tecido hepático e intestinal

Para extração do RNA, o fígado e cólon foram macerados em gral livre de RNAse, utilizando nitrogênio líquido para manter o órgão congelado. Para isso, 100mg de tecido hepático e cólon foram homogeneizados com 1 mL de reagente de TRIzol (Invitrogen, Carlsbad, CA, USA). Em seguida, os homogeneizados foram transferidos para eppendorf de 1,5 mL e agitado durante 5 minutos. Após isso, foi adicionado ao eppendorf, 200 µL de clorofórmio e feito agitação vigorosa durante 30 segundos, e em seguida, foi centrifugado a 12.000g por 15 minutos a 4°C. Em seguida, cerca de 500 µL do sobrenadante foi coletado e transferido para um novo eppendorf, juntamente com 500 µL de isopropanol para precipitação do RNA. Os eppendorfs foram agitados por inversão, incubados a temperatura ambiente durante 10 minutos e em seguida centrifugados a 12.000g por 10 minutos a 4°C. Terminada a centrifugação o sobrenadante foi descartado, e o RNA foi lavado com etanol 75% (75 mL etanol 100%/ 25 mL de água DEPC), sendo em seguida feita uma nova centrifugação a 9.500g por 5 minutos a 4°C. Por fim, o sobrenadante foi descartado por inversão, o pellet foi seco a temperatura ambiente e em seguida foi ressuspenso em 50 µL de água Mili-Q tratada com 0,01% de dietilpirocarbonato (Invitrogen Brasil Ltda.). Por fim, o RNA foi armazenado a -80°C, até a realização da etapa de síntese do cDNA.

5.7 Síntese de cDNA

O cDNA foi sintetizado utilizando-se kit M-MLV reverse transcriptase (Invitrogen Brasil Ltda.). Para isso, 2 µg de RNA foi adicionado a um tubo e tratado com 1 µL de tampão DNAse e 2 µL de DNAse. A mistura foi incubada em banho metabólico a 37°C durante 1 hora, para que ocorra a reação de transcriptase reversa e consequente síntese do cDNA. Após isso, o cDNA ficou em banho metabólico a 70°C durante 10 minutos, para interrupção do processo anterior (LIVAK; SCHMITTGEN, 2001). O cDNA foi armazenado a -80°C até o momento da realização da reação em cadeia da polimerase em tempo real (RT-PCR).

5.8 Determinação da expressão gênica de proteínas envolvidas na inflamação, estresse oxidativo, metabolismo lipídico e permeabilidade intestinal

Todos os passos a seguir foram realizados em condições livres de RNAse e DNAse. Os níveis de expressão gênica foram quantificados pelo método RT-qPCR. Para isso, foi utilizado o reagente Fast sybr green master mix da Applied Biosystems (Foster City, CA) e as análises foram realizadas no equipamento StepOne Real-Time PCR System (Thermo Fisher

Scientific). Para a realização dos ciclos de PCR, foi feita uma desnaturação inicial do cDNA a 95°C durante 10 minutos. Em seguida, foram realizados 40 ciclos, com desnaturação a 95°C durante 1 minuto, 60°C durante 1 minuto e 72°C por 2 minutos, seguido pela formação da curva analítica de dissociação. Foram utilizadas sequencias de oligonucleotideos senso e antisenso (Choma Biotechnologies) para a avaliar a expressão de PPAR-α, NFκB, Zn-SOD1, IL-1β, NRF2, AMPK, SREBP1, CPT1, no fígado e claudina, zonulina, e ocludina no cólon. A expressão relativa de níveis de mRNA foi normalizada pelo controle endógeno β-actina (Tabela 2).

Tabela 2. Sequência de primers utilizados nas análises de RT-PCR.

Genes	Oligonucleotideo (5'-3')	
	Senso	Anti-senso
β-actina	TTCGTTGCCGGTCCACACCC	GCTTTGCACATGCCGGAGCC
PPAR-α	CCTGCCTTCCCTGTGAACT	ATCTGCTTCAAGTGGGGAGA
NFκB	GCGTACACATTCTGGGGAGT	GGTACCCCCAGAGACCTCAT
Zn-SOD1	GAGCAGAAGGCAAGCGGTGAA	CCACATTGCCCAGGTCTG
IL-1β	GGGAAACAACAGTGGTCAGG	GAGCTGTCTGCTCATTCACG
NRF2	CACATCCAGACAGACACCAGT	CTACAAATGGGAATGTCTCTGC
AMPK	GTCAAAGCCGACCCAATGATA	CGTACACGCAAATAATAGGGTT
SREBP1	GCCGAGATGTGCGAACTG	GGAAGTCACTGTCTTGGTTGTT
CPT1	GTAAGGCCACTGATGAAGGAAGA	ATTTGGGTCCGAGGTTGACA
Zonulina	CCAGCTTATGAAAGGGTTGTTC	TCCTCTCTTGCCAACTTTTCTC
Claudina	GCTCTCAGAGTCCGTTGACC	CTGCCCTTTCAGGTTAGCAG
Ocludina	ATGTCCGGCCGATGCTCTC	TTTGGCTGCTCTTGGGTCTGTAT

PPAR-α: receptor alfa ativado por proliferador de peroxissoma; NFκB: fator nuclear kappa B; SOD: superóxido dismutase; TNF-α: fator de necrose tumoral alfa; IL-1β: interleucina 1 beta; NRF2: fator nuclear relacionado ao fator eritroide 2; AMPK: proteína quinase ativada por AMP; SREBP1: proteína de ligação ao elemento regulador de esteróis 1; CPT1: carnitina palmitoiltransferase 1.

5.9 Análises de peroxidação lipídica, inflamação e estresse oxidativo

5.9.1 Preparo de homogenato hepático

Para obteção do homogenato, 200 mg de fígado foram misturados com 1000 μL de tampão fosfato (50 mM) e 1 mM de EDTA (pH 7,4). A amostra foi macerada e centrifugada a 12.000 ×g, 4°C, por 10 minutos, e em seguida, o sobrenadante foi removido e armazenado em ultrafreezer (-80°C) até à análise.

5.9.2 Malondialdeído (MDA)

O MDA foi quantificado pelo método de substâncias reativas ao ácido tiobarbitúrico (TBARS) (KOHN; LIVERSEDGE, 1944; PYLES; STEJSKAL; EINZIG, 1993). A concentração de MDA foi calculada via coeficiente de absorção molar (BUEGE; AUST, 1978) e os resultados foram expressos em nanomoles de MDA por miligrama de proteína (MDA/PTN). A proteína total no homogenato de fígado foi quantificada pelo método de Bradford (BRADFORD, 1976).

5.9.3 Quantificação de IL-1β e TNF-α

Para determinar as concentrações de IL-1β e TNF-α no fígado, as amostras foram homogeneizadas por meio dos reagentes NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific Fisher, EUA). As frações nucleares de IL-1β e TNF-α foram avaliadas por imunoensaio usando os kits ELISA para camundongos IL-1β (Cat #88-6010-22; Invitrogen, EUA) e TNF-α (Cat #88-7340-22, Invitrogen, EUA), respectivamente. As concentrações de IL-1β e TNF-α foram calculadas por comparação com as curvas padrão correspondentes.

5.9.4 Superóxido dismutase (SOD)

A quantificação da SOD foi realizada em unidades relativas e uma unidade foi definida como a quantidade da enzima SOD que inibe a taxa de oxidação de pirogalol em 50%. A análise foi realizada em espectrofotômetro (Multiskan GO, Thermo Scientific) a 570

nm, e os resultados foram expressos como unidades de atividade da SOD por miligrama de proteína (MARKLUND, 1985).

5.9.5 Catalase

A catalase foi analisada de acordo com a metodologia proposta por Aebi (1984). Aos 0, 30 e 60 segundos após a reação ser iniciada, a absorbância em 240 nm foi determinada em espectrofotômetro (T70 + UV/VIS Spectrometer). A atividade enzimática foi registrada em micromoles por mililitro de amostra. A atividade da catalase foi calculada de acordo com a lei de Lambert-Beer.

5.9.6 Capacidade antioxidante plasmática

Alíquotas de plasma (10 µL) foram adicionadas aos poços de placa de 96 poços juntamente com 20 µL do reagente metamioglobina e 150 µL da solução ABTS. Em seguida, 10 µL de concentrações crescentes do padrão Trolox foram pipetados nos poços, em triplicata, para construir uma curva padrão. A placa de microtitulação foi incubada em temperatura ambiente (22°C), e então a absorbância foi lida por meio de espectrofotômetro (Multiskan GO, Thermo Scientific) a 405 nm. Os valores foram expressos em equivalentes de Trolox em mM.

5.10 Morfologia hepática e intestinal

As amostras de tecido hepático e cólon foram fixadas em formaldeído a 10% e embutidas em parafina, sendo cortadas em seções semissérias com espessura de 3 mm para coloração com hematoxilina e eosina. As análises foram realizadas e fotografadas em microscópio de campo claro (Olympus AX 70 TRF, Tóquio, Japão) com objetiva de 20×. Foram avaliados os conteúdos de vesículas lipídicas, citoplasma, infiltrado inflamatório, núcleo de hepatócitos e vasos sanguíneos. Para isso, foram selecionados dez campos hepáticos para cada animal, sendo que em cada campo histológico foi sobreposto uma rede de 266 pontos nas imagens. As interseções da rede foram analisadas até alcançar a soma de 2660 pontos por animal (CUPERTINO et al., 2013). Para a análise de cólon foram selecionados 20 campos aleatórios/animal, com base na qualidade das criptas, que apresentaram epitélio conjuntivo definido e visível para melhor medir a espessura e altura das criptas, assim como da camadas musculares circulares e longitudinais (MARTINEZ et al., 2022).

As imagens das seções histológicas (citoplasma, vesículas lipídicas, núcleo e inflamação) foram analisadas usando o software Image J® 1.48v (National Institute of Health, EUA).

5.11 Extração de DNA, sequenciamento e análise da microbiota intestinal

Um total de 200 mg do conteúdo do cólon foi submetido a protocolo de rompimento mecânico e extração com fenol/clorofórmio para extrair o DNA genômico da microbiota intestinal, conforme publicado anteriormente (STEVENSON; WEIMER, 2007). Em seguida, a concentração e a qualidade do DNA foram determinadas por espectrofotometria medindo o A260/280 e os amplicons da região 16S rRNA V3-V4 foram gerados usando o primer direto 341F (5`-CCTAYGGGRBGCASCAG-3`) e o primer reverso 806R (5`-GGACTACNNGGGTATCTAAT-3`) e um adaptador de conjunto de iniciadores de código de barras para a plataforma Illumina NovaSeq (Illumina, San Diego, Califórnia, EUA) (CAPORASO et al., 2012).

As amostras foram carregadas em uma célula de fluxo Illumina para reações de sequenciamento pareado usando a plataforma Illumina NovaSeq PE250 na Novogene Corporation no campus da Universidade da Califórnia em Davis (Sacramento, Califórnia, EUA). Os amplicons foram sequenciados em uma corrida NovaSeq 2x250bp usando primers personalizados e procedimentos de sequenciamento (CAPORASO et al., 2012). As amostras-sequências foram submetidas ao banco de dados *Sequence Read Archive* (SRA) no *National Center for Biotechnology Information* (NCBI).

Além disso, o processamento e análise dos dados foram realizados no software Mothur v.1.44.3 (SCHLOSS et al., 2009). As *reads paired-end* R1 e R2 foram unidas e as sequências menores que 380 ou maiores que 440 pb foram removidas. As sequências de quimeras foram detectadas e removidas usando UCHIME versão 4.2 (EDGAR et al., 2011) e as sequências limpas foram alinhadas com o gene 16S rRNA usando o banco de dados SILVA v.138 (QUAST et al., 2012). As sequências finais pareadas foram agrupadas em Unidades de Taxonomia Operacional (OTUs), também utilizando o banco de dados SILVA v.138, onde cada OTU caracteriza um grupo geneticamente único de organismos biológicos, e foi adotado um corte de similaridade de sequência de 97%. A cobertura de todas as amostras foi avaliada pelo *Good Coverage Estimator* (bactérias > 97%). Para corrigir o viés de amostragem

resultante de tamanhos de biblioteca de amplicon desiguais, as amostras foram normalizadas para o menor número de sequências produzidas a partir de qualquer amostra.

Em seguida, os dados foram normalizados e utilizados para calcular a diversidade α e β, assim como a abundância relativa de OTUs. A diversidade α de cada amostra foi analisada utilizando o índice de Chao1, Shannon e Simpson. Para análise da composição da comunidade microbiana, e métricas de diversidade β foram utilizados o índice de dissimilaridade de Jaccard. Os gráficos de análise de coordenadas principais (PCoA) foram realizados em matrizes de distância calculadas nas comunidades bacterianas.

5.12 Análise estatística

As análises estatísticas foram realizadas utilizando o software estatístico GaphPad Prism®, versão 7 ou IBM SPSS Statistics, versão 20. As amostras foram testadas pelo teste de normalidade Kolmogorov-Smirnov e os grupos com distribuição normal foram comparados usando ANOVA "One-way" seguida pelo teste post-hoc de Newman-Keuls. Os dados não paramétricos foram transformados para base logarítmica e submetidos ao teste *post-hoc* Duncan para comparações múltiplas. Para avaliação dos efeitos das dietas experimentais na abundância de bactérias foi realizada a análise de Kruskal-Wallis. A significância estatística da diversidade β entre os grupos amostrais foi avaliada com o teste não paramétrico *Permutation Multivariate Analysis of Variance* (Monte Carlo permutations), utilizando o software Past (versão 4.05). As frequências relativas das diferentes categorias taxonômicas obtidas foram calculadas utilizando o programa *Statistical Analysis of Metagenomic Profiles* (versão 2.1.3). Todas as análises foram realizadas considerando a classificação taxonômica em nível de gênero. Os dados foram expressos como média ± desvio padrão e considerados significantes quando $p<0,05$.

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6. RESULTADOS

6.1 ARTIGO 1: A bioactive compound digested chia protein is capable of modulating NFκB mediated hepatic inflammation in mice fed a high-fat diet

Submetido na *Food Research International* (Fator de impacto: 8.1)

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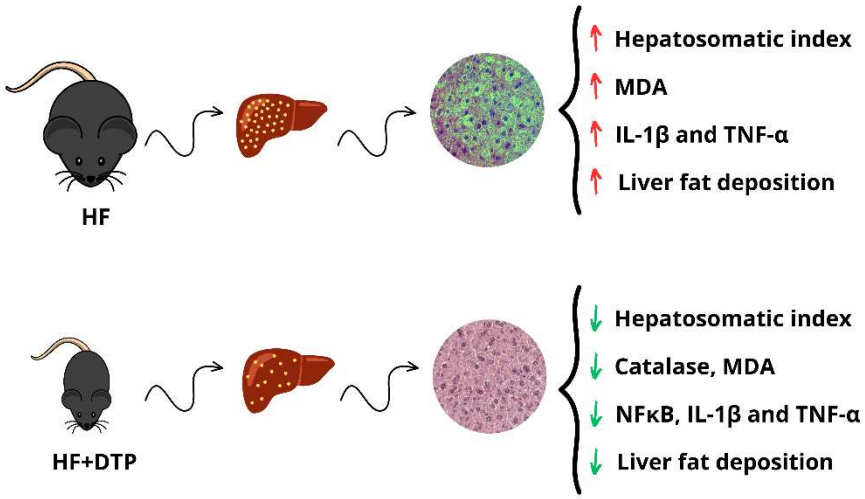
A bioactive compound digested chia protein is capable of modulating NFκB mediated hepatic inflammation in mice fed a high-fat diet



***Abstract*:** The consumption of diets high in saturated fat can induce damages in liver morphology and function, which leads to increased inflammation, oxidative stress, and hepatic steatosis. Chia seed (*Salvia hispanica* L.) is rich in protein, which provides bioactive peptides with potential benefits, including antioxidant and anti-inflammatory functions. Then, this study aimed to analyze the effect of digested total protein (DTP) of chia on inflammation, oxidative stress, and morphological changes in liver of C57BL/6 mice fed a diet rich in saturated fat. Male C57BL/6 mice (n = 8/group), 8 weeks old, were fed standard diet (AIN), high-fat diet (HF), standard diet added digested protein (AIN+DTP) or high-fat diet added digested protein (HF+DTP) for 8 weeks. In animals fed a high-fat diet, chia DTP was able to reduce weight gain, food efficiency ratio and hepatosomatic index. In addition, it presented antioxidant capacity, which reduced catalase activity and lipid peroxidation. DTP was also able to reduce hepatic inflammation by reducing p65-NFκB expression and IL-1β expression and quantification. The APSPPVLGPP peptide present in chia DTP presented binding capacity with PPAR-α, which contributed to the reduction of hepatic fat accumulation evidenced by histological analysis. Thus, chia DTP improved hepatic inflammatory and histological parameters, being an effective food in reducing the liver damage caused by a high-fat diet.

***Keywords*:** Liver steatosis; obesity; antioxidant; oxidative stress; saturated fat; chia seeds; peptides; simulated gastrointestinal digestion.

List of Abbreviation

AIN: Standard diet; **AIN+DTP**: Standard diet added digested protein; **AMPK:** AMP-activated protein kinase; **CPT1**: Carnitine palmitoyl transferase 1; **DDP IV**: Dipeptidyl peptidase IV; **DTP**: Digested total protein; **FAS**: Fatty acid synthase; **FER**: Food efficiency ratio; **GLP-1**: Glucagon like peptide; **HF**: High-fat diet; **HF+DTP**: High-fat diet added digested protein;

IL-1β: Interleukin-1 beta; **LPS**: Lipopolysaccharide; **MDA**: Malondialdehyde; p65-**NFκB**: phosphorylated65-Nuclear factor kappa B; **NRF2**: Nuclear factor erythroid 2-related factor 2; **PPAR-α**: Peroxisome proliferator-activated receptor alpha; **SOD**: Superoxide dismutase; **SREBP1**: Sterol regulatory element-binding protein 1; **TAC**: Total antioxidant capacity; **TLR4**: Toll-like receptor 4; **TNF-α**: Tumor necrosis factor alpha.

1. Introduction

A diet rich in ultra-processed foods with high levels of saturated fats and sugar is one of the main factors responsible for the elevated prevalence of chronic non-communicable diseases, such as obesity, cardiovascular diseases, non-alcoholic fatty liver disease and some types of cancer (Liu et al., 2022; Marrón-Ponce et al., 2019; Oliveira et al., 2020).

The liver is one of the organs responsible for lipid metabolism. Thus, in a chronic high-fat diet, this is one of the first organs to undergo changes in its metabolism (Koh et al., 2020; Peng et al., 2018), with accumulation of triglycerides and migration of immune cells into liver cells, which leads to the activation of inflammatory pathways with consequent production of molecules, such as tumor necrosis factor alpha (TNF-α), nuclear factor kappa B (NFκB) and interleukin-1 beta (IL-1β) (Ahmed; Robinson; O'farrelly, 2021; Tilg et al., 2021). In addition, excess hepatic triglycerides stimulate the peroxisome proliferator-activated receptor alpha (PPAR-α) to increase exacerbated lipid oxidation, which damages the mitochondrial function, leading to greater oxidation of fatty acids in peroxisomes and cytochromes, with the production of species reactive oxygen, increase in the oxidative stress and the activation of inflammatory pathways (Da Silva et al., 2019; Ipsen et al., 2018).

Thus, there is a growing search for compounds with functional properties able to reduce the damage caused by high-fat diet consumption. In this context, chia (*Salvia hispanica* L.) stands out for being a seed rich in dietary fiber, polyunsaturated fatty acids, phenolic compounds and protein (da Silva et al., 2017). This seed contains about 18% of proteins, which, after the digestion or hydrolysis, can generate peptides with antioxidant, anti-inflammatory, hypotensive, hypoglycemic and hypocholesterolemic properties (Grancieri et al., 2019a; Valenzuela Zamudio et al., 2022).

A recent study carried out with biochemical assays demonstrated that digested total protein (DTP) and digested protein fractions (DPF) from chia exhibited anti-inflammatory and antioxidant effects by their ability to eliminate superoxide, hydrogen peroxide, nitric oxide, inducible oxide nitric synthase and ciclooxigenase-2 (Grancieri et al., 2019b). Antioxidant potential was found in chia seed expeller after digestion with papain, which resulted in a potent effect on the elimination of 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Cotabarren et al., 2019).

Furthermore, the peptides formed after simulated gastrointestinal digestion demonstrated *in silico* interaction with inflammatory markers, such as NFκB, cyclooxygenase-2 and lipoxygenase-1 (Grancieri et al., 2019b). In an *in vitro* macrophage (RAW 264.7) study, those DTP and DPF reduced the inflammation induced by lipopolysaccharide (LPS) and the secretion of TNF-α, IL-6, besides blocking the NFκB pathway by preventing its translocation to the nucleus. Furthermore, DTP and DPF reduced the atherosclerosis in macrophages by inhibiting the formation of foam cells and their associated inflammation (Grancieri et al., 2019c). Additionally, in another *in vitro* study, the hydrolyzed protein from chia seed reduced the production of reactive oxygen species and nitrite, as well as the secretion of pro-inflammatory cytokines, and increased the secretion of anti-inflammatory cytokines. This effect was due to the polarization of monocytes towards a more anti-inflammatory phenotype (Villanueva-Lazo et al., 2022).

In studies carried out with C57BL/6 mice fed a high-fat diet, DTP reduced body fat, waist circumference and the area of adipocytes. Therefore, the levels of p65-NFκB, PPARγ and the expression of SREBP1 and TNF-α decreased, while the expression of adiponectin in the adipose tissue of these animals increased (Grancieri et al., 2022). This is the only study that evaluated the effects of digested chia protein on metabolic changes, caused by a high-fat diet, but only the adipose tissue was analyzed.

Thus, since the liver is an important organ for lipid and protein metabolism, as well as being susceptible to metabolic alterations due to the chronic use of high-fat diets, it should be evaluated. However, there is still no study evaluating the effects of digested chia protein on liver inflammation and oxidative stress in mice fed a high-fat diet. Then, this study aimed to analyze the effect of digested total protein (DTP) of chia on inflammation, oxidative stress, and morphological changes in liver of C57BL/6 mice fed a diet rich in saturated fat. The hypothesis of the present study is that chia digested protein, as a source of bioactive peptides, can modulate inflammation and hepatic oxidative stress in animals that consume a high fat diet.

2. Materials and Methods

2.1. Raw materials and preparation of digested total protein

Chia seeds (*Salvia hispanica* L.) cultivated in the state of Rio Grande do Sul (Brazil) were used for the production of digested total protein. The seeds were supplied by Dubai Alimentos, a company located in Ijuí, Rio Grande do Sul, Brazil. The chia used is traditional

seeds in farms from Rio Grande do Sul, Brazil, crop of 2017, July. The state of Rio Grande do Sul is located in southern Brazil, has a temperate climate, with average temperature of 26 °C and relative humidity ranging from 60 to 80%, regular rainfall, and clay soil (da Silva et al, 2017).

The seeds were immersed in distilled water for the formation of mucilage, frozen overnight (-80°C) and then lyophilized (Labconco Freeze Dryer 4.5; Kansas, MO, USA). The mucilage was manually removed with the aid of a sieve (500 μm/35 mesh), the seeds were ground in a coffee grinder (Mr. Coffee®) and sieved (500 μm/35 mesh) to make the flour uniform. Then, the flour was defatted by adding hexane and was left in a flow hood overnight. Next, the flour was stored at 4°C until the time to use (Orona-Tamayo et al., 2015).

In order to obtain the protein fraction, the flour produced above was mixed with deionized water (1:20 g/mL), and the pH was adjusted to 8 and maintained by constant agitation for 1 hour, at 35°C. Then, this mixture was centrifuged (5000g; 15 minutes; 25°C). The supernatant (concentrated protein) was collected, lyophilized, and stored at -20°C (Alves et al., 2016; Montoya-Rodríguez et al., 2014).

Protein hydrolyzed was performed using a technique that simulates gastrointestinal digestion (Megías et al., 2004). To this end, the concentrated protein was suspended in deionized water (1:20 g/mL), the pH was adjusted to 2.0 and added pepsin (1:20 g/g enzyme/protein). The reaction was kept under agitation for 2h, at 37°C. The pH was readjusted to 7.5 and pancreatin (1:20 g/g enzyme/protein) was added, and the reaction was kept for 2 hours, again, at 37°C, under agitation. The digestion was interrupted by placing the mixture in a water bath, at 75°C, for 20 minutes. The sample was centrifuged twice, at 20000g, for 15 minutes, at 4°C. The supernatant (digested total protein) was collected and then dialyzed in a 100-500 Da molecular weight exclusion membrane (Spectra/Por®, Biotech CE Membrane), lyophilized and stored at -20°C until the time to use. This final product was called digested total protein (DTP).

2.2. Identification and characterization of peptides

The protein fractions presented in the sample after hydrolysis was analyzed as describe in a previously study (Grancieri et al., 2019b). The hydrolyzed protein was analyzed by high-performance liquid chromatography–electrospray ionization–mass spectrometry (HPLC–ESI–MS) using a Q-ToF Ultima mass spectrometer (Waters, Milford, MA) to identify the generated peptides (Mojica et al., 2015). The sequence of amino acids of peptides was

identified using MassLynx V4.1 software (Waters Corp., Milford, MA). The peptides biological activity was predicted by using BIOPEP® database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep).

2.3. Animals and diets

A controlled experimental study was carried out to assess the effect of digested chia protein on histological, oxidative, and inflammatory parameters. Thirty-two male mice (C57BL/6, Inbred lineage), 8 weeks old, from the Biology Center of Reproduction of the Federal University of Juiz de Fora, Minas Gerais, Brazil, were systematically subdivided into 4 groups, each with 8 animals, and randomized by body weight. This animal model was adopted due to its ability to develop metabolic alterations and obesity when fed a high-fat diet (Aydos et al., 2019; Li et al., 2020). The animals were distributed into individual stainless-steel cages in a controlled temperature environment (22±2°C) and automatically controlled light and dark cycles of 12h. The animals received deionized water and their respective experimental diets *ad libitum*. The experimental diets were based either on the standard AIN-93M diet (Reeves et al., 1993) or high-fat diet (Research Diets, New Brunswick, NJ), with modifications. The standard diet was composed of 15.2% protein, 9.7% fat, and 75.1% carbohydrate. The high fat diet was prepared in the following proportions: 60.3% fat, 10.7% protein, and 29.0% carbohydrate. The other ingredients were added in sufficient quantities to provide the planned amounts of lipids, proteins, carbohydrates, dietary fiber and calories (Table 1).

Table 1. Composition of experimental diets.

Ingredients (g.kg^{-1})	**AIN93-M**	**HF**
Albumin*	179.71	179.71
Corn starch	425.99	113.99
Dextrinized starch	155.00	155.00
Sucrose	100.00	100.00
Lard	0.00	312.00
Soybean oil	40.00	40.00
Microcrystalline cellulose	50.00	50.00
Mineral mix	35.00	35.00
Vitamin mix	10.00	10.00

L-cystine	1.80	1.80
Choline bitartrate	2.50	2.50
Carbohydrate (%)	70.70	27.30
Protein (%)	18.70	13.30
Lipids (%)	9.30	58.50
Caloric density (Kcal/g)	3.85	5.41

Composition of experimental diets based on the standard rodent diet (AIN-93M) (REEVES; NIELSEN; FAHEY, 1993). HF: high fat diet. *Albumin based on 77.9% protein content.

The experimental groups received one of the following four diets: standard diet (AIN), high-fat diet (HF), standard diet added digested protein (AIN+DTP) or high-fat diet added digested protein (HF+DTP). The diets of all groups were offered in the form of pellets, and for the AIN+DTP and HF+DTP groups, 400mg/kg weight/day of chia DTP were added to the pellet and offered daily, based on study conducted by Lima et al (2019) and Mojica et al (2017). Thus, the animals' food intake was evaluated every day (Mojica et al., 2017). In the eighth week, after 12 h of fasting, the animals were anesthetized with isoflurane (Isoforine, Cristália®) and the blood was collected by cardiac puncture using a tube with heparin. The blood was centrifuged (4°C; 600 g) (Fanem-204, São Paulo, Brazil), and the plasma was stored at −80 °C. Liver tissue was collected and stored at −80 °C before analysis. Part of the liver was stored in formalin for histological analysis. Body weight gain and food consumption were monitored weekly during the experimental period. At the end of the experimental period, the food efficiency ratio (FER (%) = (total weight gain /total food consumption) x 100 (da Silva et al., 2016) and Lee index (cube root of the body weight/nose-to-anus length (Novelli et al., 2007) were calculated. The hepatosomatic index was calculated by liver weight (g)/body weight (g) multiplied by 100 (Moreira et al., 2022) and the % of body fat as total adipose tissue (g)/body weight (g) (Grancieri et al., 2022). The sum of the weight of retroperitoneal, abdominal and subcutaneous adipose tissue was considered as total adipose tissue. The right and left biceps muscle was collected, mixed, weighted, and stored at -80 ◦C. The AST (aspartate aminotransferase) and ALT (alanine aminotransferase) levels were measured in plasma by colorimetric methods using commercially kits (Bioclin®, Belo Horizonte, Brazil) according to manufacturer’s instructions.

All the experimental procedures with animals were performed in accordance with Directive 86/609/EEC of November 24, 1986, in compliance with the ethical principles for

animal experimentation. The study protocol was approved by the Ethics Committee of the Federal University of Viçosa (Protocol No. 01/2019).

2.4. Extraction of mRNA from liver tissue and cDNA synthesis

Liver tissue was macerated in liquid nitrogen under RNAse free conditions, and the samples were aliquoted for total RNA extraction. Total RNA was extracted with the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) (100 mg of liver were homogenized with 1 mL of TRIzol). The extracted mRNA was used to synthesize the cDNA with the M-MLV reverse transcription kit (Invitrogen Corp., Grand Island, NY) (Livak & Schmittgen, 2001).

2.5. Determination of gene expression of proteins involved in inflammation by quantitative reverse transcriptase polymerase chain reaction (RT-qPCR)

mRNA expression levels of genes in the liver that are involved in inflammation and oxidative stress were analyzed by RT-qPCR. The SYBR Green PCR master mix from Applied Biosystems (Foster City, CA) was employed, and the analyses were performed on the StepOne™ Real-Time PCR System (Thermo Fisher Scientific), by means of the measurement system involving SYBR-Green Fluorescence and Primer Express software (Applied Biosystems, Foster City, CA). The PCR cycle involved the initial denaturation at 95°C (10 min) and 40 cycles, with 1 min of denaturation (95°C), 1 min. (62°C) and 2 min. (72°C), followed by the analytical curve of dissociation. Sense and antisense primer sequences were ordered (Choma Biotechnologies) to amplify PPAR-α (ID: 2404316), p65-NFκB (ID: 376255853), Zn-SOD1 (ID: 5615763), IL-1β (ID: 376255848), NRF2 (ID: 454138112), AMPK (ID: 377251729), SREBP1 (ID: 376255850), CPT1 (ID: 04423488), β-actin (ID: 376255852). The relative expression levels of mRNA were normalized to the endogenous control β-actin (Table 2).

Table 2. Sequence of primers used in the RT-PCR analysis.

Genes	Oligonucleotide (5'-3')	
	Forward	Reverse
β-actin	TTCGTTGCCGGTCCACACCC	GCTTTGCACATGCCGGAGCC
PPAR-α	CCTGCCTTCCCTGTGAACT	ATCTGCTTCAAGTGGGGAGA
NFκB	GCGTACACATTCTGGGGAGT	GGTACCCCCAGAGACCTCAT
Zn-SOD1	GAGCAGAAGGCAAGCGGTGAA	CCACATTGCCCAGGTCTG

IL-1β	GGGAAACAACAGTGGTCAGG	GAGCTGTCTGCTCATTCACG
NRF2	CACATCCAGACAGACACCAGT	CTACAAATGGGAATGTCTCTGC
AMPK	GTCAAAGCCGACCCAATGATA	CGTACACGCAAATAATAGGGTT
SREBP1	GCCGAGATGTGCGAACTG	GGAAGTCACTGTCTTGGTTGTT
CPT1	GTAAGGCCACTGATGAAGGAAGA	ATTTGGGTCCGAGGTTGACA

PPAR-α: peroxisome proliferator-activated receptor alpha; NFκB: factor nuclear kappa B; SOD: superoxide dismutase; TNF-α: tumor necrosis factor alpha; IL-1β: interleukin 1 beta; NRF2: Nuclear factor erythroid 2-related factor 2; AMPK: AMP-activated protein kinase; SREBP1: Sterol regulatory element-binding protein 1; CPT1: Carnitine Palmitoyltransferase 1.

2.6. IL-1β and TNF-α quantification

To determine the concentrations of IL-1β and TNF-α in the liver, the hepatic tissue samples were homogenized by means of the NE-PER Nuclear and Cytoplasmic Extraction Kit reagents (Thermo Scientific Fisher, USA). The nuclear fractions of IL-1β and TNF-α were assessed by immunoassay using the mouse IL-1β (Cat #88-6010-22; Invitrogen, USA) and mouse TNF-α (Cat #88-7340-22, Invitrogen, USA), ELISA kits, respectively. The concentrations of IL-1β and TNF-α were calculated by comparison with the corresponding standard curves.

2.7. Analysis of lipid peroxidation and oxidative stress levels

2.7.1. Homogenate preparation

To obtain a liver homogenate, 200 mg of liver were mixed with 1000 µL of phosphate buffer (50 mM) and 1 mM EDTA (pH 7.4). The sample was macerated and centrifuged at 12,000 ×g, 4°C, for 10 min, and then the supernatant was collected and stored in an ultrafreezer (-80°C) until the time of analysis.

2.7.2. Malondialdehyde (MDA)

MDA was quantified by the thiobarbituric acid reactive substances (TBARS) method. In two separate 2 mL microtubes, 400 µL of liver homogenate and 400 µL of TBARs solution (15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid, and 0.25 M HCl) were aliquoted. The samples were mixed by vortexing and subjected to heating in a water bath at 95°C for 30 minutes. Following this step, 600 µL of n-Butanol were added to each tube, and the mixture was vortexed and centrifuged at 800 g for 10 minutes. The supernatant was

extracted and pipetted in triplicate onto an Elisa plate for measurement using a Multiskan GO spectrophotometer (Thermo Scientific) at a wavelength of 535 nm (Kohn & Liversedge, 1944; Pyles et al., 1993). The MDA concentration was calculated via the molar absorptivity coefficient (Buege & Aust, 1978), and the results were expressed in nanomoles of MDA per milligram of protein (MDA/PTN). Total protein in the liver homogenate was quantified by the Bradford method (Bradford, 1976).

2.7.3. Superoxide dismutase (SOD)

SOD was quantified in relative units, and one unit was defined as the amount of SOD enzyme that inhibits the pyrogallol oxidation rate by 50%. About thirty microliters of homogenate were aliquoted in the microtubes and 249 µL of Tris-HCl buffer (50 mM, pH 8.2) containing 1 mM EDTA, 6 µL of 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) (1.25 mM), and 15 µL of pyrogallol (10 mM) were added. For the standard, 6 µL of MTT, 15 µL of pyrogallol (10 mM), and 279 µL of buffer were added. To obtain the blank, 6 µL of MTT and 294 µL of buffer were applied to the wells. Then, the samples, standard, and blank were incubated at 37°C for 5 minutes. The analysis was read on a spectrophotometer (Multiskan GO, Thermo Scientific), at 570 nm, and the results were expressed as units of SOD activity per milligram of protein (Marklund, 1985).

2.7.4. Catalase

Catalase was analyzed according to the methodology proposed by Aebi (1984). About twenty microliters of the homogenate supernatant (1:10 diluted in water), 1 ml of phosphate buffer 100 mM (pH 7.2) + H_2O_2 (in each 25 ml buffer, 40 µL of H_2O_2 30%) was added in a tube. At 0, 30, and 60 s after the reaction was initiated, the absorbance at 240 nm was determined on a spectrophotometer (T70 + UV/VIS Spectrometer). Enzymatic activity was recorded in micromoles per milliliter of a sample. Catalase activity was calculated according to Lambert Beer's law.

2.7.5. Plasma total antioxidant capacity (TAC)

This analysis is based in the capacity of the sample to inhibit the ABTS oxidation by the metmyoglobin. Aliquots of plasma (10 µL) were added to the wells along with 20 µL of the reagent metmyoglobin and 150 µL of the ABTS solution. Then, 10 µL of increasing concentrations of Trolox standard were pipetted into the wells, in triplicate, to construct a standard curve. The microplate was incubated at room temperature (22°C), and then the

absorbance at 405 nm was read by means of a spectrophotometer (Multiskan GO, Thermo Scientific). The values are expressed in mM Trolox equivalents.

2.8. Potential interactions of PPAR-α and peptides from chia digested protein: *in silico* analyses

Peptides formed by the digestion of chia protein, as described by Grancieri, Martino, de Mejia (2019), were evaluated for their effects on PPAR-α marker using *in silico* analysis. The peptides were designed using Instant MarvinSketch (ChemAxon Ltd). The crystal structure file of PPAR-α was obtained from the Protein Data Bank (PDB: 1K7L). Flexible torsions, charges and grid size were assigned by AutoDock Tools, and docking calculations were performed using AutoDock Vina (Trott & Olson, 2009). The binding pose with the lowest binding energy (highest binding affinity) was selected as a representative image to be visualized in the Discovery Studio 2016 Client (Dassault Systemes Biovia Corp®). The compound Saroglitazar ((2S)-2-ethoxy-3-[4-[2-[2-methyl-5-(4-methylsulfanylphenyl)pyrrol1-yl]ethoxy]phenyl]propanoic acid) was used as pharmacological control to PPAR-α (Akbari et al., 2021), whose structure was obtained from the PubChem Website (PubChem CID: 60151560).

2.9. Histological analysis of the liver tissue

The liver tissue samples were fixed in 10% formaldehyde and embedded in paraffin and the blocks were sliced with semi serial sections of 5 mm thickness for hematoxylin and eosin staining. The analyses were performed and photographed under a bright field microscope (Olympus AX 70 TRF, Tokyo, Japan) in a 20× objective. Ten liver fields were selected for each animal, with each histological field with a 266-point reticulum on the images until reaching the sum of 1064 points per animal (Cupertino et al., 2013).

The images of the histological sections (cytoplasm, lipid vesicles, nucleus, and inflammation) were analyzed using the Image J® 1.48v software system (National Institute of Health, USA).

2.10. Statistical Analysis

The statistical analysis was performed using the GraphPad Prism® statistical software system, version 7. The samples were tested by Kolmogorov-Smirnov normality test. The groups were compared using the “One-way” ANOVA (variance analysis), followed by

Newman-Keuls *post-hoc* ($p < 0.05$). The data were expressed as mean ± standard deviation (SD), considered significant when $p < 0.05$.

3. Results

After 8 weeks of experiments, the AIN group presented the highest food intake, while the AIN+DTP group exhibited the lowest value ($p < 0.05$) (Figure 1A). The animals in the AIN+DTP group presented greater weight gain (Figure 1B) and food efficiency ratio (FER) (Figure 1C) ($p < 0.05$). The addition of digested total protein in the HF group (HF+DTP) reduced weight gain and FER when compared to the HF group (Figures 1B and C) ($p < 0.05$). The HF group had the highest percentage of body fat, followed by HF+DTP and by groups fed a normal diet (AIN and AIN+DTP), which had similar values (Figure 1D). However, the Lee index was similar among all experimental groups ($p > 0.05$), except for the AIN group, which presented a lower value, when compared to the other experimental groups ($p < 0.05$) (Figure 1E). The HF group presented a higher hepatosomatic level compared to AIN group ($p < 0.05$), and the digested protein reduced it in the HF+DTP group to the same level as the group fed with standard diet (AIN) (Figure 1F). The muscle mass was higher in groups that consumed DTP (AIN+DTP and HF+DTP) then control groups (AIN and HF groups) (Figure 1G). The AST plasmatic values were similar ($p > 0.05$) in all groups (AIN: 34.31±10.31; HF: 30.62±7.38; AIN+DTP: 38.53±5.98; HF+DTP: 40.79±11.65 U/L) and also ALT had no different ($p > 0.05$) between groups (AIN: 21.83±5.08; HF: 27.65±4.90; AIN+DTP: 27.5±8.27; HF+DTP: 25.62±5.77 U/L).

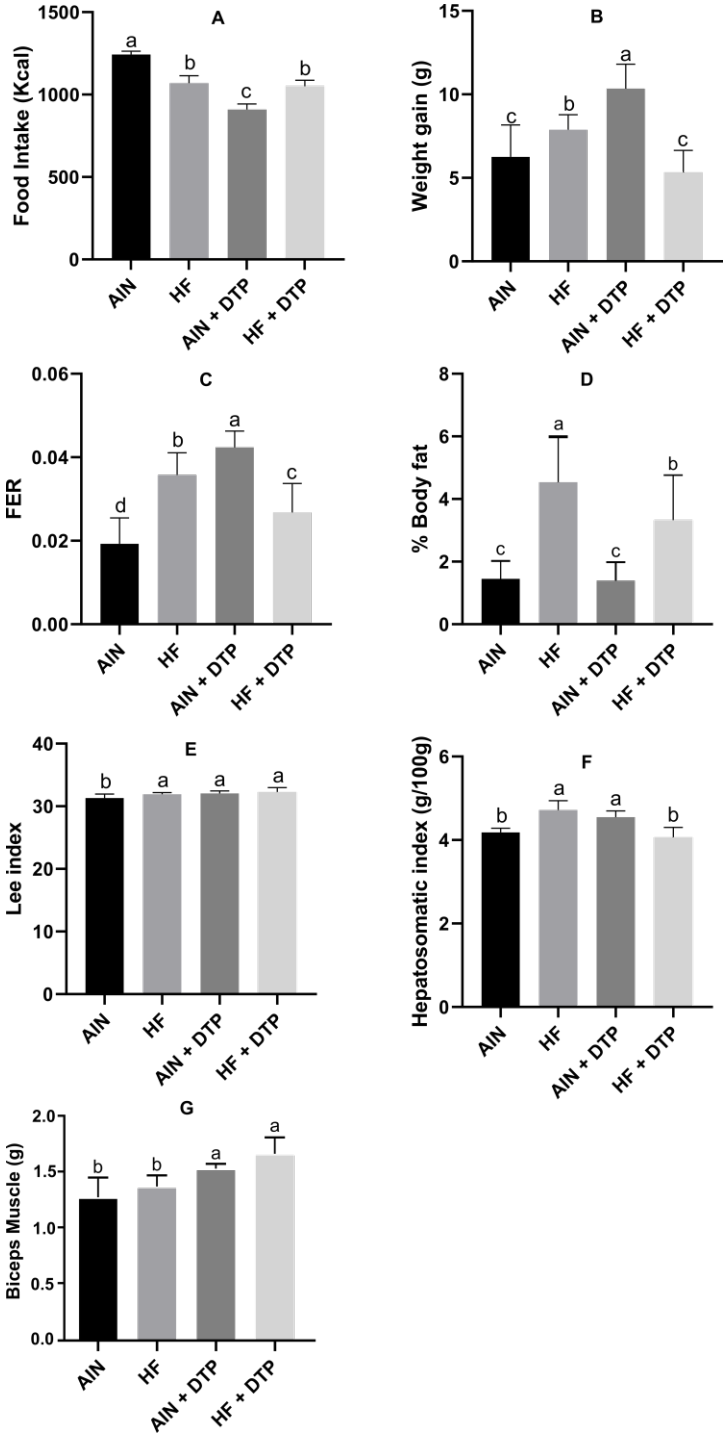


Fig. 1. Effect of digested protein from chia seeds on food consumption and body composition markers of mice fed a high-fat diet**.** FER: Food efficiency ratio. Results were expressed as means ± standard deviation. Different letters mean statistically different between the groups by ANOVA and *post-hoc* Newman-Keuls ($p<0.05$).

The high fat diet (HF group) did not change the activity of catalase, when compared to the AIN group ($p > 0.05$) (Figure 2A). Nevertheless, the addition of chia digested total protein reduced the catalase activity in the AIN+DTP and HF+DTP groups, when compared to the AIN and HF groups ($p < 0.05$) (Figure 2A). The concentration of MDA was higher in the HF than AIN group ($p < 0.05$), and the treatment with digested total protein in the HF group (HF+DTP) reduced the MDA levels ($p < 0.05$) to values comparable to those of the AIN and AIN+DTP groups (Figure 2B).

SOD quantification did not differ among the experimental groups ($p > 0.05$) (Figure 2C). High-fat diet consumption for 8 weeks did not affect the plasma total antioxidant capacity (TAC) in the HF group, compared to AIN group ($p > 0.05$) (Figure 2D). Nonetheless, the digested total protein reduced this parameter in the AIN+DTP and HF+DTP groups, compared to the groups without addition of total digested protein ($p < 0.05$) (Figure 2D).

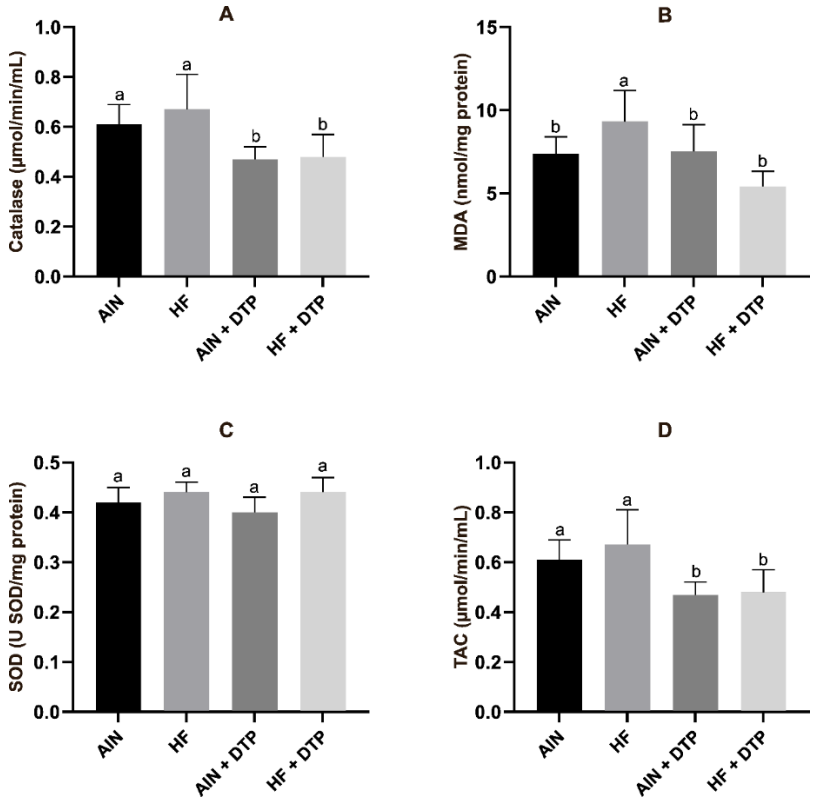


Fig. 2. Effect of chia digested protein consumption on oxidative stress in C57BL/6 mouse. (A) Catalase; (B) MDA: malondialdehyde, (C) SOD: superoxide dismutase, (D) TAC: total antioxidant capacity. Means followed by the same letter in the lines do not differ by the Newman-Keuls test, at the 5% level of significance.

NRF2 and SOD gene expression did not differ among the experimental groups ($p > 0.05$) (Figure 3A and B). p65-NFκB gene expression did not differ between the HF and AIN groups ($p > 0.05$) (Figure 3C). However, the addition of digested protein reduced the expression of this marker in the HF + DTP group compared to the HF group ($p < 0.05$) (Figure 3C). mRNA gene expression (Figure 3D) and IL-1β protein levels (Figure 3E) were lower in the AIN group, compared to the HF group ($p< 0.05$). The addition of digested protein in the standard diet (AIN + DTP) and high fat diet (HF + DTP) reduced the expression of IL-1β with expression levels of the AIN + DTP group lower than those of AIN ($p < 0.05$). The expression levels of the HF + DTP group were lower than those of the HF group ($p < 0.05$) (Figure 3D). Similar results were observed in IL-1β protein levels, in which the addition of digested chia protein reduced the IL-1β levels in the AIN+DTP and HF+DTP groups to levels lower than those of the AIN group ($p < 0.05$) (Figure 3E). The quantification of TNF-α (Figure 3F) was higher in the HF group, compared to AIN ($p < 0.05$) (Figure 3F). However, mice fed a high-fat diet with the addition of chia-digested protein (HF+DTP) presented levels of TNF-α equal to the AIN and HF group ($p > 0.05$) (Figure 3F).

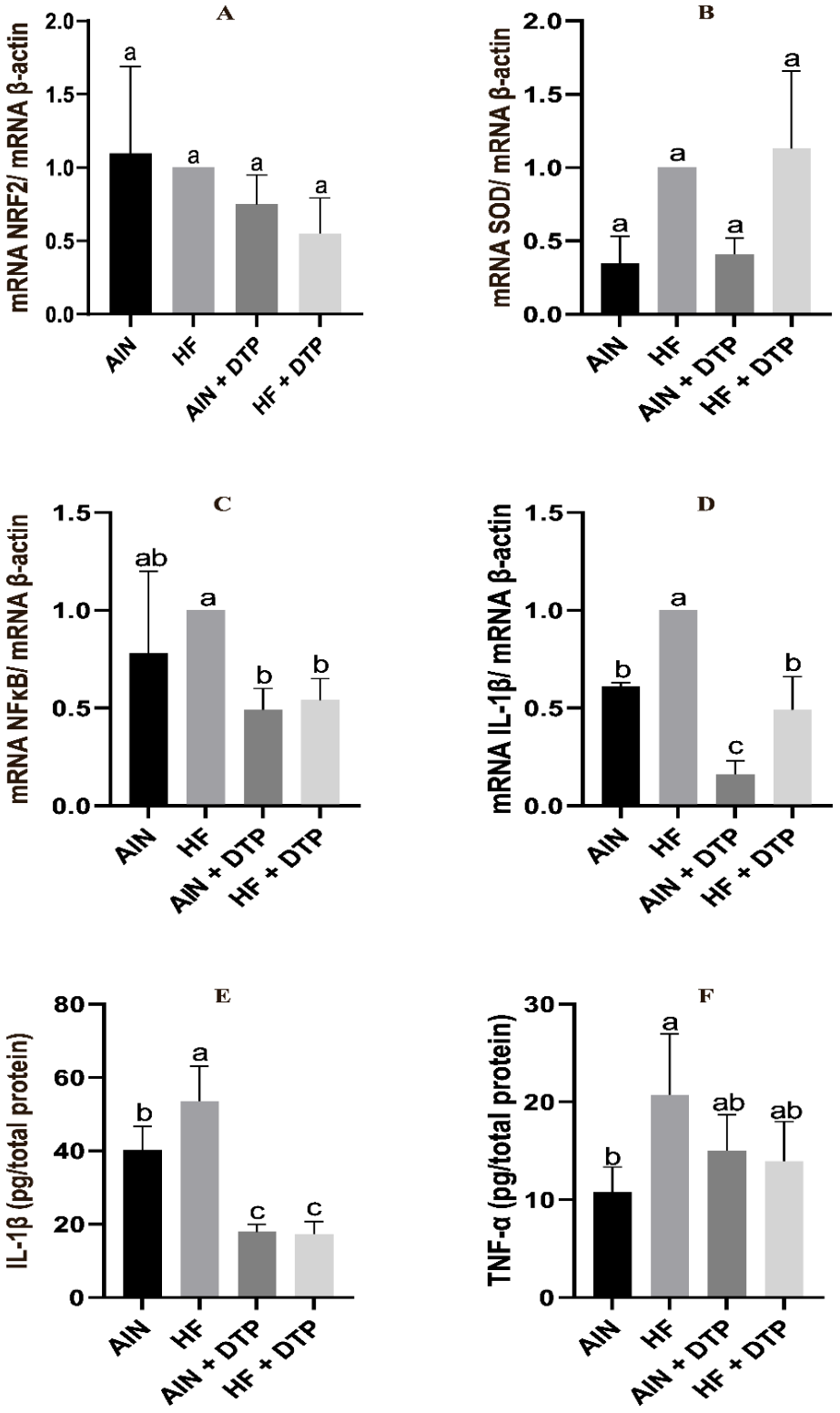


Fig. 3. Effect of chia-digested protein consumption on inflammation and oxidative stress markers in C57BL/6 mice. NRF2: Nuclear factor erythroid 2-related factor 2, SOD: superoxide dismutase, NFκB: nuclear factor kappa B, IL-1β: interleukin 8, TNF-α: tumor necrosis factor alpha. The means followed by the same letter in the lines do not differ by the Newman-Keuls test, at the 5% significance level.

The results found on the expression of genes responsible for energy metabolism are shown in figure 4. The AMPK gene expression did not differ between the AIN and HF groups ($p > 0.05$) (Figure 4A). However, the AIN+DTP group exhibited the highest gene expression of AMPK ($p < 0.05$) (Figure 4A). When we evaluated the gene expression of SREBP1, no difference was observed between AIN and AIN+DTP, or HF and HF+DTP ($p > 0.05$) (Figure 4B). The CPT1 and PPAR-α gene expression did not differ among the experimental groups ($p > 0.05$) (Figure 4C and D).

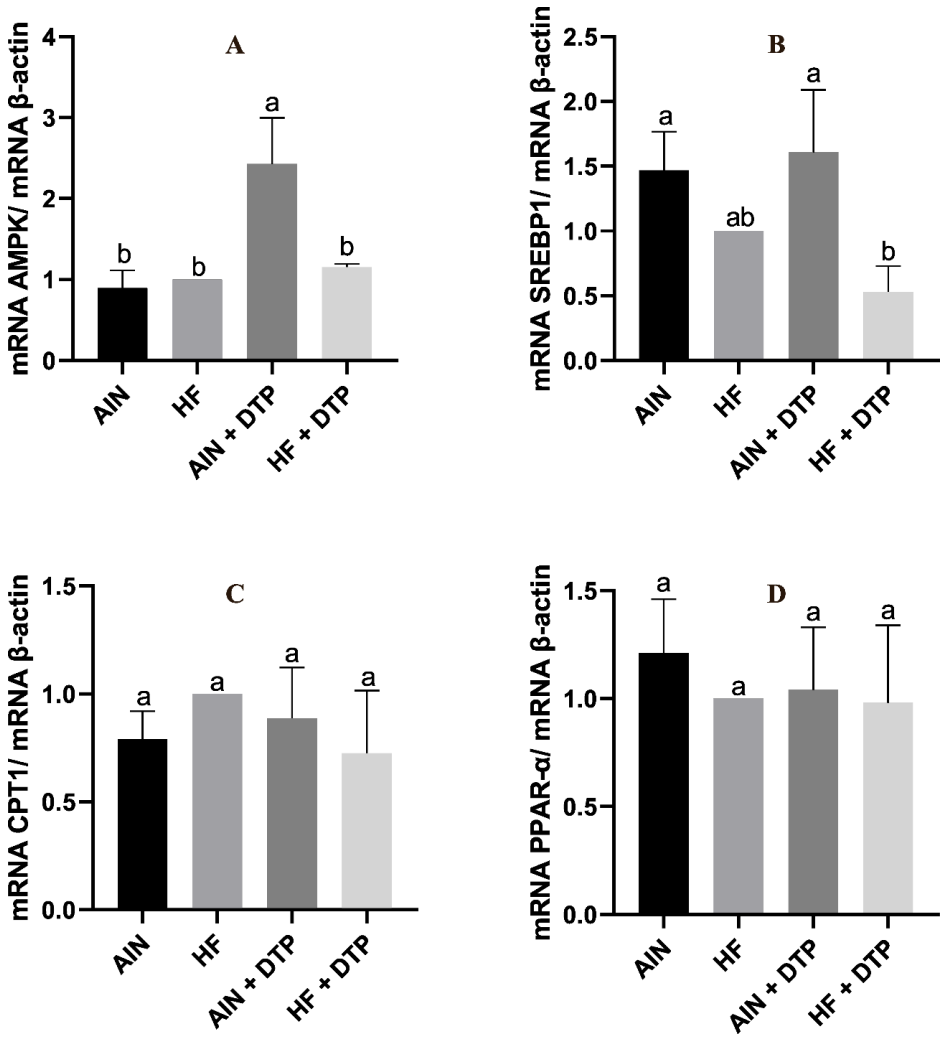


Fig. 4. Effect of chia-digested protein consumption on markers of energetic metabolism in C57BL/6 mice. AMPK: AMP-activated protein kinase, SREBP1: sterol regulatory element-binding protein 1, CPT1: Carnitine palmitoyltransferase 1, PPAR-α: Peroxisome proliferator-activated receptor alpha. The means followed by the same letter in the lines do not differ by the Newman-Keuls test, at the 5% significance level.

The *in silico* analysis demonstrated that peptides from DTP from chia seeds presented high interaction with PPAR-α, in which the peptide APSPPVLGPP allowed higher EFE (-10.2) interaction than the pharmacological control Saroglitazar (EFE= -8.4) (Table 3 and Figure 5). In addition, just one peptide presented no interaction (Table 3).

Table 3. Estimated free energy (EFE) binding and chemical interactions among the peptides present in digested protein from chia seeds with PPAR-α.

Digested protein peptides	EFE	Interacting amino acid residues
HYGGPPGGCR	-9.8	TYR A: 334, LEU A: 321, MET A: 220, VAL A: 324, LEU A: 331, SER A: 280, TYR A: 314, CYS A: 276, CYS A: 275, CYS A: 278, LEU A: 254, HIS A: 440, SER A: 280, TYR A: 314
SPPHGAGLGGGMT	-5	GLN A: 305, VAL A: 306, THR A: 288, THR A: 285, HIS A: 457, HIS A: 274, CYS A: 278
LPVFGLAAEGNVVTYLH	4.2	-
TPPGVGGFPWGGAMLGAQ	-3.9	GLU A:282, CYS A: 278, VAL A: 281, GLN A: 305, HIS A: 457, PRO A: 458, ILE A: 463, GLU A: 462, VAL A: 306, THR A: 285, ARG A: 465, LEU A: 459, THR A: 288
APSPPVLGPP	**-10.2**	LYS A: 222, LEU A: 247, PHE A: 218, ILE A: 241, ALA A: 250, TYR A: 334, ALA A: 333, MET A: 320, VAL A: 332, ILE A: 339, CYS A: 275, LEU A: 344, MET A: 330, LEU A: 321, PHE A: 318, ILE A: 317, LEU A: 254, CYS A: 276, THR A: 279, SER A: 280, PHE A: 273, HIS A: 440
NPGGGAPSGH	-6.2	ILE A: 463, GLU A: 462, ARG A: 465, LEU A: 309, LYS A: 292
LHNGGPGGEY	-8.2	MET A: 320, TYR A: 334, CYS A: 278, SER A: 280, THR A: 283, CYS A: 275, CYS A: 276, TYR A: 314, VAL A: 332
DVNPGGPAPHPPWLSVNDL	-3.9	LEU A: 302, THR A: 288, TYR A: 334, LEU A: 254, CYS A: 278, CYS A : 275, LEU A: 321, SER A:

		280, PHE A: 273, ILE A: 354, MET A: 355, CYS A: 276, ALA A: 333, VAL A: 281, HIS A: 457
TGPPRPALVFPHAVVP	-4.4	LYS A: 310, VAL A: 306, ILE A: 463, PRO A: 458, HIS A: 457, THR A: 285, VAL A: 281, THR A: 307, ARG A: 465, GLU A: 462, LEU A: 459, THR A: 288
NSSAQYSDPFLALH	-4.1	GLU A: 282, HIS A: 457, PRO A: 458, VAL A: 306, GLN A: 305, LYS A: 292, GLU A: 289
Pharmacological control	-8.4	LEU A: 456, LEU A: 460, VAL A: 444, SER A: 280, HIS A: 440, TYR A: 464, TYR A: 313, GLN A: 277, PHE A: 318, ILE A: 354, LEU A: 321, THR A: 279, PHE A: 273, CYS A: 276, MET A: 355, MET A: 330, LEU A: 344, ILE A: 339, ILE A: 272, VAL A: 332, ALA A: 333, CYS A: 275, LEU A: 254

EFE: Estimated free energy. Docking calculation were carried out using AutoDock Vina. Value in bold mean the highest interaction. Negative values mean spontaneous reaction. *Pharmacological control: Saroglitazar. ALA: alanine; ARG: arginine; CYS: cysteine; GLN: glutamine; GLU: glutamic acid; HIS: histidine; ILE: isoleucine; LEU: leucine; LYS: lysine; MET: methionine; PHE: phenylalanine; PRO: proline; SER: serine; THR: threonine; TYR: tyrosine; VAL: valine.

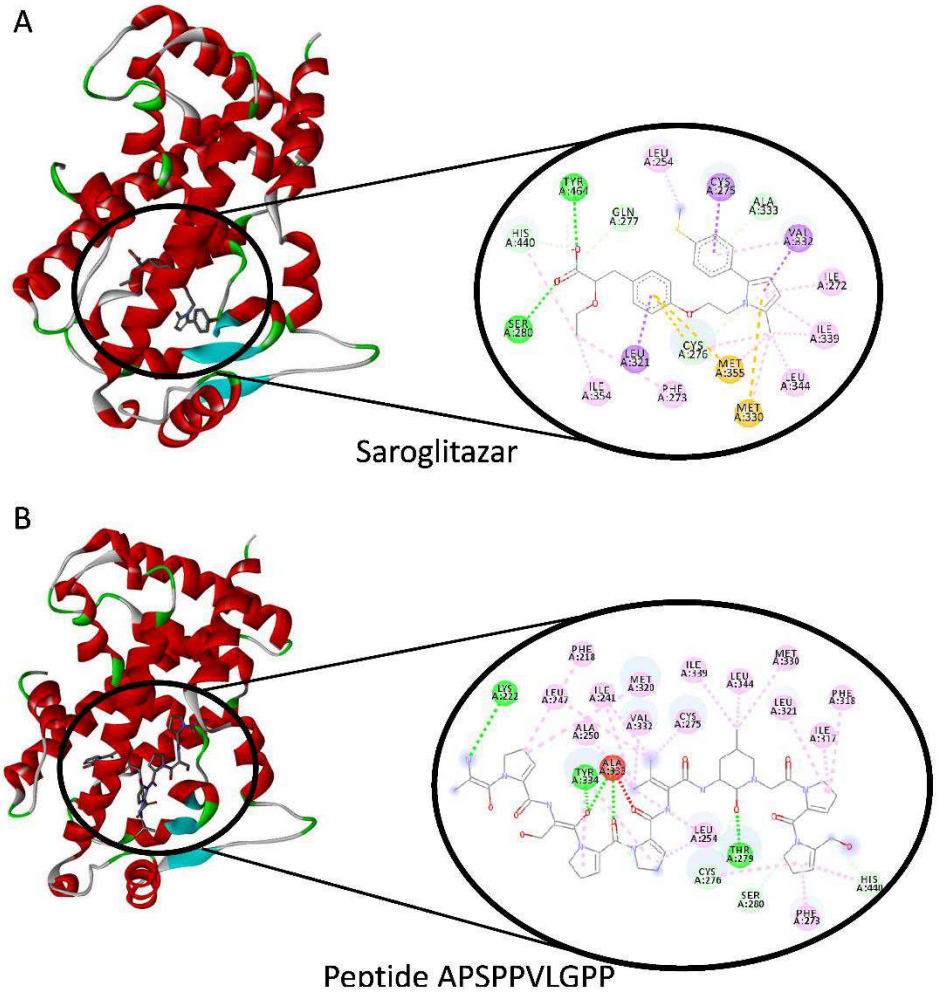


Fig. 5. The *in silico* interaction of the peptide and pharmacological control with PPAR-α. (a) Interaction of Saroglitazar pharmacological control with PPAR-α; (b) Interaction of APSPPVLGPP found in digested protein from chia seed with PPAR-α. These analyzes were performed by AutoDock Vina® and visualized by Discovery Studio 2016 Client®. EFE: Estimated free energy.

Changes in hepatic cell components were found among the experimental groups. The percentage of cytoplasm was reduced in the HF group when compared to the others ($p<0.05$). However, the percentage of fat vesicles increased in the animals of the HF group, when compared to the AIN group ($p<0.05$). Nevertheless, the addition of DTP (HF + DTP group) reduced this percentage ($p<0.05$). Despite the increased liver fat in the HF group, no differences were found in the percentage of liver inflammation among the evaluated groups ($p>0.05$) (Figure 6).

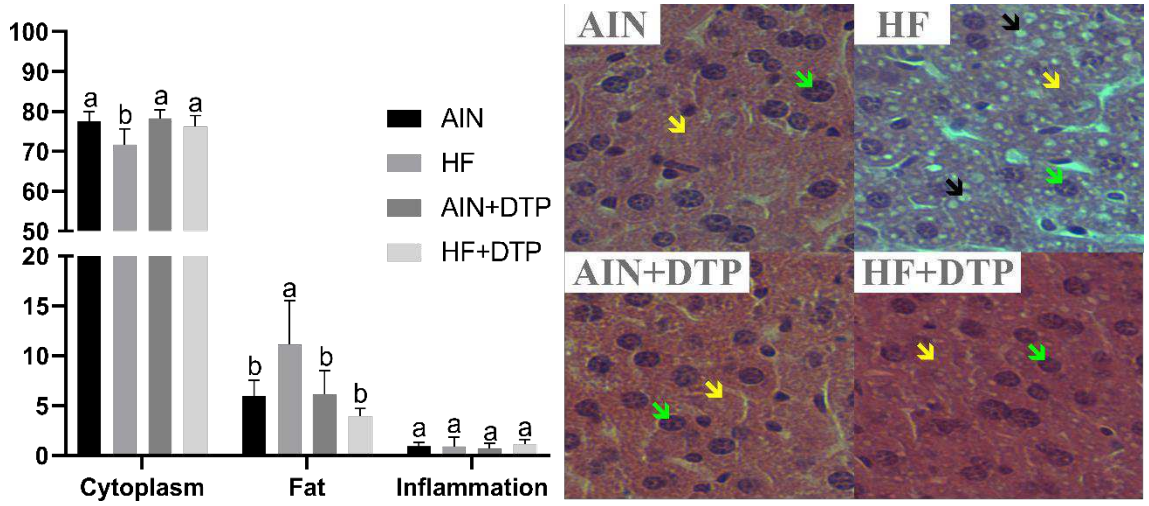


Fig. 6. Effect of chia-digested protein consumption on cellular components percentage of liver. Black arrows: fat; Green arrows: hepatocyte nucleus; Yellow arrows: cytoplasm. Staining was carried out with hematoxylin and eosin. Objective: 20x.

4. Discussion

Previous studies have already demonstrated that the digested protein of chia is rich in bioactive peptides with anti-inflammatory and antioxidant capabilities (Grancieri et al., 2019a). However, no studies have been carried out to evaluate the effect of DTP on oxidative stress and inflammation in the liver of mice fed with a high-fat diet.

In our study, despite the reduced food intake, it was observed higher weight gain in animals fed with AIN diet added with DTP, nevertheless, there was no change in the Lee index. Digestion of chia protein generates peptides that bind to the inhibitory site of the DDP IV enzyme (Grancieri et al., 2019a) which probably increased incretin GLP-1, reducing appetite and promoting weight loss (Kadouh et al., 2020; Koliaki & Doupis, 2011). Additionally, the presence of glycine-containing peptides in the hydrolyzed favor the release of GLP-1 (Grancieri et al., 2019a; Meek et al., 2016; Takeuti, 2017), which leads to an increase in AMPK, increasing oxidation of carbohydrates and lipids (Belli & Yaman, 2020, Li et al., 2021). In our study was observed weight gain in the AIN+DTP group, which is probably due to the ability of peptides from chia to stimulate muscle mass gain, which was confirmed by the increased muscle mass gain by animals fed with DTP. Furthermore, this low percentage of body fat and increased muscle mass may collaborate in less fat retention in the liver, as observed. Moreover, all groups had similar values of AST and ALT, showing that diets did not cause liver damage, despite the increase in liver fat presented by HF group (Sadulaeva et al., 2022).

The animals fed a high-fat diet added with DTP, presented reduced weight gain, which may be related to the DDP IV/GLP-1 pathway already mentioned. The lower weight gain presented by HF+DTP group reduced total body fat, which promoted low liver fat accumulation, as evidenced by histological analysis, besides reducing liver-to-body weight ratio. The accumulation of liver fat is directly related to the development or worsening of metabolic syndrome, diabetes and obesity (Lim et al., 2021; Stefan & Cusi, 2022; Subudhi et al., 2022).

Furthermore, HF+DTP had lower expression of SREBP1 then normal diets (AIN and AIN+DTP) and similar to HF group, which can be associated to the role of SREBP1 to control of lipid homeostasis in the body, acting on the expression of the genes involved in cholesterol synthesis and other lipids (Li et al., 2019; Ferré et al., 2021; Jiang et al., 2022). In addition, it stimulates the enzyme FAS, which catalyzes the synthesis of fatty acids through malonyl-CoA palmitate and acetyl-CoA (Angeles & Hudkins, 2016; Jensen-Urstad & Semenkovich, 2012). Thus, the SREBP1 gene expression did not differ between HF and HF+DTP, it was observed reduction of fat and IL-1β inflammatory protein concentration in the liver, demonstrating the protect effect of DTP in the fat diet condition.

Although no differences were found in PPAR-α gene expression in the liver, the silico docking analysis revealed the ability of the APSPPVLGPP peptide, present in digested chia protein, to bind to PPAR-α. Thus, this peptide can bind in an agonist way to PPAR-α and stimulate its activity, which increase hepatic beta-oxidation, consequently reducing the triglyceride content in that organ (Tahri-Joutey et al., 2021). In other studies, the same peptide proved to be efficient in reducing adipogenesis through its binding to FAS (Grancieri et al., 2020, 2021). However, the beneficial effects found in docking analysis and *in vitro* studies should be extrapolated to animals with caution. The bioaccessibility and bioavailability of peptides depend on the activity of proteases, peptidases, in the stomach and gut and the chemical and nutritional composition of the diets, which can reduce the bioaccessibility and bioavailability depending of its content of nutrients. In addition, the high fat content present in the HF diet reduces the absorption of these peptides and impairs their bioavailability in target organs (Sun et al., 2020), as observed in the histological analyses in the HF+DTP group in our study.

Further, the chronic use of high-fat diets leads to changes in the electron transport chain of mitochondria and hepatic beta-oxidation decrease, with consequent accumulation of

liver fat and increased products of lipid peroxidation and inflammation (Moreira et al., 2022; Souza et al., 2022; Zou et al., 2022).

In our study, the high-fat diet increased liver fat deposition, lipid peroxidation and inflammation, as evidenced by the increase in MDA, IL-1β, and TNF-α. In addition, in our study the digested protein was able to reduce the p65-NFκB gene expression activation and IL-1β concentration, suggesting its ability to reduce inflammation. Consumption of a high-fat diet reduces IκB levels, allowing the translocation of NFκB to the nucleus and consequent increase in the transcription of inflammatory mediators, such as IL-1β and TNF-α, causing liver damage (Illesca et al., 2019; Liu et al. al., 2021; Sun et al., 2021; Tapia et al., 2014). However, digested chia proteins, mainly the globulin and glutenin fractions, are able to reduce p65-NFκB phosphorylation and translocation leading to reduced inflammation (Grancieri et al., 2019a, 2021; Martino et al., 2020). A similar results were observed for other cereals such as quinoa, millet, amaranth and buckwheat (Ji et al., 2020; Moon et al., 2019; Capraro et al., 2021).

Although the literature suggests that consuming a high-fat diet (HF) may increase oxidative stress and reduce antioxidant defense, our study did not find any difference in the levels of oxidative stress caused by the HF. Our results were similar to those found in other studies (Marineli et al., 2015; Moraes et al., 2012). Consequently, we did not observe any changes in antioxidant enzymes (SOD and catalase) promoted by high-fat diet intake. This may be explained by the short duration of obesity induction, which was insufficient to alter normal oxidative metabolism (Tao et al., 2019; Ren et al., 2021; Grancieri et al., 2022). A recent study on chia seed revealed its potential to reduce lipid accumulation and liver damage through the stimulation of the Nrf2 antioxidant pathway and inhibition of the NFκB pathway (Vega Joubert et al., 2022). The Nrf2 pathway is fundamental to control oxidative stress and liver inflammation, as it stimulates the expression of antioxidant enzymes, such as SOD and catalase (Bardallo et al., 2022; Hahn et al., 2017; Kaspar et al., 2009). However, in our study, the chia DTP does not seem to act on this antioxidant pathway, since reduced catalase and TAC were observed. We believe that the increased exogenous intake of antioxidant compounds, derived from DTP, reduced need for endogenous production of antioxidant enzymes, which explains catalase and TAC decrease observed in our study.

5. Conclusion

The total digested protein from chia reduced hepatic inflammation in mice fed a high-fat diet by reducing hepatic fat accumulation and lipid peroxidation, without altering the fatty acid transport. Then, our results indicate the potential of digested chia protein to prevent liver changes, which makes it a promising ingredient for the production of nutraceuticals, besides stimulating the consumption of chia seeds.

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Author Contributions

Vinícius Parzanini Brilhante de São José: Conceptualization, Methodology, Data curation, Investigation, Formal analysis; Writing – original draft; Software, Visualization; **Mariana Grancieri**: Data curation; Methodology; Roles/Writing original draft; Writing review & editing. **Renata Celi Lopes Toledo**: Formal analysis; Funding acquisition, Methodology; Project administration; Resources. **Elvira Gonzalez de Mejia**: Resources; Methodology; Funding acquisition. **Bárbara Pereira da Silva**: Data curation; Investigation; Formal analysis; Methodology; Validation; Writing – original draft; Writing review & editing. **Hércia Stampini Duarte Martino**: Conceptualization; Data curation; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing – original draft; Writing review & editing. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest Statement

The authors declare no conflict of interest. The funders had no role in the study design; in the collection, analysis, or interpretation of data; in the manuscript writing; or in the decision to publish the results.

Institutional Review Board Statement

All procedures performed in this study involving animals were in accordance with the ethical standards of the Federal University of Viçosa. The animal study protocol was approved by the Ethics Committee on Animals Use of the Federal University of Viçosa (CEUA/UFV, protocol No 1/2019, date of approval: February 21th, 2019).

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6.2 ARTIGO 2: Effect of digested chia seed protein on the gut microbiota and morphology of mice fed a high-saturated-fat diet

Abstract

Purpose: The present study aimed to investigate the effect of digested total protein (DTP) from chia seed on the gut microbiota and morphology of mice fed with a high-fat diet.

Methods: Forty-four male C57BL/6 mice were divided into 4 groups: AIN (standard diet), HF (high-fat diet), AIN+DTP (standard diet supplemented with 400mg of digested chia seed protein), and HF+DTP (high-fat diet supplemented with 400mg of digested chia seed protein) during 8 weeks. Colon morphology, tight junction's gene expression and gut microbiota composition were evaluated.

Results: The consumption of digested chia seed protein (DTP) increased the width of crypts in AIN+DTP group, as well as the longitudinal muscular layer was increased in the HF+DTP group and the circular muscular layer was increased in the AIN+DTP and HF+DTP groups. Furthermore, the AIN+DTP group enhanced the expression of tight junction proteins, including occludin and claudin, while the AIN+DTP and HF+DTP groups increase the zonula occludens expression. The α-diversity analysis showed a reduction in bacterial dominance in the HF+DTP group. All the experimental groups were grouped in different cluster, showing differences in the microbiota community in the β-diversity analyzes. The Firmicutes/Bacteroidetes ratio did not differ among the groups. The genera *Olsenella* and *Dubosiella* were increased in the AIN+DTP group, but the *Oscillospiraceae_unclassified* was increased in the HF+DTP group. The *Alistipes* was increased, while the *Roseburia* and *Akkermansia* were decreased in the AIN+DTP and HF+DTP groups.

Conclusion: The consumption of high fat diet added with DTP from chia seed modulated the gut microbiota composition, by reducing of Simpson index and increasing of *Alistipes* genus, and the intestinal morphology increasing the circular muscle layer, counteracting the adverse effects of high-fat diet.

Keywords: Beta diversity; *Salvia hispanica*; tight junction proteins, bioactive peptide

1. Introduction

The consumption of diets high in saturated fats is one of the responsible factors, which can modify anthropometric, biochemical, hormonal, and microbiota profiles in humans. Studies have shown a strong relationship between the consumption of high-fat diets and reduction in the gut microbiota composition, richness and diversity with the development of obesity in humans and animals models (MISHIMA et al., 2023a; STANISLAWSKI et al., 2021; WAN et al., 2019). Furthermore, it is demonstrate that the Firmicutes/Bacteroidetes ratio has correlation with total fat consumption (BISANZ et al., 2019). In other animal studies, a high-fat diet reduced the abundance of *Bifidobacteria*, *Proteobacteria*, and *Akkermansia* (JU et al., 2019; SHAO et al., 2020). These bacteria are related to butyric acid short chain fatty acid production (RIVIÈRE et al., 2015), preservation of the microbiota (REN et al., 2023), cognition and cerebral energy metabolism (HIGARZA et al., 2021), and glucose homeostasis (YOON et al., 2021).

Furthermore, the consumption of high-fat diets is associated with intestinal hyperpermeability (HOU; ABRAHAM; EL-SERAG, 2011; MICHIELAN; D'INCÀ, 2015). This fact is due to the ability of high-fat diets consumption to reduce the expression of intestinal tight junctions, such as claudin and occludin, which are responsible to keep the intestinal integrity (NASCIMENTO et al., 2021; ROHR et al., 2020a). In studies conducted with mice, the use of high-fat diets led to a reduction in the expression of tight junctions, thus increasing intestinal permeability (CANI et al., 2008; KIRPICH et al., 2012; SUZUKI; HARA, 2010). Furthermore, the consumption of high-fat diets can alter intestinal morphology, reducing the length of colon villi and the muscular thickness due to the lipotoxic effects of high-fat diets (TANAKA et al., 2020; XIE et al., 2020).

In this context, recent studies have demonstrated the beneficial effects of chia seed (*Salvia hispanica* L.) in modulating intestinal permeability and microbiota (MISHIMA et al., 2022, 2023a; MORAIS et al., 2023). The hydrolysis of chia protein generates low molecular weight peptides (< 3kDa) with hydrophobic and cationic characteristics, capable of binding to negatively charged bacterial membrane, destabilizing it one and consequently improving microbiota composition (AGUILAR-TOALÁ; DEERING; LICEAGA, 2020b; KUMAR; KIZHAKKEDATHU; STRAUS, 2018). Thus, a study that performed sequential hydrolysis of chia protein using alcalase, flavorzyme, and microwave enzymes identified 16 peptides with antimicrobial potential in this seed (AGUILAR-TOALÁ; DEERING; LICEAGA, 2020b). *In ovo* study, chia soluble extract proved to be effective in improving intestinal histological

parameters, with increased villi and goblet cells (DA SILVA et al., 2019). In *in vitro* study, chia protein hydrolysate inhibited the growth of pathogenic and spoilage bacterial species such as *Escherichia coli*, *Salmonella enterica*, and *Listeria monocytogenes* (AGUILAR-TOALÁ; DEERING; LICEAGA, 2020a).

In addition, analyses of the peptide profile of chia, found the antioxidant effects to be the most prevalent, which can improve the intestinal damage caused by a high-fat diet (GRANCIERI; MARTINO; MEJIA, 2019a). In a previous *in vitro* study carried out by our research group, antioxidant and anti-inflammatory potential of digested chia seed protein was demonstrated through the reduction of TNF-α, factor nuclear kappa B (NFκB), lipoxygenase 1 (LOX-1) and reactive oxygen species (GRANCIERI; MARTINO; MEJIA, 2019d, 2019g). Subsequently, these results were confirmed in an *in vivo* study, in which mice fed a diet rich in saturated fat associated with digested chia seed protein showed less inflammation of the adipose tissue, with a reduction in TNF-α and NF-κB, in addition to increased expression of adiponectin (GRANCIERI et al., 2022). In ovo study, chia protein hydrolysate reduced the expression of alpha tumor necrosis factor (TNF-α) and increased the expression of occludin and mucin, thereby reducing inflammation and intestinal permeability. Additionally, the hydrolysate increased the abundance of *Lactobacillus*, which has immunomodulatory and anti-inflammatory effects (MISHIMA et al., 2023b).

Thus, due to the antioxidant and anti-inflammatory effects found, the digested chia protein has potential for modulating the microbiota and intestinal morphology. However, there are no studies in literature that evaluate the effects of the digested chia protein on intestinal health in animals fed a high-fat diet. Therefore, the objective of our study was to evaluate the effect of digested chia seed protein on the gut microbiota and morphology of mice fed a high-saturated-fat diet. Therefore, the present study aimed to evaluate the effect of digested chia seed protein on the intestinal microbiota and morphology of mice fed a diet rich in saturated fat.

2. Material and Methods

2.1. Preparation of digested chia protein and its bioactive peptides characterization

The chia seeds utilized in this study were sourced from Dubai Alimentos, a company situated in Ijuí, Rio Grande do Sul, Brazil. These chia seeds are of the traditional variety commonly cultivated on farms in Rio Grande do Sul, Brazil, harvested in July 2017. The region where the seeds was cultived presented a temperate climate characterized by an

average temperature of 26 °C, relative humidity levels ranging from 60 to 80%, consistent rainfall, and clay soil composition (DA SILVA et al., 2017). The chia seeds were added in distilled water (1:10, g: ml), during 1 h for mucilage formation. Then, the samples were freeze-dried (Labconco Freeze Dryer 4.5; Kansas, MO, USA) and the mucilage was manually removed from the seeds by rubbing the seeds through a sieve (500 μm / 35 mesh). The free-mucilage seeds were ground using a coffee grinder (Mr. Coffee®) and sieved (500 μm / 35 mesh) to standardize the size of grains formed. After, the chia flour (mucilage free) was degreased using hexane (1:10, g:ml) under constant stirring (60 °C, 2 h). The mixture was centrifuged (6000 g, 15 min, 4 °C) and the resulted flour was left overnight under a hood and then stored at 4 °C until use (ORONA-TAMAYO et al., 2015).

For the preparation of total chia protein, deionized water was added to the mucilage and fat-free chia flour (1:20, g:ml), the pH was adjusted to 8.0, and kept under constant stirring, during 1h at 35 °C. The mixture was centrifuged (5000 g; 15 min; 25 °C) and the supernatant collected, freeze-dried, and stored at -20 °C until use.

The total protein was underwent simulated gastrointestinal digestion. Firstly, the total protein was mixed with deionized water (1:20, g: ml), the pH adjusted to 2.0, and pepsin was added (1:20, enzyme:protein). This solution was kept under stirring for 2 h, at 37 °C. After, the pH was modified to 7.5 and pancreatin was added (1:20 enzyme: protein) and the digestion was conducted under constant agitation during 2 h, at 37 °C. At the end, the suspension was heated on a water bath (75 °C, 20 min) to stop reaction and centrifuged twice (20,000 g, 15 min, 4 °C). The supernatant was collected and dialyzed using molecular weight cut-off membrane, 100-500Da (Spectra/Por®, Biotech CE Membrane), freeze-dried and stored at −20 °C until analysis (GRANCIERI; MARTINO; MEJIA, 2019e; MEGÍAS et al., 2004).

2.2 Identification, Characterization and Bioactive Potential of Peptides from Chia

The digested total protein (DTP) obtained was analyzed to obtain the peptides characterization 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 and PDA detector wavelength at 280nm. The gradient mobile phase was A: 95% water, 5% of acetonitrile, and 0.1% of formic acid; B: A: 95% of acetonitrile, 5% of water, and 0.1% of formic acid with a volume of 200 μL/min of injection (MOJICA; CHEN; DE MEJÍA, 2015). The results were analyzed in MassLynx V4.1 software (Waters Corp., Milford, MA, USA) and the sequence of amino

acids was identified based on the accurate mass measurements, tandem MS fragmentation using the MassBank database.

The peptides with more than 90% sequence probability and antioxidant activity by BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep, accessed on February 27, 2018) were selected and their amino acids were presented as one letter nomenclature.

2.3. Animals study

Forty-four adult male C57Bl-6 mice from Reproduction Biology Center of Federal University of Juiz de Fora, Minas Gerais, Brazil were distributed in individual stainless-steel cages and kept in room with controlled temperature (22 ±2 °C) and photovoltaic period of 12 hours. The animals received deionized water *ad libitum* during all experimental time. The study was approved by Ethics Committee of the Federal University of Viçosa, Viçosa, MG, Brazil (Protocol number 01/2019). All the experimental procedures with animals were performed in accordance with Directive 86/609/EEC of November 24, 1986, in compliance with the ethical principles for animal experimentation.

The experimental diets were based on standard AIN-93M (REEVES; NIELSEN; FAHEY, 1993) or high-fat diet (Research Diets, New Brunswick, NJ), with caloric density correspond to 3.85kcal/g and 5.41kcal/g, respectively (Table 1). The diets were offered daily by pellets and the digested total protein (DTP) was incorporated on diet pellets on concentration of 400mg of DTP/kg of body weight (MOJICA et al., 2017) to simulate the physiological consumption. The food consumption was controlled to ensure that the animals consumed the entire pellet with the DTP offered. Food consumption was calculated by the amount of diet offered minus the leftovers in the feeder and in the newspaper.

Table 1. Composition of experimental diets.

Ingredients (g.kg^{-1})	**AIN**	**HF**	**AIN+DTP**	**HF+DTP**
Albumin*	179.71	179.71	179.71	179.71
Digested total protein**	-	-	0,4	0,4
Corn starch	425.99	113.99	425.99	113.99
Dextrinized starch	155.00	155.00	155.00	155.00
Sucrose	100.00	100.00	100.00	100.00

Lard	0.00	312.00	0.00	312.00
Soybean oil	40.00	40.00	40.00	40.00
Microcrystalline cellulose	50.00	50.00	50.00	50.00
Mineral mix	35.00	35.00	35.00	35.00
Vitamin mix	10.00	10.00	10.00	10.00
L-cystine	1.80	1.80	1.80	1.80
Choline bitartrate	2.50	2.50	2.50	2.50
Carbohydrate (%)	70.70	27.30	70.70	27.30
Protein (%)	18.70	13.30	18.70	13.30
Lipids (%)	9.30	58.50	9.30	58.50
Caloric density (Kcal/g)	3.85	5.41	3.85	5.41

Composition of experimental diets based on the standard rodent diet (AIN-93M) (REEVES; NIELSEN; FAHEY, 1993). HF: high fat diet. *Albumin based on 77.9% protein content.. ** Daily added to the diet at a ratio of 400mg/kg of body weight of the animal.

The animals were randomized (Excel, Microsoft) according to body weight in four groups: Normal diet group (AIN, standard diet AIN-93M); High-fat group (HF, high-fat diet); normal diet + DTP group (AIN+DTP, standard diet AIN-93M + 400mg/kg of body of DTP), and high-fat diet + DTP group (HF+DTP, high-fat diet + 400mg/kg of body of DTP). The experiment was conducted during 8 weeks. In the end, after 12 h of fasting, the animals were anesthetized with isoflurane (Isoforine, Cristália®) and blood was collected by cardiac puncture. The entire colon was collected, weight, and the colon tissue and its content were immediately frozen and stored at -80 °C. A part of proximal colon was placed in 10% formalin.

2.4. Colon histomorphology analyses

Colon fragments tissue was embedded in paraffin and histological sections of 3µm thickness were made in a non-serial manner using an automatic microtome (Reichert-Jung®), fixed on glass slides and stained with hematoxylin/eosin. Then, 20 random fields/animal were randomly selected, based on the quality of the crypts present, which should be with definite and visible connective epithelium to best measure crypt thickness, crypt height, and circular and longitudinal muscle layers (MARTINEZ et al., 2023). The slides were examined under a CX31 photomicroscope (Olympus, Japan), using 20x objective, and the measurements were

carried out using ImagePro-Plus application® version 4.5 (Media Cybernetics, Rockville USA).

2.5. mRNA extraction from colon and cDNA synthesis

About 100mg of colon tissue was macerated with liquid nitrogen and TRIzol reagent (Invitrogen, Carlsbad, CA, USA) under RNase-free conditions to obtain total mRNA. The extracted mRNA was used for cDNA synthesis using the M-MLV Reverse transcriptase kit (Invitrogen Corp., Grand Island, NY) (LIVAK; SCHMITTGEN, 2001).

2.6. Quantification of gene expression of proteins involved in intestinal integrity by quantitative reverse transcriptase polymerase chain reaction (RT-qPCR)

The mRNA gene expression in the colon involved in intestinal integrity were analyzed by RT-qPCR. The StepOne™ Real-Time PCR System (Thermo Fisher Scientific) was used for analysis, with the SYBR Green PCR master mix from Applied Biosystems (Foster City, CA) and the measurement system involving SYBR-Green Fluorescence and Primer Express software (Applied Biosystems, Foster City, CA). For this, the primers sequence, sense and antisense, was used (Choma Biotechnologies) to amplify zonulin, claudin and occludin. The endogenous control (beta-actin) was used to normalized the relative expression levels of mRNA (Table 2).

Table 2. Sequence of primers used in the RT-PCR analysis.

Genes	Oligonucleotide (5'-3')	
	Forward	Reverse
β-actin	TTCGTTGCCGGTCCACACCC	GCTTTGCACATGCCGGAGCC
Zonula occludens	CCAGCTTATGAAAGGGTTGTTC	TCCTCTCTTGCCAACTTTTCTC
Claudin	GCTCTCAGAGTCCGTTGACC	CTGCCCTTTCAGGTTAGCAG
Occludin	ATGTCCGGCCGATGCTCTC	TTTGGCTGCTCTTGGGTCTGTAT

2.7. Gut microbiota: DNA Extraction, Sequencing, and Analysis

A total of 200mg of colon content was submitted to a mechanical disruption and phenol/chloroform extraction protocol to extracted the genomic DNA of gut microbiota, as previously published (STEVENSON; WEIMER, 2007). Then, the DNA concentration and quality were determined by spectrophotometry by measuring the A260/280 and amplicons from the 16S rRNA V3-V4 region were generated using forward primer 341F (5`-CCTAYGGGRBGCASCAG-3`) and reverse primer 806R (5`-GGACTACNNGGGTATCTAAT-3`) and a barcode primer set adapter for the platform Illumina NovaSeq (Illumina, San Diego, California, USA) (CAPORASO et al., 2012).

Samples were loaded into an Illumina flow cell for paired-end sequencing reactions using the Illumina NovaSeq PE250 platform at Novogene Corporation at the campus of the University of California at Davis (Sacramento, California, USA). Amplicons were sequenced in a NovaSeq 2x250bp run using customized primers and sequencing procedures (CAPORASO et al., 2012). The samples-sequences were submitted to the Sequence Read Archive (SRA) database at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sra) under the access number PRJNA930050.

Furthermore, the data processing and analysis were performed using Mothur v.1.44.3 software (SCHLOSS et al., 2009). The R1 and R2 paired-end reads were joined and sequences shorter than 380 or longer than 440 bp were removed. Chimera sequences were detected and removed using UCHIME version 4.2 (EDGAR et al., 2011) and the cleaned sequences were aligned with the 16S rRNA gene using the SILVA v.138 database (QUAST et al., 2012). The final paired sequences were grouped into Operational Taxonomy Units (OTUs), also using SILVA v.138 database, which each OTU characterizes a genetically unique group of biological organisms, and a sequence similarity cut-off of 97% was adopted. The coverage of all samples was assessed by the Good Coverage Estimator (bacteria > 97%). To correct for sampling bias resulting from unequal amplicon library sizes, the samples were normalized for the lowest number of sequences produced from any sample (Supplementary Table S1).

Then, the normalized data was used for calculating α and β-diversity and the relative abundance of OTUs. The α-diversity of each sample was analyzed using the Chao1, Shannon, and Simpson index for microbial community composition and the β diversity metrics were calculated using the Jaccard dissimilarity index. Principal co- ordinate analysis (PCoA) plots were performed on calculated distance matrices in the bacterial communities. The statistical

significance of β diversity across sample groups was assessed with the nonparametric Permutational Multivariate Analysis of Variance (Monte Carlo permutations) test, using the Past software (version 4.05).

2.8. Statistical Analysis

Data were expressed as mean ± standard deviation (SD) or box plots. Colon histomorphometry characteristic and alpha-diversity were submitted to the Kolmogorov-Smirnov normality test followed by Analysis of Variance (ANOVA) and *post-hoc* of Newman-Keuls. The effect of experimental diets on the abundance of bacteria was analyzed by Kruskal-Wallis and H-test nonparametric test using false discovery rate by Benjamin correction for multiple comparisons (Statistical Analysis of Metagenomic Profiles, version 2.1.3). Datasets were tested for homogeneity by the Kolmogorov-Smirnov test. Nonparametric data were transformed to log10 and samples were submitted to *post-hoc* Duncan test for multiple comparison. Every analysis considered as significant level $p < 0.05$.

3. Results

3.1. Colon histomorphology

No difference was observed in crypt length among experimental groups ($p>0.05$) (Fig. 1a). However, crypt width was higher in the AIN+DTP group compared to the AIN and HF groups ($p<0.05$). The HF+DTP group presented a crypts width similar to the other experimental groups ($p > 0.05$). The HF+DTP diet did not change crypt width compared to AIN and HF groups ($p>0.05$) (Fig. 1b). The longitudinal and circular muscle layers thickness was higher in the HF+DTP group relative to HF group ($p<0.05$), become similar to the AIN group ($p>0.05$) (Fig. 1c). For the circular muscle layer, the AIN+DTP group presented the higher values compared to the others group ($p<0.05$). Furthermore, the digested chia protein also increased the circular muscle layer in the HF+DTP group compared to the HF group ($p<0.05$) (Fig. 1d).

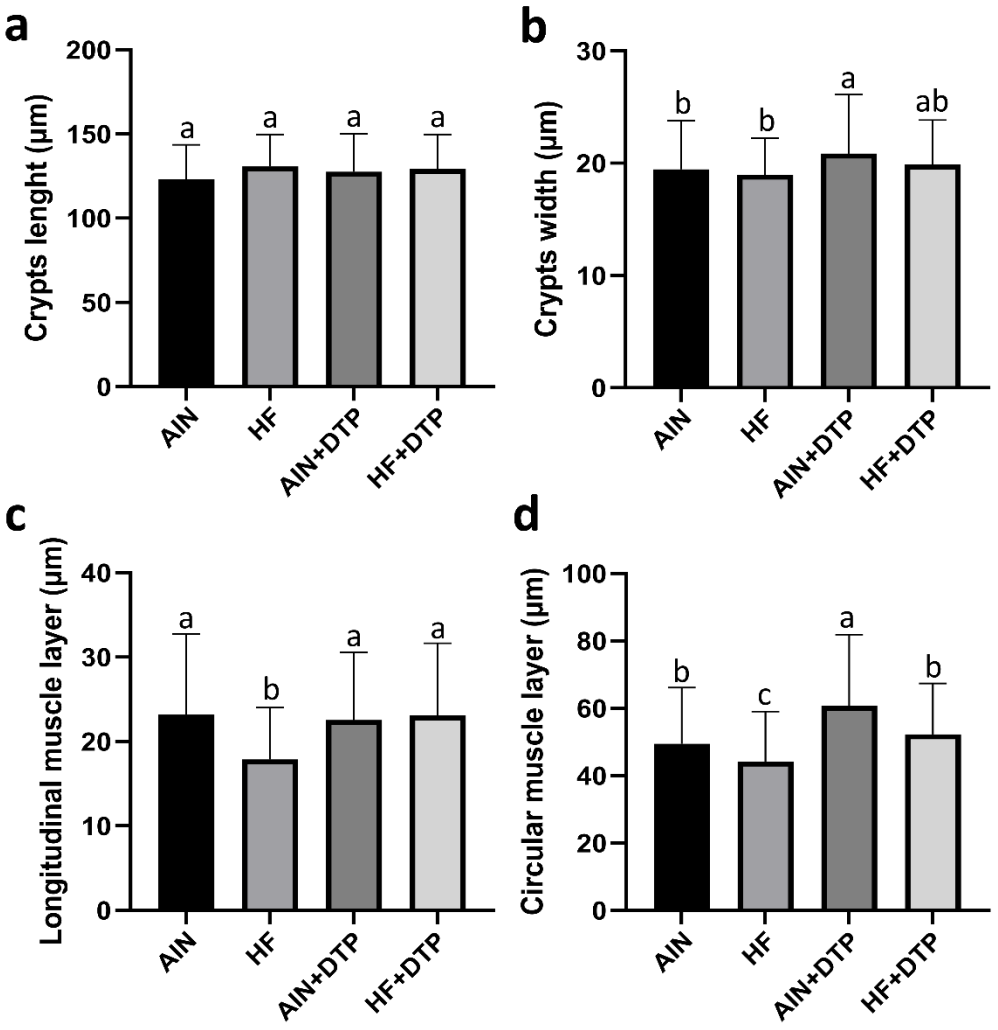

Fig. 1 Effect of chia digested protein consumption on intestinal morphology in C57BL/6 mouse. AIN: standard diet (n=11), HF: high fat diet (n=11), AIN+DTP: standard diet added digested total protein at 400 mg/kg of body of DTP (n=11); HF+DTP: high-fat diet added digested protein at 400 mg/kg of body of DTP (n=11). Means followed by the same letter did not differ by the Newman-Keuls test, at the 5% level of significance.

3.2. Gene expression of proteins of intestinal integrity

Intestinal integrity was evaluated by gene expression of the proteins occludin, claudin and zonula occludens 1 (ZO-1) (Figure 2). Gene expression of all proteins increased in HF group compared to AIN ($p<0.05$) (Fig. 2a, b and c). In addition, the AIN+DTP group increased gene expression of occludin, claudin, and ZO-1 proteins compared to AIN ($P<0.05$) (Fig. 2a, b and c). No difference was observed in HF+DTP group for occludin and claudin

proteins gene expression relative to HF group ($p>0.05$), but the ZO-1 gene expression was higher in the HF+DTP group ($p<0.05$) (Fig. 2a, 2b, 2c)

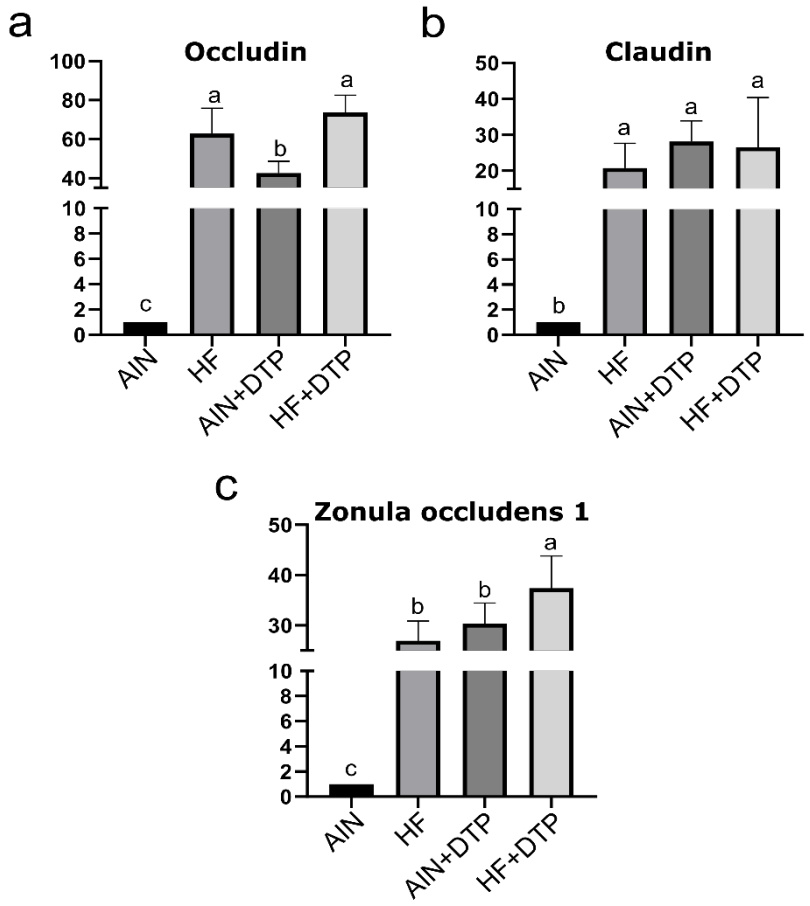

Fig. 2 Effect of chia digested protein consumption on intestinal permeability in C57BL/6 mouse. AIN: standard diet (n=11), HF: high fat diet (n=11), AIN+DTP: standard diet added digested total protein at 400 mg/kg of body of DTP (n=11); HF+DTP: high-fat diet added digested protein at 400 mg/kg of body of DTP (n=11). Means followed by the same letter did not differ by the Newman-Keuls test, at the 5% level of significance

3.3. Gut microbiota analysis

The 16S rRNA gene amplicon sequencing method (V3-V4 region) generated sequencing of the 16S rRNA gene from cecal content samples generated 6622735 raw sequences. After filtering and cleaning the sequences, 3411773 good quality sequences were obtained. The Good's coverage obtained in the samples was > 99%, thus indicating a good and complete sequencing coverage. The summary of sample sequencing data (total number of raw read, filtered read, and the normalized read counts per group) is shown in Supplementary Table 1.

The α-diversity indexes were used to assess the richness, diversity, and dominance of the microbiota by Chao1, Shannon, and Simpson index, respectively. The HF and HF+DTP groups had lowest ($p<0.05$) richness compared to AIN and AIN+DTP groups by Chao index (Figure 3a). Furthermore, the groups AIN and AIN+DTP had greater diversity than HF group ($p<0.05$) and HF+DTP was similar to other groups as verified by the Shannon index (Figure 3b). Besides, it was observed a significant effect of the HF diet ($p<0.05$) on increasing species dominance through the Simpson index. However, it was verified that the treatment with digested protein reduced this dominance in HF+DTP relative to HF group ($p<0.05$), being similar to the AIN and AIN+DTP groups ($p>0.05$) (Figure 3c).

The β-diversity analysis showed the microbial composition difference based on the overall OTUs assessed by principal coordinates analysis (PCoA) by presenting the differences in the distance metrics between treatments (PERMANOVA, $p = 0.00009$, $F = 2.146$). The PCoA analysis showed an evident distinction in bacterial communities of all groups, as the experimental groups exhibited distinct clusters among them (Figure 3D).

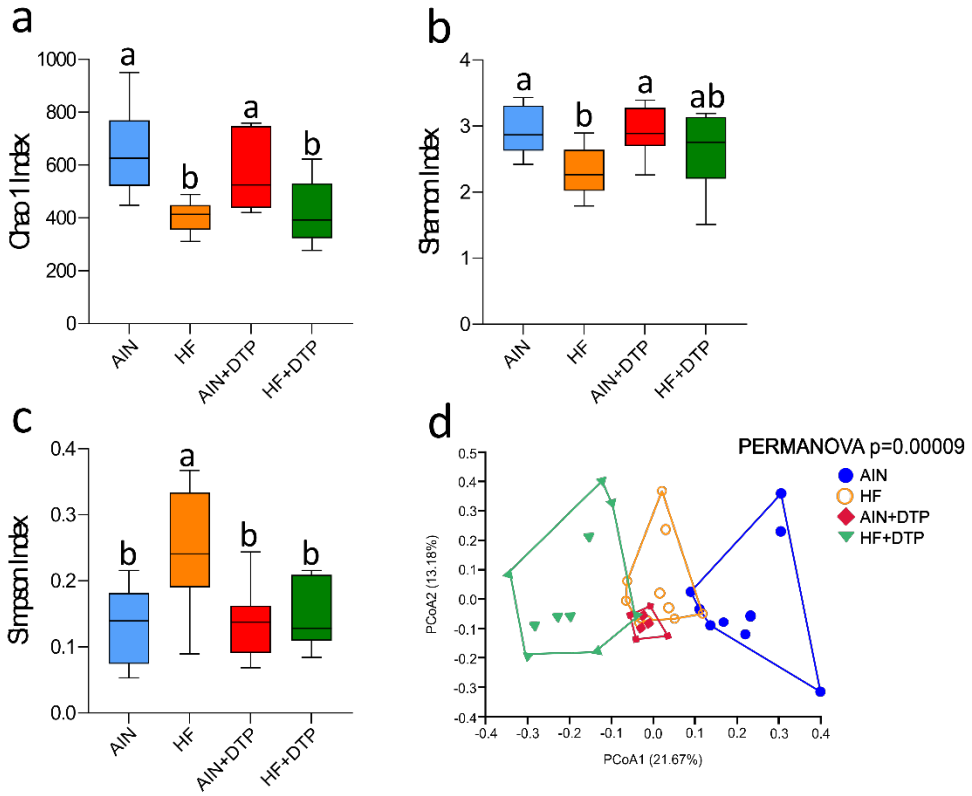

Fig. 3 Effect of chia digested protein consumption on α-diversity and β-diversity in C57BL/6 mouse. **a** Chao 1 index, **b** Shannon index, **c** Simpson index, **d** Principal Coordinates Analysis (PCoA) based on Jaccard similarity distances. AIN: standard diet (n=8), HF: high fat diet (n=8), AIN+DTP: standard diet added digested total protein at 400 mg/kg of body of DTP (n=08); HF+DTP: high-fat diet added digested protein at 400 mg/kg of body of DTP (n=08).

Groups followed by the same letter did not differ by the Newman-Keuls test, at the 5% level of significance. PCoA was analyzed by PERMANOVA test.

The taxonomic classification of the samples presented 19 phyla, 43 classes, 102 orders, 162 families, and 272 genera. The *Firmicutes* was the most abundant phylum in all experimental groups, followed by *Actinobacteriota* in groups that consumed high-fat diet (HF and HF+DTP). In the groups that received a normal diet, the *Bacteroidetes* phylum was the second most abundant, corresponding to 11.27% in the AIN group and 11.70% in the AIN+DTP group. Further, the *Firmicutes*/*Bacteroidetes* ratio was similar among experimental groups (Figure 4a) ($p>0.05$). *Clostridia*, *Lachnosspirales*, and *Lachnospiraceae* were the most abundant class, order, and family, respectively, in all groups and showed no significant difference among them ($p>0.05$).

The digested protein from chia, reduced the abundance of *Verrucomicrobiota* phylum, *Verrucomicrobiae* class and *Verrucomicrobiales* order ($p<0.05$), especially in HF+DTP group, which had the lowest value. The AIN and HF groups had similar values between them for the phylum, class and order cited before ($p>0.05$) (Figure 4b and Table S2). On the other hand, the HF+DTP had the highest value of *Desulfobacteroidete* phylum, *Desulfovibrionia* class, and *Desulfovibrionales* order ($p<0.05$), followed by AIN+DTP, which was similar to AIN group and HF group had the lowest values ($p<0.05$) (Table S2). In the family abundance, the *Rikenellaceae* had the higher value in AIN+DTP and HF+DTP groups than AIN and HF groups, mainly AIN that had the lowest abundance ($p<0.05$). The HF+DTP had the highest abundance of *Desulfovibrionaceae* compared the others group ($p<0.05$), which had similar values. The AIN group showed lowest abundance of *Atopobiaceae* family and highest abundance of *Akkermansiaceae*, while HF+DTP had the lowest value of *Akkermansiaceae* ($p<0.05$).

Related to genera, *Muribaculaceae_ge* was the most abundant genus in all experimental groups and showed no difference ($p>0.05$) among them (Figure 4c). *Olsenella* was more abundant in the AIN+DTP group compared to AIN ($p<0.05$) and the *Roseburia* genus was most abundant in AIN and HF groups, compared to AIN+DTP and HF+DTP groups ($p<0.05$). The *Dubosiella* and *Akkermansia* had lowest value in AIN and HF+DTP groups, respectively, and the *Oscillospiraceae_unclassified* and *Alistipes* had highest values in the HF+DTP group ($p<0.05$) (Figure 4c and Table S2).

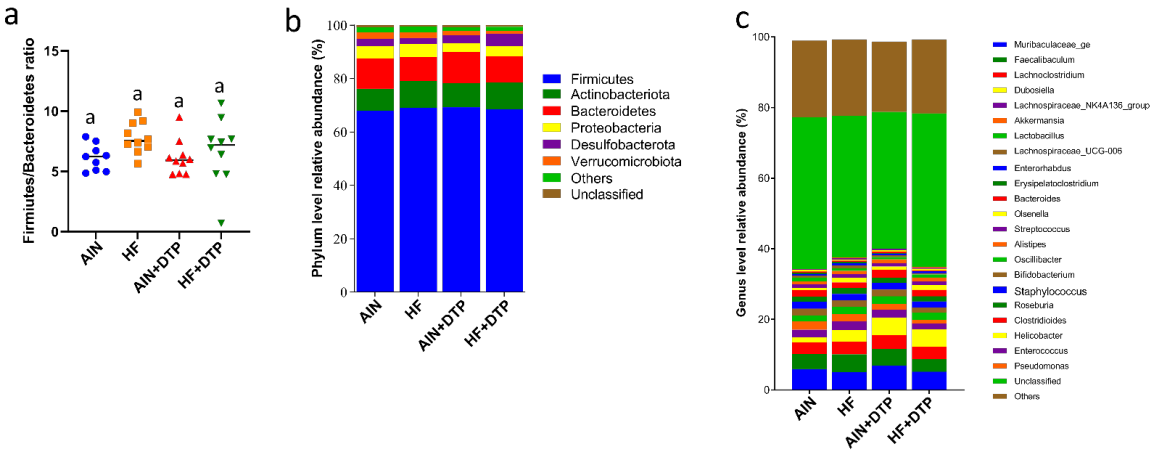

Fig. 4 Chia digested protein consumption modulate the gut microbiota in C57BL/6 mouse. **a** *Firmicutes*/*Bacteroidetes* ratio, **b** Phylum level relative abundance, **c** Genus level relative abundance. AIN: standard diet (n=8), HF: high fat diet (n=8), AIN+DTP: standard diet added digested total protein at 400 mg/kg of body of DTP (n=08); HF+DTP: high-fat diet added digested protein at 400 mg/kg of body of DTP (n=08). Means followed by the same letter did not differ by the Newman-Keuls test, at the 5% level of significance

4. Discussion

Chia seed has high protein content and provides all essential amino acids for human nutrition (SANDOVAL-OLIVEROS; PAREDES-LÓPEZ, 2013). After being digested, it releases bioactive peptides with antioxidant and antimicrobial activity can modulate the intestinal microbiota. This study shows new evidence of the properties of digested total protein from chia seed in modulating the intestinal microbiota and intestinal health in mice fed a high-saturated-fat diet. Consumption of digested chia seed protein associated with a high-saturated-fat diet increased the thickness of the intestinal muscle layer and reduced bacterial dominance, modifying the intestinal microbiota as assessed by β-diversity. Moreover, the microbiota evaluation showed that digested chia seed protein reduced the abundance of *Akkermansia* and *Roseburia* genera and increased the abundance of *Alistipes* and *Olsenella* genera.

The addition of digested chia seed protein was effective in increasing the longitudinal and circular muscle layers. This is likely due to the ability of chia protein to stimulate protein anabolism (GRANCIERI et al., 2022). This mechanism is likely due to the presence of peptides containing glycine and with inhibitory action on the enzyme dipeptidyl peptidase IV

(DPP IV), which increases the release of glucagon-like peptide 1 (GLP-1) (GRANCIERI; MARTINO; MEJIA, 2019b; MEEK et al., 2016; TAKEUTI, 2017). The release of GLP-1 stimulates the release of insulin, which in turn stimulates muscle anabolism (GONÇALVES et al., 2019; MÜLLER et al., 2019). The high-saturated-fat diet did not change the length and thickness of the intestinal villi. This result is similar to what has been reported by other studies (MARTINEZ et al., 2023; THEODORO et al., 2022). However, a reduction in the muscular layers of the HF group animals was observed. The reduction in the muscular layer is related to decreased intestinal motility, by providing a longer contact time between the mucosa and toxic molecules as free fatty acids, which are associated with increased inflammation, oxidative stress, and intestinal damage (REICHARDT et al., 2017; ROHR et al., 2020b; TANAKA et al., 2020).

Furthermore, increased expression of occludin, claudin, and ZO-1 was observed in animals fed a standard diet added of DTP and an increase in ZO-1 was observed in the high-fat added DTP group. This can be due to the profile of peptides present in digested chia seed protein, which contain fewer than 20 amino acid residues and can enter enteric cells and exert antioxidant activity (GRANCIERI; MARTINO; MEJIA, 2019h; SUN et al., 2020). Thus, can reduces intestinal inflammation and permeability (GRANCIERI; MARTINO; MEJIA, 2019c; KOU et al., 2013; MISHIMA et al., 2023b). A docking analyzes showed the peptides TGPSPTAGPPAPGGGTH, SPKDLALPPGALPPVQ and HYGGPPGGCR can bind to the inflammatory biomarkers LOX-1, toll like receptor 4 (TLR4), cyclooxygenase 2 (COX-2) and p65-NF-κB, thus reducing the inflammation (GRANCIERI; MARTINO; MEJIA, 2019g). Further, bioactive peptides can act as trophic factors for protein synthesis, improving tight junctions (BAO; WU, 2021), therefore these action in association may contribute to improve the intestinal permeability. Gene expression analysis revealed increased expression of the tight junction proteins, occludin and claudin, and the anchoring protein, zonula occludens (ZO-1) in the HF group. This result was unexpected as high-saturated-fat diets are known to increase the luminal concentration of bile acids, resulting in the dissociation of tight junction proteins and anchoring proteins, thereby increasing intestinal permeability (ROHR et al., 2020b; SUZUKI, 2020). However, the increased expression of occludin, claudin and ZO proteins may not represent an improvement in tight junctions.

The α-diversity analyses verified that the consumption of a high-fat diet reduced richness and diversity and increased the intestinal bacterial dominance, promoting a hamful intestinal health (DUAN et al., 2021; YANG et al., 2022). However, the addition of chia seed

digested protein to the HF diet was able to reduce the Simpson index. This index represents bacterial dominance, and its reduction is related to lower dominance, resulting in a microbiota with higher diversity (SIMPSON, 1949). Furthermore, an alteration in the microbiome of the different groups in this present study, was observed by a difference in the β-diversity (LIU et al., 2021). The study conducted by Aguilar-Toalá, Deering and Liceaga (2020), identified 16 peptides from chia with a potential antimicrobial effect. These peptides can bind to the membrane of bacteria, as *Eschericha coli* and *Listeria monocytogenes*, increasing the permeability and destroying the bacteria (AGUILAR-TOALÁ; DEERING; LICEAGA, 2020b). The peptide sequences from chia, YACLKVK and KLKKNL, showed the highest scores for antimicrobial activity, in addition to possessing antioxidant capacity (LEÓN MADRAZO; SEGURA CAMPOS, 2022). Thus, these are promising agents capable of altering the intestinal microbiota.

The *Actinobacteriota* was the second most abundant phylum in animals fed a high-fat diet (HF and HF+DTP). This phylum has been identified as a marker for obesity and is correlated with the development of hepatic steatosis (CHAKRABORTI, 2015; JUÁREZ-FERNÁNDEZ et al., 2021). Furthermore, the concomitant consumption of a high-fat diet and chia seed protein hydrolysate (HF+DTP) increased the abundance of the phylum *Desulfobacterota*, as well as its class (*Desulfovibrionia*), order (*Desulfovibrionales*), and family (*Desulfovibrionaceae*). Bacteria from the *Desulfovibrionaceae* family can increase the absorption of long-chain fatty acids through increasing the expression of CD36, which is associated with the occurrence of metabolic diseases (PETERSEN et al., 2019; WANG et al., 2020). However, in other study, the *Desulfovibrionaceae* family showed a positive correlation with acetate concentration, which has an anti-steatogenic effect on the liver (HONG et al., 2021; WANG et al., 2020).

Based on bioactive peptides profile with antimicrobial (YACLKVK, KLKKNL and KADVPGLKK) (AGUILAR-TOALÁ; DEERING; LICEAGA, 2020b; LEÓN MADRAZO; SEGURA CAMPOS, 2022) and antioxidant activity (TGPSPTAGPPAPGGGTH, SPKDLALPPGALPPVQ and HYGGPPGGCR) (GRANCIERI; MARTINO; MEJIA, 2019f) in chia seed protein hydrolysate we expected that it could increase the abundance of beneficial bacteria. However, the chia seed protein hydrolysate reduced the abundance of the *Akkermansia* genus, as well as its respective family (*Akkermansiaceae*), order (*Verrucomicrobiales*), class (*Verrucomicrobiae*), and phylum (*Verrucomicrobiota*). *Akkermansia* colonizes the mucosal layer of the colon, where it plays a role in the synthesis

and degradation of this layer (CROVESY; MASTERSON; ROSADO, 2020; OUWERKERK et al., 2016). However, an increase in sulfate-reducing bacteria, such as those belonging to the *Desulfovibrionaceae* family, can reduce disulfide bonds between mucins, degrading the mucus network and thus hindering the development of *Akkermansia* (CAPALDO; POWELL; KALMAN, 2017; SAYAVEDRA et al., 2021).

An increase in the abundance of the genus *Alistipes*, as well as its family *Rikenellaceae*, was observed in the HF group compared to the AIN. Consumption of chia seed protein hydrolysate (AIN+DTP and HF+DTP) promoted an increase in these bacteria, which are considered beneficial for intestinal health due to their ability to produce butyrate. Butyrate serves as an energy source for colonocytes and may have been responsible for the increased thickness of crypts in these groups (YE et al., 2021). Studies with bean protein hydrolysate and alpha-lactalbumin peptide have shown that an increase in *Rikenellaceae* enhances the production of short-chain fatty acids, reducing obesity and improving the intestinal barrier (CHEN et al., 2022; ZHAO et al., 2022). Thus, the increase in the expression of occludin, claudin, and ZO proteins observed in our study may be due to the increased abundance of *Rikenellaceae*.

Furthermore, chia seed protein hydrolysate increased the abundance of the genera *Olsenella*, which is beneficial for intestinal integrity due to their ability to produce short-chain fatty acids, contributing to the improvement of the intestinal barrier and reduction of intestinal inflammation (FORD et al., 2020; TIAN et al., 2022). Some negative points of the study were the lack of studies with DTP, making it difficult to establish the consumption dose for the animals. It is recommended to conduct studies with different doses of the protein isolate, followed by an evaluation of the ingested, excreted, and absorbed peptides. Furthermore, studies are needed to assess the ability of different bacterial species to utilize chia protein isolate and its peptides as a nutrient for their growth and development. Finally, the small cecal content produced by mice made it difficult to carry out additional analyzes.

5. Conclusion

This study provides evidence on the effects of digested chia seed protein (DTP) consumption, capable of modulating the intestinal microbiota. Thus, the DTP decreases the microbiota dominance, without changing the microbiota richness and diversity. Furthermore, despite the DTP not having increased tight junctions, the digested total protein resulting in an improvement in intestinal morphology and increased the abundance of beneficial bacteria

(*Alistipes*, *Olsenella* and *Rikenellaceae*). These results indicate a promising effect of DTP on intestinal health. However, future studies need be conducted to observed the effects of different doses of DTP and time of intervention.

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Supplementary material

Table S1. Sequencing data at the end of 8 weeks of treatment, according to each experimental group**.**

Groups	Good's coverage	Raw Sequences	After filtering and cleanup		Normalized reads	
		Reads	Reads	OTUs	Reads	OTUs
AIN	0.9991 ± 0.0002	174577 ± 13417	92736 ± 9850.51	286 ± 28.75	63108.56 ± 8.69	286 ± 28.75
HF	0.9992 ± 0.0002	167716 ± 8345	91985.5 ± 12153.81	275.9 ± 37.05	63115 ± 7.32	275,9 ± 37.05
AIN+DTP	0.9991 ± 0.0003	169630 ± 12472	85716.3 ± 10245.51	272.1 ± 23.82	63106.9 ± 5.28	272,1 ± 23.82
HF+DTP	0.9991 ± 0.0003	167808 ± 11758	80013.1 ± 10850.85	272.9 ± 32.95	63110,1 ± 7.50	272,9 ± 32.95

Values expressed as mean ± standard deviation, n=10 animals/group. AIN: normal diet group; HF: high-fat diet group; AIN+DTP: Normal diet plus digested protein from chia seed group; HF+DTP: high-fat diet plus digested protein from chia seed group; OUTs: Operational Taxonomic Units.

Table S2. Relative abundance of different taxonomic levels at the end of 8 weeks of treatment, according to each experimental group.

Classification	Groups	AIN	HF	AIN+DTP	HF+DTP
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Phylum	*Verrucomicrobiota_filo*	2.44 ± 0.54 ^{a}	2.10 ± 0.65 ^{a}	1.63 ± 0.34 ^{b}	0.95 ± 0.28 ^{c}
	Desulfobacterota_filo	2.80 ± 0.65 ^{bc}	2.27 ± 0.52 ^{c}	2.93 ± 0.54 ^{b}	4.70 ± 1.50 ^{a}
	F_B_filo	6.15 ± 1.09 ^{a}	7.79 ± 1.29 ^{a}	6.14 ± 1.46 ^{a}	7.29 ± 1.82 ^{a}
Class	*Verrucomicrobiae*	2.44 ± 0.54 ^{a}	2.10 ± 0.65 ^{a}	1.63 ± 0.34 ^{b}	0.95 ± 0.28 ^{c}
	Desulfovibrionia	2.77 ± 0.59 ^{bc}	2.27 ± 0.52 ^{bc}	2.93 ± 0.54 ^{b}	4.70 ± 1.50 ^{a}
Order	*Verrucomicrobiales*	2.37 ± 0.57 ^{a}	2.10 ± 0.65 ^{a}	1.63 ± 0.34 ^{b}	0.95 ± 0.28 ^{c}
	Desulfovibrionales	2.77 ± 0.59 ^{bc}	2.27 ± 0.52 ^{bc}	2.93 ± 0.54 ^{b}	4.70 ± 1.50 ^{a}
Family	*Atopobiaceae*	0.74 ± 0.31 ^{b}	1.93 ± 0.49 ^{a}	1.48 ± 0.58 ^{a}	2.05 ± 0.79 ^{a}
	Akkermansiaceae	2.29 ± 0.64 ^{a}	2.10 ± 0.65 ^{ab}	1.63 ± 0.34 ^{b}	0.95 ± 0.28 ^{c}
	Desulfovibrionaceae	2.77 ± 0.59 ^{b}	2.27 ± 0.52 ^{b}	2.93 ± 0.54 ^{b}	4.70 ± 1.50^{a}
	Rikenellaceae	0.79 ± 0.25 ^{c}	1.06 ± 0.27 ^{b}	1.36 ± 0.26 ^{a}	1.45 ± 0.29 ^{a}
Genera	*Olsenella*	0.66 ± 0.25 ^{c}	1.25 ± 0.23 ^{a}	1.00 ± 0.28 ^{b}	1.30 ± 0.23 ^{a}
	Dubosiella	1.38 ± 0.46 ^{b}	3.27 ± 2.59 ^{a}	5.03 ± 1.89 ^{a}	4.89 ± 1.09 ^{a}
	Roseburia	0.35 ± 0.03 ^{a}	0.37 ± 0.05 ^{a}	0.14 ± 0.17 ^{b}	0.04 ± 0.11 ^{b}
	Akkermansia	2.29 ± 0.64 ^{a}	2.10 ± 0.65 ^{ab}	1.63 ± 0.34 ^{b}	0.95 ± 0.28 ^{c}
	Oscillospiraceae_unclassified	1.10 ± 0.54 ^{b}	0.77 ± 0.32 ^{b}	0.78 ± 0.29 ^{b}	1.76 ± 0.51 ^{a}
	Alistipes	0.79 ± 0.25 ^{c}	0.90 ± 0.20 ^{b}	1.07 ± 0.15 ^{ab}	1.18 ± 0.13 ^{a}

7. CONCLUSÃO GERAL

Neste estudo foi demonstrado como a proteína digerida da semente de chia pode afetar a inflamação, estresse oxidativo, acúmulo de gordura hepática, microbiota intestinal e morfologia hepática e intestinal de camundongos alimentados com dieta padrão ou dieta rica em gordura saturada.

O consumo de 400mg/kg de proteína digerida de chia (DTP) associado a dieta hiperlipídica apresentou efeitos no fígado, visto que reduziu o acúmulo de gordura hepática, com consequente redução do indice hepatossomatico, reduzindo a inflamação, por meio da menor expressão de NFκB e IL-1β. Além disso, a proteína digerida de chia reduziu a peroxidação lipídica hepática, reduzindo MDA, com consequente redução da atividade da enzima catalase e redução da capacidade antioxidante total.

Além disso, a DTP associada a dieta hiperlipidica foi capaz de aumentar as espessuras das camadas musculares do cólon e a expressão das proteínas das *tight-junctions*, assim como reduzir a dominância bacteriana pelo índice de Simpson. Ademais, os melhores efeitos foram observados quando a DTP foi associada a dieta padrão, com aumento da espessura de criptas e camada muscular circular, aumento de claudina, ocludina e ZO-1 e aumento da abundância de *Alistipes, Olsenella* e *Rikinellaceae*, considerados benéficos.

Assim, concluímos que a proteína digerida da semente de chia é fonte de peptídeos bioativos eficazes na redução da inflamação e acúmulo de gordura hepática. No entanto, os resultados relacionados a microbiota intestinal necessitam serem mais investigados, com variação na dose de DTP e o tempo de intervenção.

8. CONSIDERAÇÕES FINAIS

Nós sugerimos que a proteína digerida da chia seja utilizada em estudos clínicos como medida coadjuvante para o tratamento da doença hepática gordura não alcoolica, permitindo avaliar se os benefícios encontrados em modelo animal serão refletidos em humanos. Além disso, para o presente estudo da microbiota intestinal, sugerimos a realização de análises de predição funcional, do perfil de ácidos graxos de cadeia curta, marcadores inflamatórios e análises de correlação, para melhor compreensão dos mecanismos pelos quais a proteína de chia atua na saúde intestinal. Ademais, a realização de estudos que avaliem a microbiota por meio de sequenciamento *shotgun* devem ser realizados, para uma compreensão mais aprofundada a nível de espécie das alterações causadas pelos peptídeos presentes na proteína digerida da semente de chia. Sendo assim, esse estudo serve de base cientifica para futuras

aplicações da proteína digerida da semente de chia, seja para o desenvolvimento de nutracêuticos, seja para incentivar o aumento do consumo da chia pela população em geral.

ANEXOS

Anexo I: Aprovação CEUA

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº01/2019, intitulado "**Efeito protetor da proteína total digerida de semente de chia (*Salvia hispanica L.*) na adipogênese, inflamação e estresse oxidativo em camundongos alimentados com dieta hiperlipídica**", coordenado pela professora Hércia Stampini Duarte Martino do Departamento de Nutrição e Saúde, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTI, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTI, portanto sendo aprovado por esta Comissão em 21/02/2019, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 01/2019, named **"Protein effect of whole digested protein from chia seed (Salvia hispanica L.) on adipogenesis, inflammation and oxidative stress in mice fed a hyperlipid diet"**, is in agreement with the a actual Brazilian legislation (Lei Nº 11.794, 2008), Normative Resolutions edited by CONCEA/MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific Purposes and Teaching) and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI therefore being approved by the Committee on February 21, 2019 valid for 12 months.

Prof.ª Átima Clemente Alves Zuanon
Presidente
Comissão de Ética no Uso de Animais – CEUA/UFV