VIOLETA NUNES DE MORAIS

FARINHA E ÓLEO DE CHIA (*Salvia hispanica* L.) MODULAM A SAÚDE INTESTINAL EM RATOS COM ALTERAÇÕES METABÓLICAS

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: Hércia Stampini Duarte Martino

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BIOGRAFIA

VIOLETA NUNES DE MORAIS nasceu na cidade de Vitória, Espírito Santo, em 26 de julho de 1997, filha de Eunice Marini Nunes e Paulo Edison de Morais. Em maio de 2015, ingressou no Curso de Nutrição da Universidade Federal do Espírito Santo do *campus* de Alegre/ES, graduando-se Nutricionista em agosto de 2019. Recebeu título de destaque acadêmico por seu alto desempenho durante o curso de nutrição.

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Apresentou trabalhos advindos do Trabalho de Conclusão de Curso (TCC), de forma oral, e em banner, no IV Congresso Nacional de Alimentos e Nutrição, em maio de 2019, Ouro Preto/MG.

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Apresentou um trabalho sobre o óleo de chia no formato de banner no 2º Congresso Internacional de Compostos Bioativos, em outubro de 2022, Campinas/SP.

Concluiu o curso de inglês ofertado pelo Centro de línguas da Universidade Federal de Espírito Santo, em dezembro de 2022.

RESUMO

MORAIS, Violeta Nunes de, M.Sc., Universidade Federal de Viçosa, março de 2023. **Farinha e óleo de chia (*Salvia hispanica* L.) modulam a saúde intestinal em ratos com alterações metabólicas.** Orientadora: Hércia Stampini Duarte Martino. Coorientadoras: Bárbara Pereira da Silva, Mariana Grancieri e Izabela Maria Montezano de Carvalho.

O consumo elevado de dietas ricas em gordura saturada, frutose e pobres em nutrientes, desencadeia alterações metabólicas e disbiose intestinal na população. A farinha e o óleo de chia (*Salvia hispanica.* L.) são fontes ácido graxo ômega 3 (ω-3), fenólicos, peptídeos bioativos, e vitaminas. Ademais a farinha de chia possui elevado teor de fibra alimentar. Estas frações da chia promovem ação antioxidante, anti-inflamatória e anti-glicêmica, podendo apresentar potencial em promover a saúde intestinal. Estudos que abordam essa temática são incipientes. Diante disso, objetiva-se analisar o efeito da farinha e do óleo de chia nos marcadores de saúde intestinal em ratos *Wistar* submetidos à alteração metabólica. Realizou-se o experimento com 40 ratos *Wistar* machos adultos. Na primeira etapa (8 semanas) os animais receberam dieta padrão (AIN-93M) (n=10) e dieta com alto teor de gordura e frutose (HFHF) (n=30). Na segunda etapa (10 semanas) os animais foram alocados em quatro grupos e receberam dieta HFHF (n=10), dieta HFHF + farinha de chia (CF) (n=10), e dieta HFHF + óleo de chia (CO) (n=10), e dieta AIN-93M (n=10). Realizou-se a eutanásia por pulsão cardíaca ao final das 18 semanas. Coletou-se o colón descendente, o conteúdo cecal e os tecidos adiposos. Analisou-se variáveis de saúde intestinal, sendo estas, ácidos graxos de cadeia curta (SCFAs), imunoglobulina A (IgA), pH do conteúdo cecal, histomorfometria, permeabilidade intestinal e o sequenciamento 16SrRNA da microbioma intestinal. Utilizou-se os softwares *GraphPad Prism*, *Statistical Analysis of Metagenomic Profiles* (STAMP) e *Statistical Package for Social Science* (SPSS) para rodar a estatística, $p < 0,05$. Os testes utilizados foram *Simple Analysis of Variance* (ANOVA) com *post-hoc Newman-Keuls* ou *Duncan* e o teste *Kruskal-Wallis* com *post-hoc* Dunn ou correção de Bonferroni. Os grupos CF e CO aumentaram a ingestão de ω-3, os níveis de IgA, o número de células caliciformes, a espessura e profundidade das criptas, a camada muscular longitudinal e o peso do ceco, além de reduzir o pH do conteúdo do ceco, o peso corporal e o índice de massa corporal (BMI). Somente o CO aumentou a camada muscular circular e reduziu a adiposidade. Os tipos de SCFAs, a abundância relativa de bactérias, a beta e alfa diversidade, o LEfSe e o KEGG foram distintos entre estes grupos. CF apresentou dissimilaridade microbiana, tendo o maior índice de Shannon e menor índice de Simpson enquanto o índice de Chao (riqueza microbiana) foi similar entre os grupos; aumentou a

abundância de *Monoglobus sp., Lachnospiraceae sp.* e *Prevotellaceae sp.,* e a concentração de ácido acético e butírico; na *Linear discriminant analysis Effect Size* (LEfSe) teve a maior predominância de gêneros em relação aos grupos controles; na análise predição funcional da *Kyoto Encyclopedia of Genes and Genomes* (KEGG) teve significância em dezesseis vias metabólicas identificadas. O CO aumentou a abundância de Firmicutes, *Lactobacillus sp., Faecalibacterium sp.* e *Erysipelatoclostridium sp.* e o teor de ácido propiônico, apresentando ausência de significância nas análises do LEfSe, KEGG e de alfa e beta diversidade. Conclui-se que a farinha e o óleo de chia melhoram a saúde intestinal, atuando de maneira distinta na microbiota intestinal, mas de forma positiva, sendo eficazes na modulação da microbiota intestinal, morfologia e funcionalidade intestinal, em um modelo de alteração metabólica induzido pela dieta HFHF.

Palavras-chave: Microbiota. Ácidos graxos de cadeia curta. Histomorfometria intestinal. IgA. PH cecal. Microbioma intestinal.

ABSTRACT

MORAIS, Violeta Nunes de, M.Sc., Universidade Federal de Viçosa, March 2023. **Chia flour and oil (*Salvia hispanica* L.) modulate intestinal health in rats with metabolic disorders**. Adviser: Hércia Stampini Duarte Martino. Co-advisers: Bárbara Pereira da Silva, Mariana Grancieri and Izabela Maria Montezano de Carvalho.

The high consumption of diets rich in saturated fat, fructose and poor in nutrients triggers metabolic alterations and intestinal dysbiosis in the population. Chia flour and oil (*Salvia hispanica*. L.) are sources of omega 3 fatty acid (ω-3), phenolics, bioactive peptides, and vitamins. In addition, chia flour has a high content of dietary fiber. These fractions of chia promote antioxidant, anti-inflammatory and anti-glycemic action, and may have potential to promote intestinal health. Studies that address this theme are incipient. Therefore, the objective is to analyze the effect of chia flour and oil on intestinal health markers in Wistar rats submitted to metabolic alteration. The experiment was carried out with 40 adult male Wistar rats. In the first stage (8 weeks) the animals received a standard diet (AIN-93M) (n=10) and a high-fat and high-fructose diet (HFHF) (n=30). In the second stage (10 weeks) the animals were divided into four groups and received a HFHF diet (n=10), a HFHF diet + chia flour (CF) (n=10), and a HFHF diet + chia oil (CO) (n=10), and AIN-93M diet (n=10). Euthanasia was performed by cardiac impulse at the end of 18 weeks. Descending colon, cecal contents and adipose tissues were collected. Intestinal health variables were analyzed, namely, short-chain fatty acids (SCFAs), immunoglobulin A (IgA), cecal content pH, histomorphometry, intestinal permeability and 16SrRNA sequencing of the intestinal microbiome. GraphPad Prism, Statistical Analysis of Metagenomic Profiles (STAMP) and Statistical Package for Social Science (SPSS) software were used to run the statistics, $p < 0.05$. The tests used were Simple Analysis of Variance (ANOVA) with post-hoc Newman-Keuls or Duncan and the Kruskal-Wallis test with post-hoc Dunn or Bonferroni correction. The CF and CO groups increased ω-3 intake, IgA levels, the number of goblet cells, crypt thickness and depth, longitudinal muscle layer and cecum weight, in addition to reducing the pH of the cecum content , body weight and body mass index (BMI). Only CO increased circular muscle layer and reduced adiposity. The types of SCFAs, relative abundance of bacteria, beta and alpha diversity, LEfSe and KEGG were different between these groups. CF showed microbial dissimilarity, having the highest Shannon index and lowest Simpson index while the Chao index (microbial richness) was similar between groups; increased the abundance of *Monoglobus sp*., *Lachnospiraceae sp*. and *Prevotellaceae sp*., and the concentration of acetic and butyric acid; in the Linear Effect Size

Discriminant Analysis (LEfSe) it had the highest predominance of genders in relation to the control groups; in the functional prediction analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) it had significance in sixteen identified metabolic pathways. CO increased the abundance of Firmicutes, *Lactobacillus sp.*, *Faecalibacterium sp.* and *Erysipelatoclostridium sp*. and the propionic acid content, showing no significance in the LEfSe, KEGG and alpha and beta diversity analyses. It is concluded that chia flour and oil improve intestinal health, acting differently on the intestinal microbiota, but in a positive way, being effective in modulating the intestinal microbiota, morphology and intestinal functionality, in a model of metabolic alteration induced by HFHF diet.

Keywords: Microbiota. Short chain fatty acids. Intestinal histomorphometry. IgA. Cecal pH. Gut microbiome.

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LISTA DE ABREVIATURAS E SIGLAS

Adipor2: do inglês: *Adiponectin receptor protein 2*

SCFAs: Ácidos Graxos de Cadeia Curta; do inglês: *Short Chain Fatty Acids*

AIN-93M: dieta padrão para murino; do inglês: *murino standard diet*

Akt: do inglês: *Protein Kinase B*

ANOVA: do inglês: *Simple Analysis of Variance*

AMPK: do inglês: *AMP-Activated Protein Kinase*

CF: farinha de chia; do inglês: *chia fluor*

CO: óleo de chia; do inglês: *chia oil*

Cpt1a: do inglês: *Carnitine Palmitoyltransferase 1ª*

FDR: do inglês: *False Discovery Rate*

GK: do inglês: *Glucokinase*

GLP-1: do inglês: *Glucagon-Like Peptide-1*

GLUT4: do inglês: *Glucose transporter type 4*

GPCRs: do inglês: *G-Protein-Coupled Receptors*

H_2SO_4: do inglês: *Sulfuric Acid*

ANOVA: do inglês: *Simple Analysis of Variance*

HFHF + CF: do inglês: *high-fat high-fructose plus chia flour*

HFHF + CO: do inglês: *high-fat high-fructose plus chia oil*

HFHF: dieta rica em gordura e frutose; do inglês: *high-fat high-fructose*

HPLC: do inglês: *High Performance Liquid Chromatography*

IgA: Imunoglobulina A; do inglês: *Immunoglobulin A*

BMI: Índice de Massa Corporal; do inglês: *Body Mass Index*

INSR: Insulin Receptor

KEGG: do inglês: *Kyoto Encyclopedia of Genes and Genomes*

LEfSe*:* do inglês: *Linear discriminant analysis Effect Size.*

MAPK: do inglês: *Mitogen-activated protein kinase*

miRNA: do inglês: *Micro-Ribonucleic Acid*

MLCK: do inglês: *Myosin Light Chain Kinase*

NCBI: do inglês: *National Center for Biotechnology Information*

NF-Kb: do inglês: *Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells*

OTUs: do inglês: *Operational Taxonomy Units*

PCoA: do inglês: *Principal Coordinate Analysis*

PERMANOVA*:* do inglês*: Permutational Multivariate Analysis of Variance*

PFK: do inglês: *Phosphofructokinase*

PGC 1-α: do inglês: *Peroxisome Proliferator-Activated Receptor*

PK: do inglês: *Phosphokinase*

PPAR-ɤ: do inglês: *Peroxisome Proliferator-Activated Receptor 1-* ɤ

PYY: do inglês: *Peptide* YY

STAMP: do inglês: *Statistical Analysis of Metagenomic Profiles*

SPSS: do inglês: *Statistical Package for Social Science*

SRA: do inglês: *Sequence Read Archive*

Srebf[1:] do inglês: *Sterol regulatory element-binding transcription factor 1*

TLR: do inglês: *Receptor Toll-like*

TLR4: do inglês: *Toll-like receptor-4*

TNF: do inglês: *Tumor Necrosis Fator*

TNF-α: do inglês: *Tumor Necrosis Factor-Alpha*

UCP-1: do inglês: *Uncoupling Protein 1*

ω-3: Ômega 3; do inglês: *Alpha-linolenic acid*

ω-6: Ômega 6; do inglês: *linoleic acid*

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1 INTRODUÇÃO GERAL

A população mundial encontra-se cada vez mais sedentária e consumindo dietas ricas em gordura, sal e açúcar, denominada dieta ocidental (Maurya *et al.*, 2023). Esses fatores conduzem à maior incidência de alterações metabólicas, marcadas pela inflamação crônica (Sabir *et al.*, 2022; Siqueira *et al.*, 2022), como a disbiose, marcada pelo desequilíbrio da comunidade bacteriana intestinal, elevada permeabilidade, degradação dos enterócitos, redução da síntese de imunoglobulina A (IgA), ácidos graxos de cadeia curta (SCFAs) e muco (Arima *et al.*, 2022; Liang *et al.*, 2021; Ou *et al.*, 2022).

Os compostos bioativos vêm sendo estudados como fontes para prevenção e tratamento da disbiose. As frações da chia se destacam na sua composição nutricional, sendo a farinha de chia (CF) fonte de fibras alimentares e Ômega 3 (ω-3), enquanto o óleo de chia (CO) é fonte de ω-3 (Moreira *et al.*, 2022). Além de ambos apresentarem peptídeos bioativos e fenólicos em sua matriz (Enes *et al*., 2020; Grancieri *et al*., 2022).

Evidências cientificas apontam o potencial modulador da chia atrelada a dieta ocidental em modelo animal, como, o aumento da superfície, largura e comprimento do vilo, e diâmetro e número de células caliciformes (Silva, da *et al.*, 2019a), melhora da síntese de ácidos graxos acético e butírico, reduz o pH cecal, e aumenta a proliferação de cepas bacterianas do gênero *Bacteroides sp.* e das famílias Muribaculaceae e Lachnospiraceae (Mishima *et al*., 2022a). O diferencial do presente estudo é o tipo de dieta proposto, dieta rica em gordura e frutose (HFHF) e HFHF com CF ou CO e, o modelo animal, ratos *Wistar* adultos machos com disfunções metabólicas).

Diante disto, as frações da chia podem atuar de forma distintas em um mesmo desfecho, visto que o ambas apresentam composições diferentes. Entretanto o ácido alfa linolênico, as fibras alimentares, os peptídeos bioativos e fenólicos presentes nessas matrizes alimentares podem modular de forma positiva a microbiota intestinal, e promover a saúde intestinal.

A hipótese do presente estudo é que CF e CO atrelado a dieta HFHF podem atuar como alimentos funcionais auxiliando na prevenção e tratamento da disbiose, aumentando a síntese de SCFAs e de bactérias probióticas, reduzindo as bactérias patogênicas e o pH cecal, melhorando a morfologia intestinal, permeabilidade e o teor de IgA em ratos *Wistar* machos adultos com disfunções metabólicas.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar o efeito da farinha e do óleo de chia (*Salvia hispanica* L.) na saúde intestinal de ratos *Wistar* com alterações metabólicas promovida pela ingestão de dieta rica em gordura e frutose (HFHF).

2.2 Objetivos específicos

- Quantificar o consumo alimentar e as variáveis murinométricas (Índice de massa corporal (BMI), peso, porcentagem total de tecido adiposo) em ratos *Wistar* alimentados com dieta rica em gordura e frutose adicionada de farinha ou óleo de chia;
- Avaliar as variáveis de saúde intestinal (IgA, SCFAs, peso do ceco, pH das fezes do ceco, permeabilidade, histomorfometria intestinal) dos ratos *Wistar* alimentados com dieta rica em gordura e frutose adicionada de farinha ou óleo de chia;
- Analisar alfa e beta diversidade da comunidade bacteriana de ratos *Wistar* alimentados com dieta rica em gordura e frutose adicionada de farinha ou óleo de chia.

3 REFERENCIAL TEÓRICO

3.1 Alterações metabólicas causadas frente ao consumo de dieta rica em gordura e frutose

Atualmente, o hábito alimentar ocidental tende ao consumo de dietas com elevado teor de gordura, frutose, sal e açúcar, que acarreta o ganho de peso, hiperfagia e elevado risco de desenvolvimento de obesidade e outras doenças crônicas não transmissíveis, incluindo câncer, diabetes e doenças cardiovasculares (Attal *et al*., 2021 Zou *et al*., 2021; Maurya *et al*., 2023).

A dieta rica em gordura é composta, em sua maioria, por gorduras saturadas (origem animal e vegetal) e *trans* (produtos industrializados), que ocasionam estresse oxidativo, desequilíbrio na regulação intestinal, disfunção hepática e neuroinflamação (Maredia, Hamilton e Thanos, 2021; Wang *et al*., 2021; Sabir *et al.,* 2022). Outro tipo de dieta é a rica em frutose, advinda, principalmente, do xarope de milho e produtos alimentícios industrializados. Seu consumo pode acarretar a síndrome metabólica, deposição de gordura intra-hepática, intolerância à glicose e a resistência à insulina (Bunbupha *et al*., 2021; Rønnevik e Gudbrandsen, 2020).

Segundo Hanousková *et al*., (2019), a ingestão de bebidas ricas em frutose (23 g de frutose e 19 g de sacarose/l) ocasionou expressão aberrante de diversos *Micro-Ribonucleic Acid* (miRNAs) que atuam no metabolismo lipídico, hipercolesterolemia hepática, modificação de adipócitos e doença hepática gordurosa não alcoólica em camundongos machos C57BL/6. A administração de dieta HFHF em ratos *Wistar* contendo 4% de óleo de soja, adicionado de 30% de banha de porco e 20% de frutose por 18 semanas, ocasionou deposição de gordura (ganho de peso), níveis elevados de glicose, triglicerídeos, aspartato e alanina aminotransferase, proteínas pró-inflamatórias (*Toll-like receptor-4* (TLR4) e *Tumor Necrosis Fator* (TNF)), óxido nítrico e ácido úrico, além de causar esteatose hepática grau 2 e reduzir proteína quinase ativada por *AMP-Activated Protein Kinas*e (AMPK) e o *Insulin Receptor* (INSR) (Enes *et al.*, 2020; Moreira *et al.*, 2022).

As pesquisas que visam reverter ou melhorar o quadro de disfunções metabólicas, devido à ingestão de elevados teores de gordura e frutose, analisam principalmente a ação de compostos bioativos e produtos funcionais, sendo os modelos animais os mais utilizados (Lee *et al*., 2020; Mishra e Ghosh, 2020; Mishima *et al*., 2022b). O consumo de farinha de sorgo integral aquecida a seco, da farinha de milheto germinada, da CF e CO promoveu a redução da esteatose hepática, da lipogênese e do estresse oxidativo, além de melhorar o metabolismo da glicose e a modulação da adiposidade em ratos que recebiam dieta rica em gordura e frutose (Moreira, 2019; Enes *et al.*, 2020; Medina Martinez *et al*., 2021; Theodoro *et al*., 2021).

3.2 Chia: Farinha e Óleo

A chia (*Salvia hispanica* L.) é uma planta herbácea da família Lamiaceae, originária do norte da Guatemala e do sul do México, também cultivada na Argentina, Peru, Paraguai, Equador, Nicarágua, Bolívia, Brasil e Austrália, locais com clima tropical e subtropical, sendo utilizada como alimento há mais de 5.000 anos (Bermejo, Hoummadi e Munné-Bosch, 2021; Silveira Ramos, da *et al.*, 2021). O mercado da chia vem crescendo na Europa, devido à adoção de padrões alimentares vegetarianos e saudáveis pela população (Ferreira *et al.*, 2023).

A semente é pequena, oval, lisa, coloração branca, cinza, marrom e preta, com cerca de 1,9 a 2,0 mm de comprimento, 1,00 a 1,4 mm de largura e 0,8 a 1,0 mm de espessura, possuindo mucilagem de solubilidade e viscosidade elevada, sendo estável em altas temperaturas até 244°C (Goh *et al*., 2016; Timilsena *et al*., 2016; Ghafoor *et al*., 2022). Os teores dos nutrientes da chia variam de acordo com o clima, altitude, solo, forma de cultivo, radiação do sol, entre outros (Brandán, Izquierdo e Acreche, 2022; Cabrera-Santos *et al*., 2021). A chia é consumida *in natura*, hidratada em líquido (água, bebida vegetal, leite e iogurte), em forma de farinha ou óleo (Copado *et al.*, 2021; Ferraro *et al.*, 2022; Silva *et al.*, 2022).

A farinha de chia é constituída por 3,2% carboidratos, 20,7% proteínas, 27,2% lipídios, sendo 10,3% gorduras saturadas, 7,5% monoinsaturadas e 82,2% de poliinsaturadas, e 37,9%, fibra alimentar total com 34,48% de fibra alimentar insolúvel e 3,43% de fibra alimentar solúvel, e fenólicos, principalmente ácido rosmarínico, ácido ferúlico e ácido cafeico (Enes *et al.*, 2020; Moreira *et al.*, 2022) Enquanto o óleo de chia é composto por 7,6% de ácido palmítico, 2,7% de ácido esteárico, 7,5% de ácido oleico, 19,9% de ácido linoleico, 62,3% de ácido alfa linolênico, e fenólicos (Moreira et al., 2022; Oliveira-Alves *et al*., 2017).

Por ser rica em óleo e fibra alimentar, a chia apresenta elevada viscosidade, sendo utilizada para substituir a farinha branca, a gordura saturada e os ovos em receitas de sorvetes, panquecas, batidas, bolos, pães, mingau e biscoitos. Viabilizando a criação de preparações sem glúten, veganas e com maior perfil de lipídio poliinsaturado (Falco, de, Amato e Lanzotti, 2017; Hrnčič *et al*., 2020; Velotto *et al*., 2021; Hsieh, Lin e Kuo, 2022; Katunzi-Kilewela *et al*., 2022).

As frações da chia vem sendo correlacionadas com a promoção da saúde (Enes *et al*., 2020; Moreira *et al*., 2022; Mishima, *et al*., 2022a; Mishima, *et al*., 2022b). A CF auxilia na regulação dos níveis de mRNA de AMPK e *Protein Kinase B* (AKT) (Enes *et al*., 2020). Além de reduzir a deposição de gordura corporal, principalmente no fígado, por inativação da expressão de *Sterol regulatory element-binding transcription fator-1* (Srebf^{1}) e ativação da via de Carnitine Palmitoyltransferase 1^{a}(Cpt1a) (Moreira *et al.*, 2022). O CO promove o escurecimento do tecido adiposo subcutâneo por elevação do *Uncoupling Protein- 1* (UCP-1),

do *Peroxisome Proliferator-Activated Receptor-* ɤ (PPAR-ɤ), coativador gama 1-alfa do receptor ativado por *Peroxisome Proliferator-Activated Receptor* (PGC 1-α), que induz à biogênese mitocondrial (Fonte-Faria *et al.*, 2019; Moreira *et al.*, 2022; Souza, de *et al.*, 2020). Regula o gene *Adiponectin receptor protein 2* (Adipor2), responsável por atenuar a síndrome metabólica e o teor de leptina (Moreira *et al.*, 2022). Eleva os teores de Akt, *Glucose transporter type 4* (GLUT4), AMPK, INSR, *Glucokinase* (GK), *Phosphokinase* (PK) e Phosphofructokinase (PFK), modulando o metabolismo da glicose em vias dependentes e independentes da insulina (Enes *et al.*, 2020; Moreira *et al.*, 2022). Estudos sobre a ação da farinha e óleo de chia na saúde intestinal são incipientes, no entanto evidenciaram que a farinha de chia melhora a morfologia intestinal, comunidade microbiana, o pH do conteúdo cecal e a síntese de SCFAs (Da Silva, *et al*., 2019; Mishima *et al*., 2022a; Mishima *et al*., 2022b).

O potencial da farinha e óleo de chia na restauração da homeostase intestinal pode ter *link* direto com as modificações benéficas nos marcadores hepáticos, de glicose e de lipídio, observadas após a ingestão dessas frações de chia (Enes *et al.*, 2020; Moreira *et al.*, 2022). Visto que a microbiota e o fígado regulam um ao outro, via veia porta, no ciclo da homeostase da glicose (os ácidos bilhares conjugado e desconjugado eleva a liberação de *Glucagon-Like Peptide-1* (GLP-1) e na metabolização de lipídio (os enterócitos intestinais mediam a absorção pós-prandial de nutrientes, regulando o armazenamento de triglicerídeos e a síntese de quilomícrons) (Nawrot *et al.*, 2021).

3.3 Saúde Intestinal

O intestino é dividido em delgado e grosso, sendo um órgão promotor do bem-estar mental e metabólico, visto que apresenta eixos de ligação com diversos órgãos, destacando-se o eixo intestino-cérebro, responsável pela regulação da motilidade gastrointestinal (Ni *et al*., 2021; Petrella *et al*., 2021). Um intestino humano saudável apresenta mais de 100 trilhões de microrganismos com elevada diversidade de genes, funções metabólicas eficientes e estruturas histológicas íntegras (Chen *et al.*, 2020; Yang, G. *et al.*, 2021).

O intestino delgado inicia no piloro e termina na válvula ileocecal, sendo constituído pelo duodeno, jejuno e íleo, que apresentam lúmen espesso revestido com três camadas: epitélio, lâmina própria e muscular da mucosa, além de pregas proeminentes e predominância de enterócitos (Qi *et al.*, 2020). No entanto, há outras células, como células enteroendócrinas - detectam nutrientes e metabolitos microbianos por diferentes *G protein-coupled receptors* (GPCRs), secretando hormônios peptídicos (com função endócrina pancreática, na motilidade gastrointestinal e no apetite). A proliferação e a diferenciação das células epiteliais ocorrem

constantemente por meio das células-tronco intestinais, que se situam na base da cripta e, após, a diferenciação se desloca para a região apical da vilosidade (Baulies *et al.*, 2020).

O intestino grosso inicia no íleo distal e finaliza no ânus, sendo dividido em apêndice, ceco, cólon, reto e canal anal. O cólon é subdividido em segmentos ascendente, transversal, descendente e sigmoide (Berthold; Jones; Udalova, 2021). A região colorretal é constituída de epitélio colunar simples com borda em escova fina, elevado teor de células caliciformes, ausência de vilosidades e renovação epitelial intensa (Sokolis e Sassani, 2013). O epitélio é constituído das camadas: serosa, muscular longitudinal, intermuscular, muscular circular, submucosa e mucosa (Alazzouni *et al.*, 2021; Jee *et al.*, 2021). O intestino grosso é responsável pela absorção de água, eletrólitos e vitaminas, além de ser um canal estrutural que viabiliza o transporte de compostos residuais para fora do corpo (Siri *et al.*, 2019). A população microbiana, a espessura do muco, a produção de SCFAs e o pH se elevam ao sair do intestino delgado para o cólon.

A microbiota intestinal é uma estrutura funcional e organizada, constituída de células epiteliais intestinais, incluindo enterócitos, células caliciformes, células de Paneth e células enteroendócrinas, apresentando complexos juncionais: junções apertadas, junções de aderência e desmossomos, sendo revestidas com muco sintetizado pelas células caliciformes, células secretoras de IgA (auxilia na síntese de linfócitos T reguladores) e moléculas de defesa, como os peptídeos antimicrobianos, além de ter células intraepiteliais, linfócitos e células dendríticas (Caruso, Lo, Núñez, 2020; Popkes, Valenzano, 2020; Park *et al*., 2021). A mediação da membrana funcional é feita pelo sistema imune, população microbiana, mecanismo de permeabilidade celular e mecanismo de motilidade (Zhang *et al.*, 2021). Diversos fatores externos e internos, como, idade, genética, uso de medicamentos, fatores ambientais, aleitamento materno, presença de patologias, e fatores psicológicos, como o stress e a ansiedade mediam a conformação da microbiota intestinal (Liu, Cai e Qin, 2022; Yang, J. *et al.*, 2021).

Na junção da membrana apical-lateral se encontram os complexos de junção apical, que atuam na união intensa entre as células, que limita a difusão livre de íons e pequenas moléculas, sendo a primeira linha de defesa contra invasão de bactérias patogênicas, toxinas e antígenos (Cremonini *et al*., 2018; Xing et al., 2020). É composto por junções apertadas (do inglês: *Tight Junction*): proteínas transmembrana (ocludina, tricelulina e claudinas) e proteínas adaptadoras: zonulina (zonulina tipo 1, 2 e 3), caderinas (proteínas-transmembrana: E-caderina e nectina), proteínas-esqueleto (α-catenina, β-catenina, γ-catenina e afadina) e os desmossomos (Alvarez *et al.*, 2019; Cray *et al.*, 2020; Odenwald e Turner, 2016; Shukla *et al.*, 2018). As junções apertadas regulam a permeabilidade por regulação do *Nuclear Factor Kappa-Light-Chain-*

Enhancer of Activated B Cells (NF-κB), via *Myosin Light Chain Kinase* (MLCK) e *Mitogen-Activated Protein Kinase* (MAPK) (Che et al., 2020). Sua interrupção está associada à com o intestino gotejante (do inglês, *Leaky Gut*), inflamação e disbiose (Gao *et al*., 2022; Snelson *et al*., 2021).

Quando a população de bactérias patogênicas é mais elevada do que as bactérias probióticas, ocorre a disbiose intestinal. Essa modificação leva à ativação da via de sinalização do *Receptor Toll-like* (TLR), eleva a concentração de lipopolissacarídeos e ácidos graxos livres (regulam a expressão de TLR), secretando citocinas pró-inflamatórias, como, interleucina 1β, interleucina 6 e *Tumor Necrosis Factor*- α (TNF-α) (Duan *et al.*, 2018). Esse quadro leva à degradação das células epiteliais intestinais, elevação da permeabilidade, inativação de vias que promovem a restauração da saúde intestinal, elevando o risco de desenvolvimento de câncer (principalmente de cólon), insuficiência hepática aguda, doenças cardiovasculares, doenças inflamatórias do intestino, obesidade e neuroinflamação (Dey *et al.*, 2019; Zhang *et al.*, 2021). A disbiose se desenvolve devido a hábitos alimentares não saudáveis, contato com poluentes ambientais, procedimentos cirúrgicos, tratamentos medicamentosos, presença de patologias e alergias (Pan *et al.*, 2021; Schneider *et al.*, 2021).

Os compostos bioativos, como fibras alimentares, ω-3, fenólicos e peptídeos bioativos presentes na farinha e óleo de chia, auxiliam na prevenção e tratamento da disbiose (Ghafoor *et al*., 2022; Mishima *et al*., 2022a; Mishima *et al*., 2022; Rocchetti *et al*., 2022). Visto que a síntese de SCFAs é ocasionada pelo consumo de prebióticos que fermentados no intestino grosso por bactérias anaeróbicas do cólon, resultando em ácido lático, dióxido de carbono, hidrogênio e SCFAs, como, ácido acético, propiônico e butírico (Grancieri *et al*., 2017; Ma *et al*., 2021; Scortichini *et al*., 2020). A absorção de SCFAs leva à redução do pH do lúmen, elevando proliferação de bactérias benéficas e fornece energia para os colonócitos, regulando as células Langerhans, que secretam GLP-1 e *peptide* YY (PYY), auxiliando no gasto de energia, na redução da ingestão alimentar e melhora do metabolismo de glicose e insulina (Cao *et al*., 2022). Além de aumentar a síntese de IgA, que atua na proteção da microbiota contra antígenos (Markowiak-Kopeć e Śliżewska, 2020; Ikeda *et al*., 2022; Wen *et al*., 2022).

Variáveis de saúde intestinal são: Os SCFAs que são quantificados por meio das amostras fecais, por análise de cromatografia gasosa, *High Performance Liquid Chromatography* (HPLC) ou combinado a espectrometria de massa (triplo, quadrupolo ou quadrupolo de armadilha linear), os principais ácidos analisados são o propiônico, butírico e acético (Chen *et al.*, 2021; Fang *et al.*, 2022). A permeabilidade intestinal (*in vitro e in vivo*) que é feita por coleta de urina (administração da lactulose-manitol) ou soro com marcadores

radiomarcados administrados entericamente, quantificados por HPLC ou espectrofotômetro de fluorescência (Stewart, Pratt-Phillips e Gonzalez, 2017; Herrera-Cazares *et al*., 2021; Teskey *et al*., 2021). O pH do lúmen intestinal é quantificado pelas amostras fecais por utilização de pHmetro (Martinez *et al.*, 2023; Mishima, Silva, da, *et al.*, 2022). A morfologia intestinal que é determinada pela análise histológica, onde placas com tecidos orgânicos pigmentados são observadas no microscópio de luz para obtenção de imagens histológicas (Soares *et al.*, 2021). Por meio das imagens e dos *softwares* quantifica-se a histomorfometria (Martinez *et al.*, 2023; Medina-Reyes *et al.*, 2020). O IgA intestinal é quantificado em amostra fecal ou sérica, por kit de ELISA para IgA em ratos ou humanos(Goguyer-Deschaumes *et al.*, 2022; Yang *et al.*, 2020). A composição do microbioma intestinal obtido a partir do sequenciamento de DNA das amostras fecais, por meio do método de Amplicon RNA ribossômico 16s (mais acessível) ou Sequenciamento Shotgun do genoma inteiro por Metagenômica ou Metatranscriptômica, ambos necessitam que seus sequenciamentos sejam analisados por *software* de bioinformática (Lakshmanan *et al.*, 2021; Liu, Cai e Qin, 2022).

Á comunidade bacteriana intestinal vem sendo avaliada por meio da β-diversidade que corresponde a variabilidade na composição da comunidade bacteriana dos grupos experimentais, avaliada por meio da quantificação da dissimilaridade entre elas, por análise de *Principal Coordinate Analysis* (PCoA) (Walters e Martiny, 2020; Thielemann *et al*., 2022). α-diversidade que é avaliada pelos índices de Chao, Shannon, e Simpson, quantifica a riqueza, diversidade e predominância de espécies (Han *et al.*, 2023; Korczak *et al.*, 2023). Da predição funcional por meio do *Kyoto Encyclopedia of Genes and Genomes* (KEGG), correlaciona os dados advindos de dezesseis bancos genômicos aos funcionais, definindo as vias metabólicas que os microrganismos participam (Kanehisa *et al.*, 2023; Kanehisa, Sato e Kawashima, 2022; Zhao *et al.*, 2023). E por meio da análise *Linear discriminant analysis Effect Size* (LEfSe) que é um algoritmo que quantifica a estatística, modelagem linear e visualização das *Operational Taxonomy Units* (OTUs) de acordo com duas ou mais classes (Segata *et al.*, 2011; Thielemann *et al.*, 2022).

O presente estudo utilizou o mesmo desenho experimental dos estudos de Enes *et al*. (2020) e Moreira *et al*. (2022). Ambos os estudos evidenciam efeitos benéficos da farinha e óleo de chia nas vias glicolíticas, lipídicas e hepática. Sendo vias que atuam na regulação da funcionalidade intestinal. Portanto, a presente proposta é a continuidade destes estudos, investigando a ação da farinha e do óleo de chia nas variáveis de saúde intestinal.

4 METODOLOGIA GERAL

Este estudo experimental faz parte de um projeto guarda-chuva que originou dois trabalhos originais, sendo o primeiro de Enes *et al*., (2020) que avaliaram os efeitos da farinha e do óleo de chia no metabolismo hepático da glicose em modelo *in vivo* e *in vitro.* E o segundo de Moreira *et al*., (2022) que analisaram a ação da farinha e óleo de chia na atividade antioxidante hepática e em marcadores inflamatórios de ratos *Wistar* com disfunções metabólicas. Neste trabalho, que é o terceiro derivado do experimento animal, será realizado uma investigação dos efeitos da farinha e do óleo de chia na modulação e saúde intestinal.

O estudo foi submetido à Comissão de Ética no Uso de Animais da Universidade Federal de Viçosa (CEUA / UFV). Para o 1° artigo original utilizou-se o protocolo n °. 31/2018, com data da aprovação em 26 de abril de 2018 (ANEXO A) (Enes, 2020), e para o 2° artigo original utilizou-se o protocolo n°. 89/2018, data da aprovação: 21 de fevereiro, 2019 (ANEXO B) (Moreira, 2019). Os procedimentos experimentais com os animais foram realizados em consonância com os princípios éticos na experimentação animal.

Realizou-se o cálculo do número amostral para determinar a quantidade ideal de animais em cada grupo experimental. Seguiu-se a equação proposta por Fontelles *et al*., (2010):

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

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.

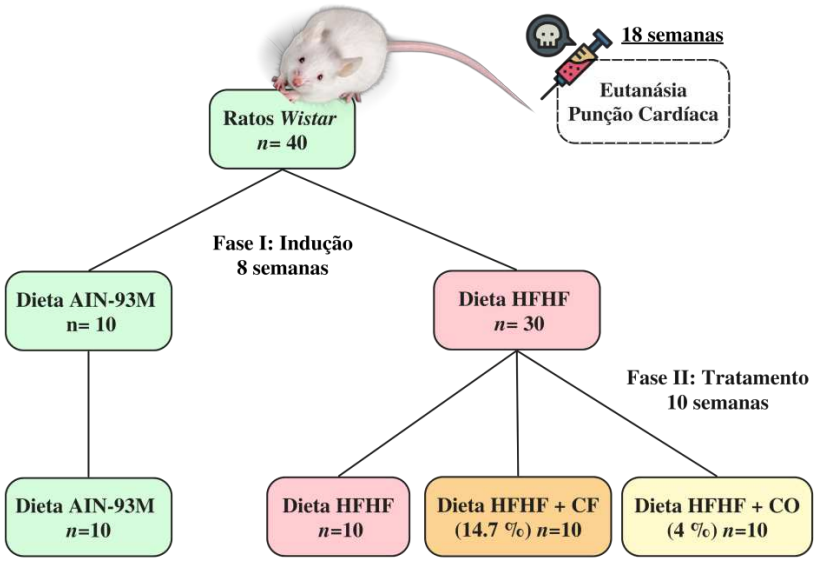
$z\frac{a}{2}$: Erro alfa do tipo I: determinado por meio da tabela de valores críticos da distribuição normal gaussiana.

Nenhum experimento prévio foi realizado para o cálculo do número de repetições ideal, visto que seria necessária a utilização de animais. Portanto, utilizou os dados de estudo prévio de Marineli *et al*., (2015), que induziu alterações metabólicas nos animais por meio da dieta HFHF, e o cálculo foi realizado a fim de confirmar o número de animais. Considerou-se α=5%, e, portanto, um $z\frac{a}{2}$=1.96, conforme utilizado nos estudos de saúde (Fontelles *et al.*, 2010). O

número de repetições calculado foi 9,42; portanto, considerando possíveis perdas, solicitamos 10 animais por grupo. Fizeram-se necessários 40 animais, uma vez que o estudo contou com 4 grupos experimentais. Os animais foram randomizados através do peso corporal.

Inicialmente, 40 ratos *Wistar* foram divididos em quatro grupos experimentais por randomização de acordo com o peso corporal (**Figura 1**). Na 1° fase, durante 8 semanas, foram induzidas as mudanças metabólicas por meio da ingestão de dieta HFHF e na 2° fase foi oferecidos aos animais óleo ou farinha de chia, durante 10 semanas, dependendo de qual grupo eles estivessem alocados. O grupo controle recebeu dieta padrão AIN-93M em ambas as fases. As dietas experimentais foram balanceadas, o teor de fibra presente na farinha de chia determinou a quantidade de celulose que seria utilizada nas demais dietas e a quantidade de farinha e óleo de chia se baseou na quantidade total de óleo de soja, no intuito de substituir 100% este ingrediente na dieta (**Tabela 1**). Determinou-se a concentração de ω-3 e ω-6 nas dietas por meio da soma destes compostos presentes na banha de porco, no óleo de soja, e no óleo advindo da farinha de chia (Moreira, 2019; Enes, 2020).

Figura 1- Desenho experimental do estudo.



O experimento foi dividido em duas fases. Fase I: animais separados em 2 grupos: controle recebeu dieta padrão (AIN-93M, n=10) e o grupo HFHF (n=30) que recebeu AIN-93M com alto teor de gordura (34%) e frutose (20%), por 8 semanas. Fase II: o grupo HFHF foi dividido em HFHF (controle positivo, n=10); HFHF+CF (HFHF com 14,7% de farinha de chia, n=10) e HFHF+CO (HFHF com 4% de óleo de chia, n=10). O grupo controle continuou com dieta AIN-93 na fase II por 10 semanas. Na 18 semana os animais foram eutanasiados Fonte: autor.

Tabela 1- Composição nutricional das dietas (g/kg)

Ingredientes	AIN-93M	HFHF	CF	CO
Albumina*	136,4	136,4	101,8	136,4
Amido de milho	463,5	135,0	116,8	135,0
Amido destronizado	155,0	45,0	45,4	45,0
Sacarose	100,0	28,6	29,3	28,6
Banha de porco	-	310,0	310,0	310,0
Óleo de soja	40,0	40,0	-	-
Óleo de chia	-	-	-	40,0
Farinha de chia	-	-	147,3	-
Frutose	-	200,0	200,0	200,0
Celulose	55,8	55,8	-	55,8
Mix mineral	35,0	35,0	35,0	35,0
Mix vitamínico	10,0	10,0	10,0	10,0
L-cistina	1,8	1,8	1,8	1,8
Bitartarato de colina	2,5	2,5	2,5	2,5
Macronutrientes				
Carboidrato (%)	77.4	30.1	31	30,1
Proteína (%)	12,9	9.1	9,2	9,1
Lipídio (%)	9,7	59,8	60,4	59,8
Densidade calórica (kcal.g-1)	3,7	5,3	5,2	5,3
Ácidos Graxos (g/kg)				
ω-3**	3,3	10,2	31,8	31,8
ω-6**	20,2	58,8	46,5	46,5
Razão ω-6:ω-3	6,1:1	5,8:1	1,5:1	1,5:1

AIN-93M (Reeves, Nielsen e Fahey, 1993): grupo de dieta padrão; HFHF (Marineli *et al.*, 2015): grupo de alto teor de gordura e alto teor de frutose; CF: grupo farinha de chia - HFHF com 14,7% (p/p) de farinha de chia; CO: grupo óleo de chia - HFHF com 4% (p/p) de óleo de chia. * A quantidade foi calculada com base no teor de proteína igual a 88% para fornecer 12 g de proteína.100 g-1 de dieta (Moreira, 2019).** Ácidos graxos do óleo de chia foram determinados por cromatografia gasosa (Moreira, 2019).

Na 17th semana coletou-se a urina de 24 horas para a análise da permeabilidade intestinal. Ao final das 18 semanas os animais foram eutanasiados e coletou-se o colón descendente, o conteúdo cecal e o tecido adiposo. A partir das fezes do ceco foram quantificadas as concentrações de SCFAs e IgA, o pH, assim como para o sequenciamento de

DNA da microbiota intestinal por Amplicon RNA ribossômico 16s. Utilizou-se o colón descendente para a histomorfometria das criptas, tecido muscular e células caliciformes, analisando por microscópio de luz junto a *softwares Image* J e pro. Quantificou-se o tecido adiposo total para obter a porcentagem de adiposidade corporal. A partir do peso e comprimento corporal determinou-se o peso total e o índice de massa corporal. A ingestão alimentar total e as calorias ingeridas foram obtidas a partir do consumo alimentar semanal. A quantificação da ingestão semanal de ω-3 e ω-6 foi baseada no consumo total de dieta ingerida.

Os dados foram tabulados no *Notepad++*, *Excel*, e *Word*. Todas as variáveis, menos os dados da microbioma foram quantificadas por teste de normalidade de *Kolmogorov-Smirnov*, seguido de *one-way Simple Analysis of Variance* (ANOVA) e teste *post-hoc* de *Newman-Keuls* ou *Kruskal-Wallis* com teste de comparação múltipla de Dunn no *software GraphPad Prism* versão 9.0 (*GraphPad Software*, São Diego, CA).

A partir dos dados de sequenciamento do microbioma realizou-se as análises de α-diversidade (Chao, Shannon e Simpson), β diversidade (PCoA de dissimilaridade de *Jaccard*, com análise não paramétrica de semelhanças (PERMANOVA) de 10.000 permutações, feito no *software Past* 4.12.), relação Firmicutes/Bacteriodetes, gráficos de abundância relativa de filo e gênero, correlação de Pearson, KEGG e LEfSe. A estatística de todos os dados de microbioma foram submetidos a *false discovery rate* (FDR) no *software* STAMP versão 2.1.3. Obteve-se as letras estatísticas no *software Statistical Package for Social Science* (SPSS) versão 20.0, por *one-way* ANOVA com *post-hoc* Duncan ou *Kruskal-Wallis* com correção de Bonferroni. Estabeleceu-se uma significância estatística de $p < 0,05$.

Utilizou-se *software GraphPad Prism* versão 9.0 (*GraphPad Software*, São Diego, CA) para a elaboração dos gráficos de relação Firmicutes/Bacteroidetes, Filo, Gênero, correlação de Pearson, e dos índices de Chao, Shannon e Simpson. Obteve-se a figura do LEfSe na Galaxy (https://huttenhower.sph.harvard.edu/galaxy/), e a figura do KEGG foi feita no Excel.

A partir da dissertação originou-se dois artigos originais, sendo um sobre a ação da farinha de chia na modulação intestinal, e o outro sobre o óleo de chia na saúde intestinal. As análises de KEGG e LEfSe do óleo de chia foram sem significância, sendo estes resultados excluídos no artigo. A metodologia detalhada se encontra em ambos os artigos.

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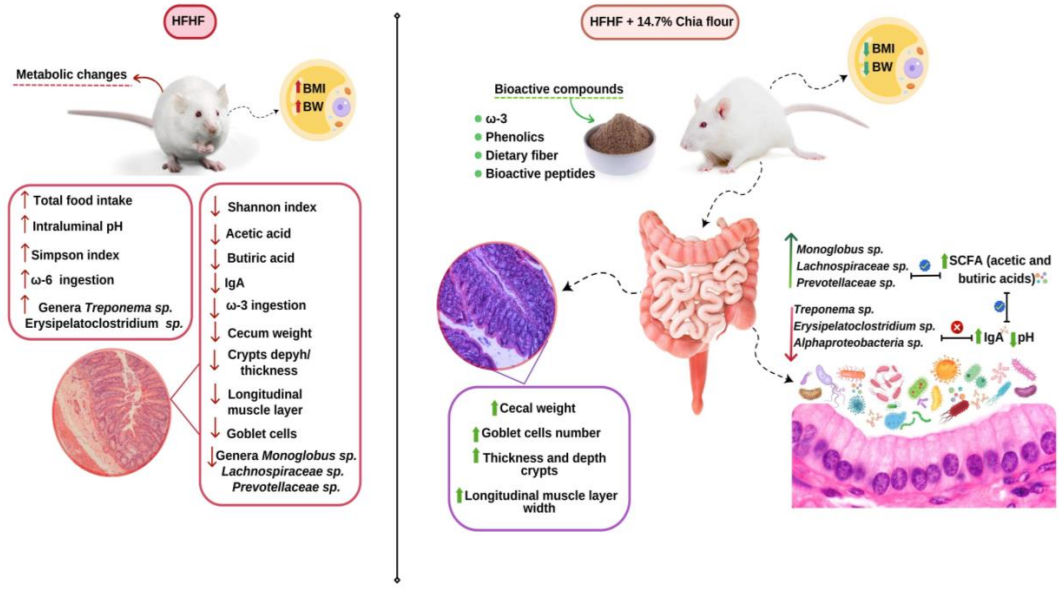
6 RESULTADOS E DISCUSSÃO

6.1 Artigo 1: CHIA (*Salvia hispanica* L.) FLOUR MODULATES THE INTESTINAL MICROBIOTA IN *WISTAR* RATS FED A HIGH-FAT AND HIGH-FRUCTOSE DIET

ABSTRACT

A diet rich in sugar and fat can promote metabolic disorders development, especially in the intestine. Chia flour (*Salvia hispanica*. L) is a source of dietary fiber, alpha-linolenic fatty acid (ALA), bioactive peptides, and phenolics, promoting health benefits. This study aimed to analyze chia flour's effect on gut microbiota modulation and intestinal health in adult male *Wistar* rats fed a high-fat and high-fructose diet (HFHF). Male *Wistar* rats (n = 10/group) were fed the diets standard (AIN-93M) or HFHF (31% saturated fat and 20% fructose) in the first phase to induce metabolic disorders. In the second phase, the rats were fed AIN-93M, HFHF, or HFHF plus 14.7% c chia flour (HFHF + CF) for 10 weeks. The consumption of chia flour increased the ALA (3.24 ± 0.24) intake and significantly improved immunoglobulin A (IgA) levels (1126.00 ± 145.90), goblet cells number (24.57 ± 2.76), crypt thickness (34.37 ± 5.86), crypt depth (215.30 ± 23.19), the longitudinal muscle layer (48.11 ± 5.04), cecum weight (4.39 ± 0.71), Shannon index ($p < 0.05$), and significantly increased the production of acetic (20.56 ± 4.10) and butyric acids (5.96 ± 1.50), *Monoglobus sp., Lachnospiraceae sp.,* and *Prevotellaceae sp.* abundance. Furthermore, chia significantly reduced the cecal pH content (7.54 ± 1.17), body mass index (0.62 ± 0.03) and weight (411.00 ± 28.58), and Simpson index ($p < 0.05$). Therefore, chia intake improved intestinal health parameters and functionality in rats with metabolic disorders, which demonstrates to be an effective strategy for gut microbiota modulation.

Keywords: Chia flour; Bioactive compounds; Short-chain fatty acids; Intestinal morphology; Immunoglobulin A; Cecal pH content; Diversity analysis; Functionality analysis.



Graphical Abstract: Effect of chia flour intake in modulation and health intestinal in adults *Wistar* rats with metabolic changes. The consumption of HFHF promote metabolic changes. Nonetheless, the chia flour modulates the bacterial community, improves intestinal morphology and functionality. AIN-93M: standard diet; HFHF: high-fat high-fructose diet; HFHF + CF: high-fat high-fructose with chia flour diet; BMI: body mass index; BW: body weight; IgA: immunoglobulin A; SCFAs: short-chain fatty acids.

1 INTRODUCTION

The Western diet is based on ultra-processed foods rich in fat, fructose, salt, and sugar, which contribute to hyperphagia, obesity, intestinal dysbiosis, inflammatory bowel diseases, and other noncommunicable diseases, such as diabetes, and cardiovascular diseases (Woodie et al., 2020; Attal et al., 2021; Maurya et al., 2023; Newsome et al., 2023). The intestinal dysbiosis caused by ultra-processed food intake impairs the intestinal membrane through microbial community modification, leads to an increase of pathogenic bacteria proliferation, and a reduction of probiotic bacteria (Kang et al., 2023; Lee et al., 2023; Saranya & Viswanathan, 2023). Besides, dysbiosis can modify the enterocyte morphology of villus and crypts and reduce mucus synthesis, which may increase the passage of pathogens and liposaccharides and decrease secretory IgA, which forms part of the first line of defense on the intestinal surface against pathogens (Ou et al., 2022; Lu et al., 2023).

The consumption of bioactive compounds promotes the restoration and maintenance of healthy intestinal homeostasis, assisting the short-chain fatty acids (SCFAs) synthesis, the

beneficial bacteria strains proliferation, and the improvement of intestinal bacterial morphology (Liang et al., 2021; Mishima et al., 2022a). The chia flour (*Salvia hispanica* L.) is the source of bioactive compounds, dietary fiber (37.9% total dietary fiber = 34.5% insoluble dietary fiber, 3.4% soluble dietary fiber), unsaturated fatty acids (27.5% lipids = 82.2% polyunsaturated fatty acids and 7.5% monounsaturated fatty acids), proteins (20.7%), bioactive peptides and phenolics compounds (rosmarinic, ferulic and caffeic acids) (Da Silva et al., 2017; Enes et al., 2020; Amaya Cano et al., 2021; Grancieri et al., 2022; Moreira et al., 2022).

Chia flour soluble extracts (0.5%) by intra-amniotic administration improve intestinal morphology since it increases the villus surface area, villus length, villus width, and the goblet cells diameter and number and promotes the growth of *Bifidobacterium sp*. and *Lactobacillus sp.* genera and reduces *E. coli sp.* and *Clostridium sp.* (Da Silva et al., 2019). The consumption of chia flour with a standard diet by adult female ovariectomized *Wistar* rats increased acetic and butyric acids concentrations longitudinal and circular muscle layers, and crypt thickness, decreased the cecal pH content, and enriched *Bacteroides sp.* genus and Muribaculaceae and Lachnospiraceae families (Mishima et al. 2022a). Moreover, chia flour added to a standard diet increased SCFAs production, faecal moisture, and circular muscle layer width in young male rats without metabolic disorders (Mishima et al. 2022b).

In this present study, adult male *Wistar* rats were submitted to metabolic disorders (liver steatosis - grade 2, insulin resistance, and high triglyceride and uric acid levels) (Enes et al., 2020; Moreira et al., 2022) by a high-fat and high-fructose (HFHF) diet to investigate chia flour effects on the functionality, morphology, and gut microbiota modulation. Our hypothesis is that chia flour intake increases SCFAs production, decreases cecal pH content, increases the beneficial bacterial strain proliferation, improves intestinal morphology and IgA levels in rats with metabolic disorders caused by HFHF diet consumption.

2. Material and Methods

2.1. Samples

Chia seeds were obtained in Rio Grande do Sul, Brazil. After harvesting, the seeds were stored in a freezer, at -18 ºC in vacuum packaging. The chia seeds were ground in a blender (PHILIPS WALITA® model RI2035 500W) to obtain the flour and prepare the experimental diet. The flour was stored at -18°C in a black polypropylene bag to prevent lipid oxidation.

2.2. Animals and Experimental Diets

Thirty adult male *Wistar* rats (*Rattus norvegicus*), aged 45 to 50 days, were obtained from the Central Animal House of the Center for Biological Sciences and Health, Federal University of Viçosa, Minas Gerais, Brazil. The sample calculation equation was determined by Marineli et al., (2015). During the experiment, the animals were kept in individual stainless-steel cages, under controlled temperature (22 °C ± 2°C), light/dark cycle (12h), and received deionized water and diets *ad libitum*. The animals were fed a standard diet (AIN-93M; n = 10; initial body weight 156.0 ± 17.0 g) (Reeves et al., 1993) and an HFHF diet (n = 20; initial body weight: 156.5 ± 17.9 g), for 8 weeks, to induce metabolic disorders (Moreira et al., 2022). The HFHF diet comprised fructose 20%, lard 31%, and soybean oil 4% (Marineli et al., 2015). After 8 weeks, the AIN-93M group (n=10) was maintained, and the HFHF group (n = 20) was randomized into the groups: HFHF (n=10) and HFHF plus chia flour (HFHF + CF): animals fed HFHF diet + 14.7% chia flour, n=10) (**Table 1**). In the HFHF + CF diet, 4% of soybean oil from HFHF was replaced by 4% of lipid from chia flour, equivalent to 14.7% of chia flour, which contains 5.58% of flour's fiber. In the diets, AIN-93M and HFHF, microcristalline cellulose (5.58%) was used as a fiber source.

Table 1 - Nutritional composition of diets.

Ingredients (g/kg)	**AIN-93M**	**HFHF**	**HFHF + CF**
Albumin*	136.4	136.4	101.8
Maize starch	463.5	135.0	116.8
Dextrinized starch	155.0	45.0	45.4
Sucrose	100.0	28.6	29.3
Soybean oil	40.0	40.0	-
Cellulose	55.8	55.8	-
Mineral Mix	35.0	35.0	35.0
Vitamin Mix	10.0	10.0	10.0
Choline Bitartrate	2.5	2.5	2.5
L-cystine	1.8	1.8	1.8
Lard	-	310.0	310.0
Fructose	-	200.0	200.0
Chia flour	-	-	147.3

Macronutrients			
Carbohydrate (%)	77.4	30.1	31.0
Protein (%)	12.9	9.1	9.2
Lipid (%)	9.7	59.8	60.4
Caloric density (kcal/g)	3.7	5.3	5.2
Fatty acids (g/kg)			
ω-3	3.3	10.2[#]	31.8[#]
ω-6	20.2	58.8[#]	46.5[#]
Ratio ω-6:ω-3	6.12:1	5.77:1[#]	1.46:1[#]

*The amount was calculated based on the protein content equal to 88% to provide 12 g of protein. Chemical composition of chia flour (g/100g) used to calculate the diet: carbohydrates: 3.2g; total dietary fiber: 37.9g; lipids: 27.7g; protein: 20.7g; moisture: 6.9g; ALA: 2.49g; and ω-6: 0.79 g (Moreira et al., 2022). # (Fonseca; Gutierrez, 1974). AIN-93M (Reeves et al., 1993), standard diet; HFHF (Marineli et al., 2015), high-fat and high-fructose diet; HFHF + CF, HFHF with 14.7% of chia flour diet; ALA, Alpha-linolenic acid.

At the end of the 18th week, the animals were euthanized by cardiac puncture after anesthesia with isoflurane 5% (Isoforine, Cristália®). The cecum content, colon, and adipose tissue were collected, weighed, immediately frozen with liquid nitrogen, and stored at -80°C for subsequent analyses. The ascending large colon was collected and stored in a 10% formalin solution for histological analyses.

The study was approved by the Ethics Committee for the Use of Animals of the Federal University of Viçosa (CEUA/UFV), protocol N° 31/2018. All experimental procedures with animals were carried out in accordance with Directive 86/609/EEC of November 24, 1986, in compliance with the ethical principles for animal experimentation.

2.3. Food Intake and Biometric Measurements

Food intake and body weight were weekly measured. The body mass index (BMI) was calculated as body weight/ length^2 (Shah; Braverman, 2012). Adiposity percentage was calculated by the sum of epididymal, abdominal, and retroperitoneal tissues, and divided by final weight, multiplied by one hundred (Medina Martinez et al., 2022). The ALA and linoleic

fatty acid (ω-6) intake was calculated by the weekly, multiplied by the omega content in 1 kg of diet, and divided by 1000.

2.4. DNA Extraction and Sequencing

The genomic DNA was extracted from cecal content samples by mechanical disruption and phenol/chloroform extraction protocol, and each sample was treated with RNAse (Stevenson & Weimer, 2007). The Illumina MiSeq platform was used to load the samples into an Illumina flow cell, for paired-end sequencing reactions, at the Argonne National Laboratory (Lemont, Illinois, USA) (Caporaso et al., 2012). The PCR amplicon libraries targeted the hypervariable V4 - region of 16S rRNA gene, using 515F (5'GTGYCAGCMGCCGCGGTAA3') and 806R (GGACTACNVGGGTWTCTAAT3') primers and a barcoded primer set for the Illumina MiSeq platform (Illumina, San Diego, California, USA) (Caporaso et al., 2011). The customized sequencing primers were used, and procedures followed the 151bp x 12bp x 151bp MiSeq run parameter (Caporaso et al., 2012).

In this study, the sequences obtained in each sample were submitted to the Sequence Read Archive (SRA) in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sra) under the accession number PRJNA926948, and the data analyses were carried out by the Mothur software (1.47.0) (Schloss et al., 2009). UCHIME was used to detect and remove Chimera sequences (Edgar et al., 2011). The taxonomic classification and alignment of the sequences with the 16S rRNA were performed using SILVA database v.138.1 (Quast et al., 2013).

The Operational Taxonomic Units (OTUs) were grouped with a 97% sequence similarity cut-off. The coverage of all samples was assessed by the Good's coverage estimator (Bacteria > 97%). The samples were normalized for the lowest number of sequences produced by any sample (**Supplementary Table S1**). The standardized data table was used for calculating alpha and beta-diversity and the relative abundance of OTUs. The Chao, Shannon and Simpson indexes estimate alpha-diversity. Beta-diversity was determined by Principal Coordinate Analysis (PCoA), based on the Jaccard dissimilarity index. The metagenome functional predictive analysis was performed with the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) Software (Douglas et al., 2020). The abundance was normalized by the 16S rRNA gene copy number and identified by Greengenes database (DeSantis et al., 2006). Normalized data were used to run the Kyoto Encyclopedia of Genes and Genomes (KEGG), Linear discriminant analysis Effect Size (LEfSe), and alpha and beta-diversity analyses.

2.5. Intestinal Health

2.5.1 Cecum Content pH

The cecal content was diluted with distilled water at a 1:10 (w/v) ratio and vortexed until complete homogenization. Then, the pH was read by glass electrode insertion (Grancieri et al., 2017).

2.5.2 Intestinal Permeability

The animals were fasted for 12 h, and 2 mL of a solution containing 200 mg of lactulose and 100 mg of mannitol was administered by gavage. Subsequently, the animals were placed in metabolic cages and fasted for 5 hours. The urine samples were collected for 24 hours, labeled according to the experimental group, and stored at -80°C (Song et al., 2011). Then samples were centrifuged (4°C, 12,000 x g, 10 min), the supernatants were collected and filtered through 0.45 mm Millipore filters, and 1.5 mL was placed in vials for high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) at 55°C and pressure of 1920 psi under isocratic conditions. Chromatography consisted of the degasser system (Model DGU-14A), pump (Model LC-10AT), automatic injector (Model SIL-20A), column oven (Model CTO-10AS), and UV-Vis detector (model SPD-10AV) connected in series with a refractive index detector (model RID-10A). An Aminex HPX-87H analytical column (300 cm x 8.7 mm - brand BIO-RCalifornia, USA) was used (Grancieri et al., 2017). Sulfuric acid (0.005 mM) was used in the mobile phase with a flow of 0.6 ml/min, and 20 μL of sample was injected (De Sá et al., 2011). The lactulose and mannitol (Sigma-Aldrich, São Paulo/SP, Brazil) were used as internal standards. The peak areas were obtained and converted into g/L according to the percentage of urinary excretion of lactulose and mannitol and the ratio of lactulose/mannitol (De Sá et al., 2011).

2.5.3 Short-Chain Fatty Acids Concentration (SCFAs)

Cecal content (400 mg) was homogenized in Milli-Q water (1 mL), vortexed and centrifuged at 12,000 x g for 10 min, and the supernatant was collected and evaluated according to the methodology proposed by Siegfried et al. (1984).

The samples were analyzed by HPLC, using a 3000 Dual detector HPLC apparatus (Dionex Corporation, Sunnyvale, CA, USA), coupled to a Shodex RI-101 refractive index (IR) detector, maintained at 40ºC and Rezex ROA ion exclusion column, 300 × 7.8 mm (Phenomenex Inc. Torrance, CA, USA), maintained at 40°C. Sulfuric acid (5 mM) was used at a flow rate of 0.7 mL/min. Acetic, propionic, and butyric acids were used as standard curves (Sigma-Aldrich, São Paulo/SP, Brazil).

2.5.4 Histological Analyses

Semi-serial histological sections of 3 μm thick ascending large colon fragments were obtained using an automatic microtome (Reichert Jung®, Germany) and stained using the eosin/hematoxylin technique. The slides were analyzed under a light microscope (Olympus AX 70 TRF, Tokyo, Japan) with a 10× objective. Twenty random fields per animal were selected for longitudinal muscle layer width and quantifying the number of goblet cells from the crypt (Da Silva et al., 2019). The values were obtained using the Image Pro Plus® (version 4.5) (Media Cybernetics, Rockville, USA) and ImageJ® (National Institutes of Health, USA) software.

2.5.5 Immunoglobulin A (IgA)

Mucosal immunity was assessed based on the concentration of IgA in the cecum content. Briefly, cecal content (200 mg) was added in 800 μl of phosphate-buffered saline solution and vortexed, and the concentration was measured using an ELISA kit (Cat.N° EA0032Ra). The absorbance was read at 450 nm (Multiskan Microplate Photometer, Thermo Fisher Scientific, MA, USA). and the values were expressed in ng/ml (Vaz-Tostes et al., 2014).

2.6. Statistical Analyses

Data of food consumption, body weight, intestinal permeability, cecal pH content, colonic morphometric characteristics, and SCFAs concentrations were analyzed using the Kolmogorov-Smirnov normality *test*, followed by one-way analysis of variance (ANOVA) and *post hoc* Newman-Keuls *test*. Non-parametric and independent samples were submitted to Kruskal-Wallis and *post hoc* Dunn's *test*. Pearson correlation test assessed correlations among biological markers and gut microbial. The analyses were performed in GraphPad Prism (version 9.0).

The Chao, Shannon, and Simpson indexes were used for alpha-diversity. Beta-diversity was assessed by PCoA based on the Jaccard dissimilarity index in the Past software (version 4.12). Differences among beta-diversity values were analyzed by the Pairwise Permutational multivariate analysis of variance (PERMANOVA) *test*. Microbiota data were corrected for multiple comparisons using the false discovery rate (FDR) by Benjamini–Hochberg in the Statistical Analysis of Metagenomic Profiles (STAMP) software (version 2.1.3). The statistical analyses were performed by IBM SPSS Statistics (version 20.0). The data were analyzed by one-way ANOVA and Duncan *post hoc test*. Non-parametric data were submitted to Kruskal-Wallis with Bonferroni correction. The graphics of Phylum, Genera, Firmicute/Bacteriodetes ratio, Pearson Heatmap, and Chao, Shannon, and Simpson indexes were obtained by GraphPad Prism (version 9.0). The KEGG heatmap was generated by Excel. The figure LEfSe was

produced by Galaxy (https://huttenhower.sph.harvard.edu/galaxy/). The significance level was $p < 0.05$.

3. Results

3.1 Effect of chia flour on body weight gain and food intake

The groups HFHF and HFHF + CF presented lower food intake and higher caloric density ingestion ($p < 0.05$) than the AIN-93M group. Body weight, BMI, and ω-6 intake ($p < 0.05$) presented lower values in groups fed with HFHF + CF and standard diet (AIN-93). The adiposity increased ($p < 0.05$) in the HFHF group compared to AIN-93M. Although the HFHF + CF group did not differ from the HFHF group, it was similar to the AIN-93M group ($p > 0.05$). Chia flour increased the ALA consumption compared to the AIN-93M and HFHF groups ($p < 0.05$) (**Table 2**).

Table 2 - Chia flour effect on food intake and adiposity in *Wistar* rats (n =10) for 10 days.

Variables	AIN-93M	HFHF	HFHF+CF
Total food intake (g)	1367.00 ± 69.07^{a}	1046.00 ± 130.20^{b}	1052.00 ± 65.01^{b}
Caloric intake ($kcal.g^{-1}$)	4.85 ± 0.24^{b}	5.50 ± 0.68^{a}	5.53 ± 0.34^{a}
Final body weight (g)	390.00 ± 31.36^{b}	461.80 ± 29.78^{a}	411.00 ± 28.58^{b}
Total adiposity (%) *	5.48 ± 0.89^{b}	7.30 ± 1.57^{a}	6.36 ± 0.62^{ab}
Body mass index (kg/m^2)	0.61 ± 0.05^{b}	0.71 ± 0.07^{a}	0.62 ± 0.03^{b}
Weekly ω-3 intake (g)	0.45 ± 0.02^{c}	1.05 ± 0.10^{b}	3.24 ± 0.24^{a}
Weekly ω-6 intake (g)	2.77 ± 0.10^{c}	6.06 ± 0.63^{a}	4.74 ± 0.35^{b}

AIN-93M, standard diet; HFHF, high-fat and high-fructose diet; HFHF + CF, HFHF with chia flour diet. *The total adiposity was determined by the sum of the epididymal, abdominal, and retroperitoneal tissues, divided by the final weight, multiplied by one hundred. Different lower-case letters in the same line: groups were significantly different ($p < 0.05$). Data analyzed by ANOVA with Newman-Keuls *post-hoc* or Kruskal Wallis with Dunn *test*.

3.2 Effect of chia four on intestinal health

Chia flour consumption (HFHF + CF group) positively upregulated the butyric acid and IgA concentrations when compared to the AIN-93M and HFHF groups ($p < 0.05$). Chia flour (HFHF + CF) increased the acetic acid content, the number of goblet cells, crypt thickness,

crypt depth, longitudinal muscle layer width, and cecum weight compared to the HFHF group ($p < 0.05$), and it was similar to AIN-93M (**Figure 1**). Furthermore, chia consumption reduced cecal pH content compared to the control groups ($p < 0.05$). The propionic acid content, circular muscle layer width, and mannitol/lactulose ratio ($p > 0.05$) were similar among the three experimental groups (**Table 3**).

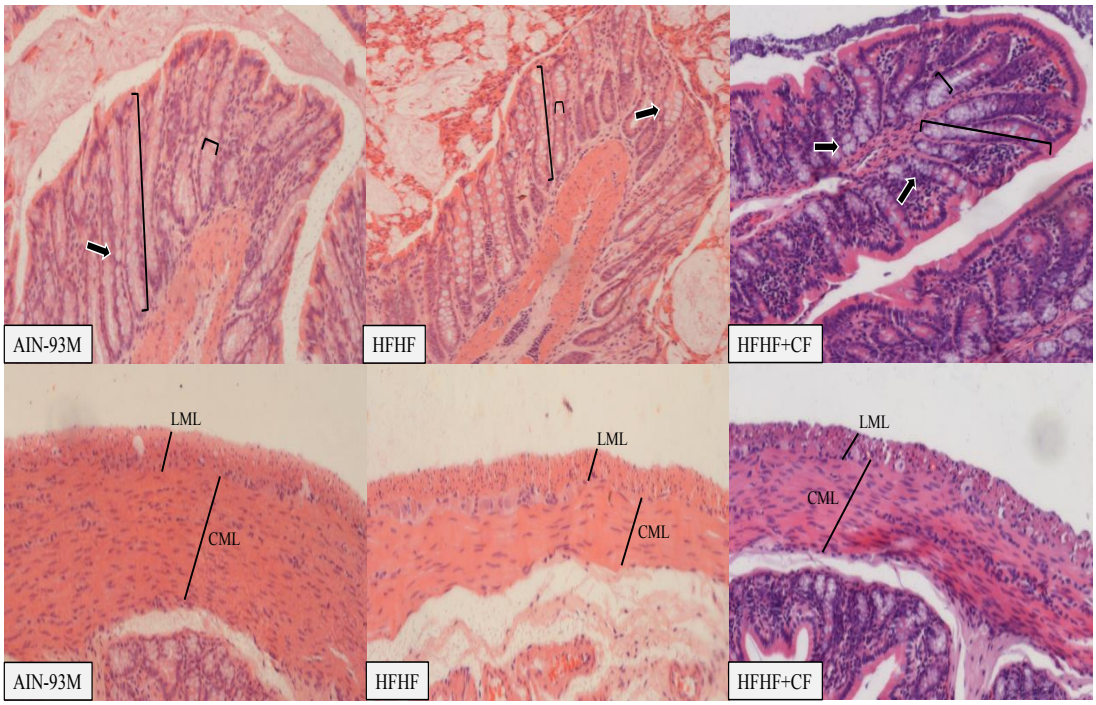


Fig. 1. Effects of chia flour intake on colonic histomorphometry characteristics in adult *Wistar* rats fed HFHF diet. n=10 animals/group. AIN-93M, standard diet; HFHF, high-fat and high-fructose diet; HFHF + CF, high-fat and high-fructose with chia flour diet; CML, circular muscle layer; LML, longitudinal muscle layer. Black arrows refer to goblet cells in the crypt. Black brackets refer to the crypt's depth and width. Staining was carried out with hematoxylin and eosin. Bar: 100 μm.

Table 3 - Effect of chia flour intake on intestinal health in *Wistar* rats (n =10) for 10 days.

Variables	**AIN-93M**	**HFHF**	**HFHF+CF**
SCFAs (mM)			
Acetic acid	21.36 ± 5.11[a]	13.71 ± 2.90[b]	20.56 ± 4.10[a]
Propionic acid	4.58 ± 1.13 [a]	4.35 ± 1.55 [a]	6.46 ± 2.45[a]

Butyric acid	2.51 ± 0.77[b]	3.60 ± 1.01[b]	5.96 ± 1.50[a]
Cecal faeces pH	9.01 ± 0.40[a]	9.17 ± 0.25[a]	7.54 ± 1.17[b]
Cecum weight (g)	4.66 ± 0.83[a]	3.58 ± 0.60[b]	4.39 ± 0.71[a]
IgA (ng/ml)	851.50 ± 106.20[b]	786.50 ± 89.50[b]	1126.00 ± 145.90[a]
Number of goblet cells	24.80 ± 0.88[a]	19.55 ± 2.66[b]	24.57 ± 2.76[a]
Crypt thickness (μm)	38.62 ± 3.15[a]	22.94 ± 3.22[b]	34.37 ± 5.86[a]
Crypt depth (μm)	201.20 ± 16.27[a]	167.10 ± 4.52[b]	215.30 ± 23.19[a]
CMLW (μm)	125.70 ± 38.28[a]	101.50 ± 43.10[a]	158.30 ± 15.49[a]
LMLW (μm)	49.61 ± 7.86[a]	33.88 ± 4.02[b]	48.11 ± 5.042[a]
Mannitol/Lactulose (%)	1.79 ± 0.24[a]	1.85 ± 0.68[a]	1.90 ± 0.29[a]

AIN-93M: standard diet; HFHF: high-fat and high-fructose diet; HFHF + CF: HFHF with chia flour diet; LMLW: Longitudinal muscle layer width; CMLW: Circular muscle layer width. Different letters in the same line: groups are significantly different ($p < 0.05$). Data analyzed by ANOVA with Newman-Keuls *post-hoc* or Kruskal Wallis with Dunn *test*. Histomorphometry measurements were performed on ascending large colon crypt.

3.3 Chia flour changes the intestinal microbiota pattern promoted by the HFHF diet.

The sequencing of the 16S rRNA gene generated 810.021 raw sequences, with lengths ranging from 151 bp to 300 bp. After filtering and cleaning the sequences, 611.323 good quality sequences were obtained. The Good's coverage obtained in the samples was > 99%, which indicates good coverage of the sequencing.

The PCoA represented approximately 48.91% of the dissimilarity in bacterial species composition. The Permutational Multivariate Analysis of Variance (PERMANOVA) presented statistical differences in the distance metrics among treatments (PERMANOVA, $p < 0.05$) (**Figure 2A**). The clustering of the bacterial community differed among the three groups ($p < 0.05$).

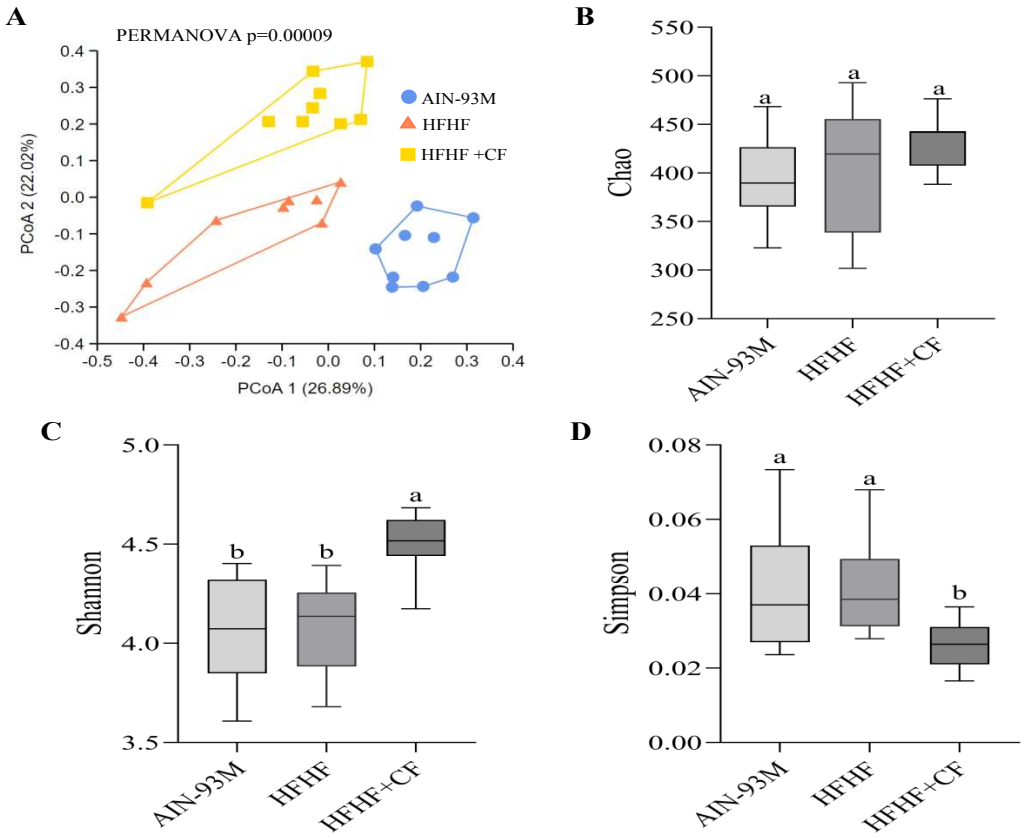


Fig. 2. Effect of chia flour intake on beta and alpha diversity. (**A**) Beta diversity estimated by PCoA based on Jaccard similarity distance of cecal microbial communities in adult *Wistar* rats fed a high-fat and high-fructose diet. Each dot refers to one animal and the colors refer to the experimental groups. The *p*-value among the groups was obtained by PERMANOVA. Alpha diversity was estimated by the indices: (**B**) Chao, (**C**) Simpson and (**D**) Shannon, by one-way ANOVA, followed by the Ducan *post-hoc* test ($p < 0.05$). AIN-93M, standard diet (n = 9); HFHF, high-fat and high-fructose diet (n = 8); HFHF + CF, HFHF with chia flour (n = 9).

The microbial richness estimated by the Chao index did not differ among the groups ($p>0.05$) (**Figure 2B**). The Shannon index indicated an increase in species diversity in the HFHF + CF group ($p < 0.05$) compared to the HFHF and AIN-93M groups (**Figure 2C**). The AIN-93M and HFHF groups showed an increase in the Simpson index, while the HFHF + CF group was able to reduce the dominance of species in their bacterial community ($p < 0.05$) (**Figure 2D**).

The samples presented 19 phyla, 30 classes, 70 orders, 108 families, and 204 genera. The Firmicutes/Bacteroidetes ratio was similar among the groups ($p > 0.05$) (**Fig. 3A**). All

groups presented eight predominant phyla, including Firmicutes (HFHF + CF: 73%; AIN-93M and HFHF: 69%), Bacteroidetes (HFHF + CF and AIN-93M: 16%; HFHF: 17%), Desulfobacterium (HFHF + CF: 4.2%; AIN-93M: 4.4%; HFHF: 4.9%), Actinobacteria (HFHF + CF: 2.9%; AIN-93M: 3.7%; HFHF: 3.4%) and Proteobacteria (HFHF + CF: 1.9%; AIN-93M: 2.7%; HFHF: 2.4%) (**Fig. 3B**).

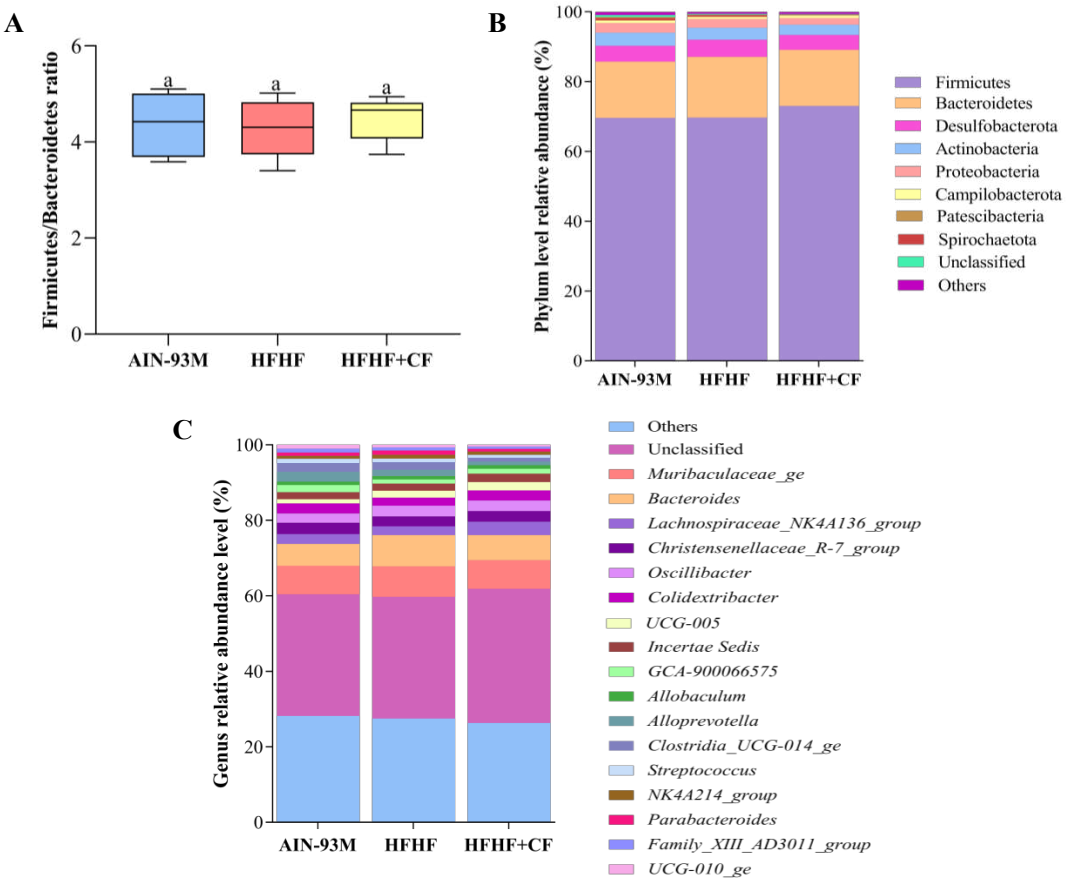


Fig. 3. Effect of chia flour intake on cecal microbiota relative abundance at phylum and genera levels, at the end of treatment (10 weeks). (**A**) Firmicutes to Bacteroidetes ratio; (**B**) Bacterial composition at phylum level; (**C**) Bacterial composition at the genera level. n=10 animals/group. Phyla with abundance > 0.3% and genera with abundance > 0.96%. The data were analyzed by the Dunn's test with FDR and Bonferroni corrections. AIN-93M, standard diet; HFHF, high-fat and high-fructose diet; HFHF + CF, HFHF with chia flour diet.

The groups presented 17 predominant genera, including *Muribaculaceae sp.* (HFHF + CF and AIN-93M: 7.5%; HFHF: 8%), *Bacteroides sp.* (HFHF + CF: 6.7%; AIN-93M: 5.8%;

HFHF: 8.3%), *Lachnospiraceae NK4A136 sp.* (HFHF + CF: 3.5%; AIN-93M: 2.6%; HFHF: 2.3%), *Christensenellaceae R-7 sp.* (HFHF + CF: 2.8%; AIN-93M: 3%; HFHF: 2.6%) and *Oscillibacter sp.* (HFHF + CF and HFHF: 2.8%; AIN-93M: 2.4%) (**Fig. 3C**).

The HFHF + CF group exhibited a higher abundance of *Monoglobus sp.* and *Lachnospiraceae sp.* genera ($p < 0.05$) than the AIN-93M and HFHF groups. Further, the HFHF + CF group presented a high abundance of *Prevotellaceae sp.* genus ($p < 0.05$) relative to the HFHF group and low Patescibacteria phylum, *Alphaproteobacteria sp*. genus ($p < 0.05$) abundance relative to the AIN-93M group. In addition, the HFHF + CF group presented the lowest abundance of Spirochaetota Phylum and *Treponema sp.* genus ($p < 0.05$), compared to the AIN-93M and HFHF groups and *Erysipelatoclostridium sp.* genus ($p < 0.05$), compared to HFHF.

3.4. Correlation analysis

The Pearson correlation assessed the relationship among changes in intestinal microbial abundance, intestinal health markers, and adiposity parameters. *Monoglobus sp.* was positively correlated with IgA ($r = 0.68$; $p < 0.05$). *Prevotellaceae sp.* was positively correlated with crypt thickness ($r = 0.71$; $p < 0.05$). *Lachnospiraceae sp.* was positively correlated with butyric acid concentration ($r = 0.50$), crypt depth ($r = 0.54$), and longitudinal muscle layer width ($r = 0.58$) ($p < 0.05$), but inversely correlated with cecal pH content ($r = 0.50$; $p < 0.05$). IgA presented a positive correlation with acetic acid concentration ($r = 0.50$; $p < 0.05$), which was positively correlated with butyric acid content ($r = 0.46$; $p < 0.05$). The number of goblet cells was inversely correlated with BMI ($r = -0.66$) and positively correlated with crypt thickness ($r = 0.77$) and longitudinal muscle layer width ($r = 0.73$) ($p < 0.05$). Longitudinal muscle layer width was inversely correlated with BMI ($r = -0,66$; $p < 0.05$). Total body weight was positively correlated with BMI ($r = 0,59$; $p < 0.05$) (**Figure. 4**).

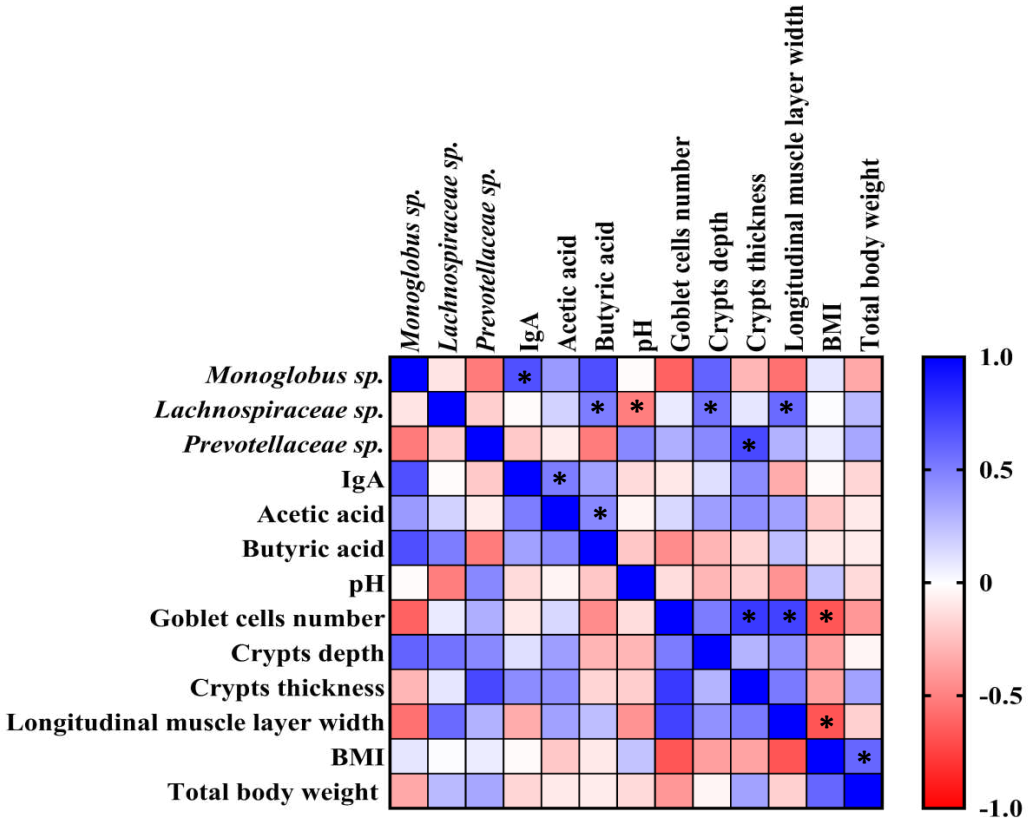


Fig. 4. Heatmap of Pearson correlation among microorganisms (gender level) and intestinal health markers. Blue is a positive correlation and red is a negative correlation. Correlation matrix *($p < 0.05$). n=10 animals/group. BMI, body mass index; IgA, immunoglobulin A. The specific bacteria, such as *Monoglobus sp., Lachnospiraceae sp.* and *Prevotellaceae sp.* were selected due to their abundance increase in the chia flour group.

3.5. Chia flour effect on the dominant cecal microbiota

All OTUs were analyzed to identify dominant cecal microbiota and intestinal biomarkers using the taxonomy. There were 18 dominant OTUs with an effect size > 4 %. The HFHF + CF group exhibited higher bacterial taxa relative to control groups, with a larger effect size of the *Lachnospiraceae sp.* genera. The *Selimonas sp*. was the dominant genus in the microbiota of the HFHF group. *Allobaculum sp.* (**Figure 5**) was the dominant community in the AIN-93M group.

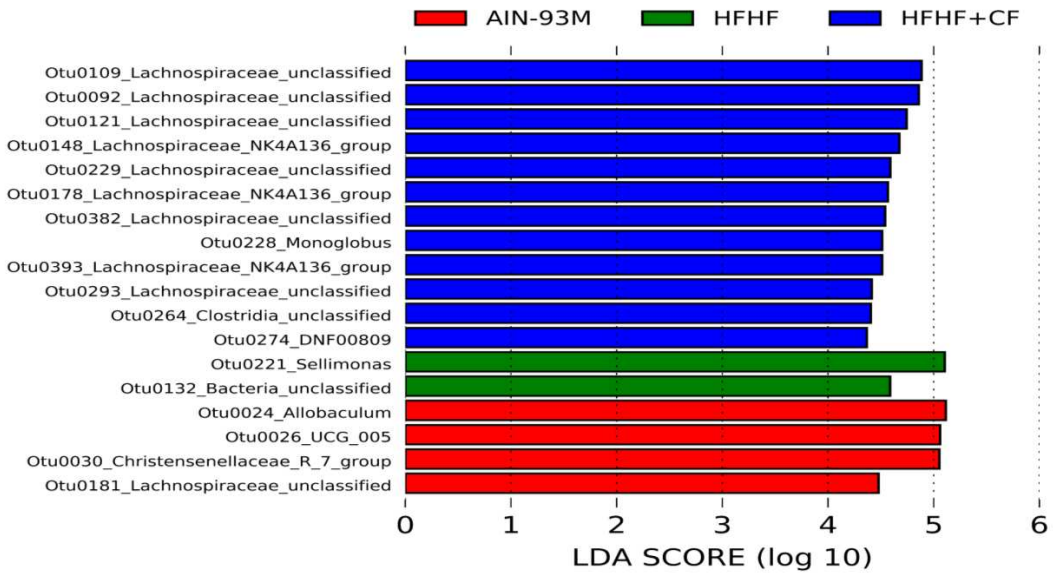


Fig. 5. Histogram of linear discriminant analysis effect size (LEfSe) method to compute linear discriminant analysis (LDA) scores of differences in dominant microorganisms between groups. AIN-93M, standard diet (n = 9); HFHF, high-fat and high-fructose diet (n = 8); HFHF + CF, HFHF with chia flour diet (n = 9). Significant differences were considered with $p < 0.05$.

3.6. Chia flour effect on Kyoto Encyclopedia of Genes and Genomes (KEGG)

347 metabolic pathways were observed in all bacterial communities of the experimental groups and 17 pathways presented significance ($p < 0.05$) (**Figure 6**). The HFHF + CF was significant in 15 pathways, such as demethylmenaquinol-9 biosynthesis; menaquinol-9 biosynthesis; 4-hydroxyphenylacetate degradation; menaquinol-6 biosynthesis I; chitin derivatives degradation; demethylmenaquinol-6 biosynthesis I; menaquinol-10 biosynthesis; methylphosphonate degradation I; TCA cycle VII compared with the AIN-93M and HFHF groups. The thiamin salvage II, (Kdo) 2-lipid A biosynthesis, L-methionine biosynthesis III, enterobacterial common antigen biosynthesis, L-arginine, putrescine, and 4-aminobutanoate degradation, and L-arginine and L-ornithine degradation compared with the AIN-93M group ($p < 0.05$) (**Figure 6**). The HFHF + CF and AIN-93M groups were significant in demethylmenaquinol-6 biosynthesis II. The AIN-93M was significant in glycolysis V (Pyrococcus).

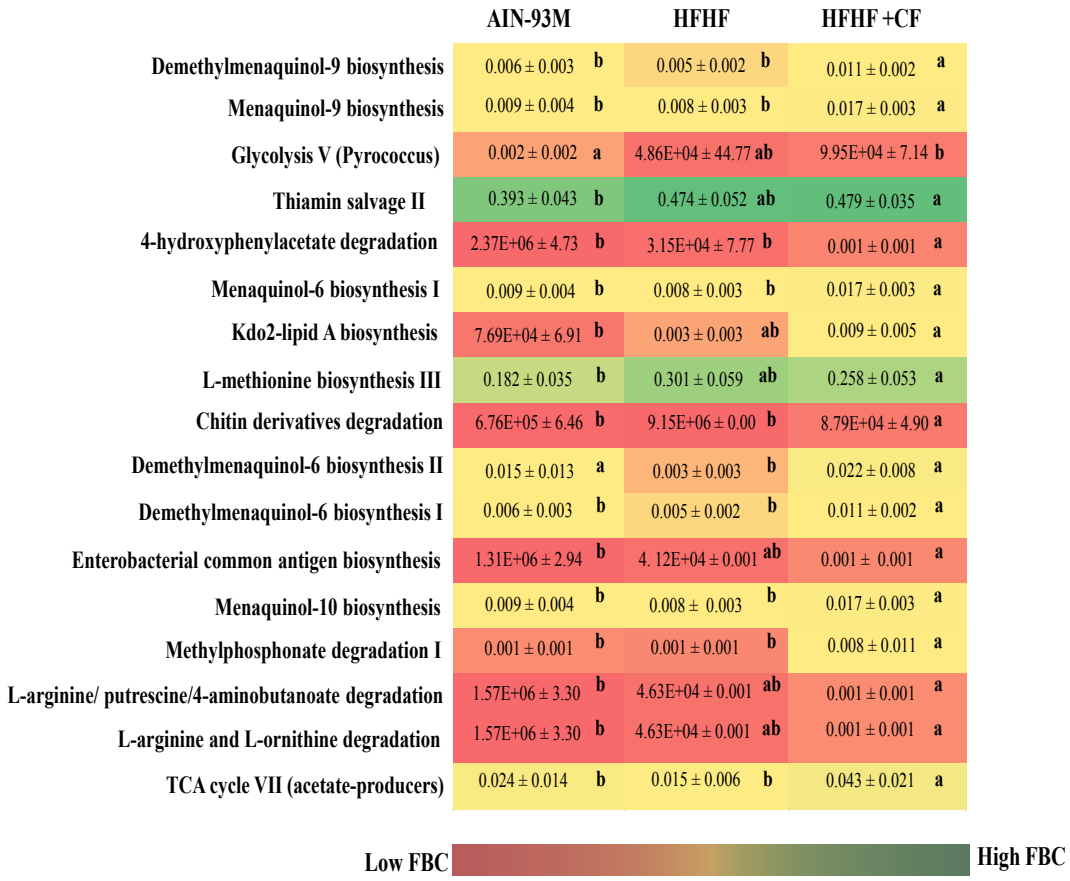


Fig. 6. Heatmap of the effect of chia flour consumption on the relative significance of differentially enriched microbial metabolic pathways in cecal microbiota in mice fed a high-fat and high-fructose diet. Different lower-case letters in the same line: groups are significantly different ($p < 0.05$) by SPSS. Used Kruskal Wallis with false discovery rate (FDR) by Benjamini–Hochberg. Green color indicates higher functional bacterial capacity (FBC); yellow indicates medium FBC; red indicates lower FBC. AIN-93M, standard diet (n = 9); HFHF, high-fat and high-fructose diet (n = 8); HFHF + CF, high-fat and high-fructose with chia flour diet (n = 9); FBC, functional bacterial capacity.

4. Discussion

Our study investigated the effects of chia flour on gut health in adult *Wistar* rats with metabolic disorders caused by the consumption of a HFHF diet. Chia flour reduced food intake, body weight, BMI, and cecal pH content and increased cecal weight. It also increased SCFAs concentration, IgA content, probiotic bacterial strains, and reduced pathogenic strains. Further,

chia flour enhanced the number of goblet cells, thickness, and depth crypt, besides the longitudinal muscle layer width in the cecum.

The groups that received the HFHF diet presented lower food intake and higher energy intake than the AIN-93M group. This fact can be due to the high energy density observed in the HFHF diet, which could have to promote high satiety (Enes et al., 2020; Medina Martinez et al., 2021; Moreira et al., 2022). The HFHF + CF diet offered 14.7g of chia flour/100g diet. This amount replaced 100% of the recommended oil and dietary fiber for rodents (AIN93-M) (Reeves et al, 1993). Considering the recommendation of 14g of dietary fiber/1000 kcal for human consumption (Dahl et al., 2015), to supply 100% of the human dietary fiber recommendation, it would be necessary to consume 73.8 g of chia flour/day, which is a high amount for human consumption. However, other studies must test different doses to validate the consumption of chia flour for humans.

The HFHF + CF diet reduced body weight gain and BMI. It could be explained by chia chemical composition due to its dietary fiber, bioactive peptides, and alpha-linolenic acid (ALA) (Da Silva et al., 2017; Enes et al., 2020; Grancieri et al., 2022; Moreira et al., 2022). These compounds may contribute to body weight loss (Enes et al., 2020; Fonte-Faria et al., 2019; Kobyliak et al., 2020; Moreira et al., 2022). These data were different from those of Miranda et al. (2019), in which male Swiss mice fed with a high-fat diet with 3% chia flour presented similar body weight compared to the high-fat group.

The beta-diversity analysis presented differences in the intestinal bacterial community in the HFHF + CF group without a change in the Firmicutes/Bacteroidetes ratio. These results indicated that the dietary fibers and ALA present in chia flour may increase intestinal fermentation, probiotic bacteria proliferation, and microbial species diversity. Mishima, et al., (2022b) observed differences in beta diversity in young male rats fed with a high-fat diet (64% fat), with or without chia flour (41.6% flour). Further, no changes in Firmicutes/Bacteroidetes ratio or alpha diversity were reported in these animals.

The HFHF + CF group improved the cecal pH content, cecal weight, butyric and acetic acids content, and the histomorphometry variables. These factors promoted the proliferation of probiotic strains, such as *Monoglobus sp*., *Lachnospiraceae sp*., and *Prevotellaceae sp*. The bioactive compounds from chia flour, such as dietary fibers, and phenolics compounds, including rosmarinic, ferulic, and caffeic acids (Da Silva et al., 2017; Moreira et al., 2022), may be a substrate to fermentation in the colon, besides providing energy for colonocytes and enabling probiotic strain proliferation (Markowiak-Kopeć & Śliżewska, 2020). Further, person analysis demonstrated a correlation between *Lachnospiraceae sp.* and the content of acetic and

butyric acids and between crypt depth and longitudinal muscle layer, thus evidencing the probiotic activity of these bacteria and the synergy among SCFAs. It also indicated the abundance of *Prevotellaceae sp.* correlated with crypt thickness, highlighting the functional effect of these bacteria on intestinal morphology. Similar results were observed in ovariectomized adult female *Wistar* rats fed a high-fat (51% fat) and 23.2% chia flour diet (Mishima et al., 2022a).

The intake of HFHF + CF reduced the richness of pathogenic bacteria, such as Patescibacteria, Spirochaetota, *Treponema sp., Erysipelatoclostridium sp.* and *Alphaproteobacteria sp.* It is an important health effect since chia flour increased IgA concentration, which may promote humoral immune response mediation (Ma et al., 2021; Tang et al., 2022). Furthermore, the bioactive peptides in chia flour may act in the anti-inflammatory pathway and inactivate pro-inflammatory cytokines (Grancieri et al., 2022), which favors IgA production. The Person's correlation in our study demonstrated that *Monoglobus sp.* correlated with IgA and IgA correlated with acetic acid, which indicates a prebiotic effect of chia flour.

The LEfSe indicated a predominance of *Sellimonas sp*. in the HFHF group, since it acts in the transport of amino acids and carbohydrates, as well as energy production and conversion, thus promoting intestinal homeostasis (Muñoz et al., 2020; Shen et al., 2022). This can be justified by the presence of ALA in the HFHF diet, which may act as an energetic substrate that enables the production of SCFAs and the proliferation of this bacterial strain (Kim et al., 2020). The HFHF + CF group presented *Lachnospiraceae sp.* predominance, which assisted to reduce primary bile acids through conversion into SCFAs and/or secondary bile acids, could lead to intestinal homeostasis (Wan et al., 2022). The AIN-93M group presented the predominance of *Allobaculum sp.*, a bacterium that has a negative correlation with inflammation, insulin resistance, and obesity (Thomaz et al., 2020; Wang et al., 2020; Zhao et al., 2023).

The KEGG microbial metabolic pathway shows that the HFHF + CF upregulated the pathways associated with Thiamin (vitamin B1) salvage, which is fundamental in energy metabolism, being a cofactor of several enzymes (Jenkins et al., 2007; H. J. Kim et al., 2020; *Thiamine Salvage II | Pathway - PubChem*, n.d.); L-methionine biosynthesis III is an essential proteinogenic amino acid, which helps in protein synthesis, DNA methylation, rRNA and xenobiotics, besides promoting the cysteine, phospholipid, and polyamine biosynthesis (*L-Methionine Biosynthesis III | Pathway - PubChem*, n.d.; Mota-Martorell et al., 2021); TCA cycle VII (acetate-producers) is a catabolic pathway by aerobic respiration. Acetic acid bacteria oxidize ethanol into acetate (Kim et al., 2022; TCA Cycle VII (Acetate-Producers) | Pathway - PubChem, n.d.).

The limitation referred to the DNA sequencing analysis, performed only after treatment, since the sequencing performed both before and after the treatment is important for obtaining more reliable data about the bacterial community. Then, new studies should be conducted to elucidate that gap.

5. Conclusion

Chia flour consumption associated with a HFHF diet improved probiotic bacterial strain proliferation, immune system, SCFAs synthesis, intestinal morphology, and cecal pH content, and reduced body weight. The results obtained in the present study evidence the functional potential of chia flour to modulate intestinal health, and it is an alternative to control metabolic diseases caused by the consumption of unbalanced diets.

Supplementary Material

Table S1. Sequencing data in baseline at the end of the 10-week treatment, according to each group.

Treatment	Good's coverage	Raw Sequences	After Filtering and Cleaning		After Normalization	
		Reads	Reads	OTUs	Reads	OTUs
AIN-93M	0.99 ± 0.001	27923 ± 4549	21447 ± 3440	353 ± 43	17717 ± 13	352 ± 44
HFHF	0.99 ± 0.001	26414 ± 5420	20287 ± 3868	336 ± 51	17711 ± 11	357 ± 47
HFHF+CF	0.99 ± 0.004	26664 ± 8087	19397 ± 5770	377 ± 58	17717 ± 18	404 ± 35

Values expressed as average ± standard deviation, n=10 animals/group. AIN-93M: standard diet; HFHF: high-fat and high-fructose diet; HFHF+CF: HFHF with chia flour diet; OTUs: Operational Taxonomic Units.

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6.2 Artigo 2: CHIA OIL (*Salvia hispanica* L.) IMPROVES INTESTINAL MICROBIOTA AND MORPHOLOGY, PROPIONIC ACID, AND IGA CONCENTRATIONS, AN *IN VIVO* STUDY

ABSTRACT

Aim: To investigate the effects of chia oil on short-chain fat acids content, immunoglobulin A (IgA) levels, intestinal permeability and morphology, and gut microbiota in *Wistar* rats fed a high-fat and high-fructose diet (HFHF). **Methods:** *Wistar* rats were separated into two groups and received diets: standard diet (AIN-93M, n = 10) and HFHF (n = 20) during eight weeks (phase I, metabolic disorders induction). After that, we maintained the AIN-93M group, while the HFHF group was divided into two: HFHF (n = 10) and HFHF plus chia oil (HFHF+CO) group (n = 10) for ten weeks (phase II, chia oil treatment). **Results:** Chia oil consumption increased alpha-linolenic acid intake, IgA levels, propionic acid production, cecum weight, goblet cell number, thickness, depth of intestinal crypts, and the circular and longitudinal colon muscle layers and decreased cecum pH content. No change was observed in the alpha and beta diversity between the HFHF and HFHF+CO groups. The HFHF+CO diet increased the relative abundance of *Lactobacillus sp., Faecalibacterium sp*., and *Erysipelatoclostridium sp.* genera, compared to the AIN-93M group. No difference was observed in the intestinal permeability among the groups. **Conclusion:** Consumption of chia oil increases propionic acid and IgA concentrations and improves intestinal microbiota and morphology of rats fed a HFHF diet.

Keywords: short-chain fatty acids; α-diversity; β-diversity; goblet cells; correlation analysis; intestinal health.

1. INTRODUCTION

Unhealthy Western lifestyles, increased consumption of diets rich in saturated fat, fructose corn syrup, salt, and sugar, and lack of physical activity contribute to the development of non-communicable diseases (NCDs) worldwide [1].

One of the metabolic disorders caused by Western diets is the activation of chronic inflammation and immune system imbalance, which culminates in a pro-inflammatory process [2, 3]. This food pattern leads to intestinal disorders known as dysbiosis, which is a homeostatic breakdown in the intestine's physical-functional mucosa, presenting a higher abundance of pathogenic bacteria and lower production of short-chain fatty acids (SCFAs) [4]. This condition causes an increase in intestinal barrier permeability, damage to epithelial cells, inflammation,

and low nutrient absorption [5]. Changes to healthy eating habits, such as adding foods rich in bioactive compounds, can prevent or reduce intestinal dysbiosis [6, 7].

The chia seed is composed of 34%, of which 7.6% is palmitic acid, 2.7% stearic acid, 7.5% oleic acid (ω-9), 19.9% linoleic acid (ω-6), 62.3% linolenic acid (ω-3), and phenolics [8–10]. Studies have demonstrated that adding chia oil to a high-fat and high-fructose (HFHF) or high-fat diets improved glucose and insulin tolerance [9], liver health, the antioxidant system, and fatty acid oxidation [10] in *Wistar* rats and inflammation in C57BL/6 mice [11]. Besides, ω-3 consumption can contribute to intestinal homeostasis [12], since it could restore the SCFAs content through the microbial community and improve intestinal tight junctions, permeability, and morphology [13–16]. Thus, the oil from chia, a source of alpha-linolenic acid (ALA), could modulate intestinal health.

The present study upholds the hypothesis that chia oil promotes the proliferation of beneficial bacterial strains, increases SCFAs synthesis, reduces cecal pH, improves intestinal morphology and permeability, IgA production, and anti-inflammatory activity in *Wistar* rats with metabolic disorders. Therefore, the present study aimed to investigate the effects of chia oil on intestinal permeability, cecal pH, IgA, SCFAs concentrations, intestinal morphology, and microbial community in *Wistar* rats fed a high-fat and high-fructose (HFHF) diet.

2. MATERIAL AND METHODS

2.1. Chia oil

Chia seeds produced in Rio Grande do Sul, Brazil were ground in a blender (Philips Walita® model RI2035 500W) for 2 min at high speed. Chia flour was cold pressed in a hydraulic press (Carver Laboratory Press, ModelC 22400-36 - USA) with an 8% extraction efficiency rate. The oil was filtered and centrifuged at 1,050 x g, for 15 min at 7 °C. The extracted oil was stored in a photoprotector amber glass at -20°C, to avoid fatty acid oxidation until the weekly experimental diet preparation.

2.2. Animals and Experimental Diets

Male *Wistar* rats (*Rattus norvegicus*), 45–50-day old (n=30), were kept in individual stainless-steel cages and received deionized water and diets *ad libitum*, under controlled temperature (22°C ± 2°C; 12 h light/dark cycle). The study was approved by the Ethics Committee for the Use of Animals of the Federal University of Viçosa (CEUA/UFV), protocol No. 89/2018. All experimental procedures with animals were carried out in accordance with Directive 86/609/EEC of November 24, 1986, and the ethical principles for animal studies.

The sample size calculation was performed according to Marinelli et al. [18], which induced metabolic disorders in the animals by consuming a HFHF diet and treated with the addition of chia oil (HFHF+CO). The rats were randomized by body weight, and ten animals were considered in each group. In Phase I, the animals consumed a standard diet (AIN-93M; n = 10; initial body weight 156.0 ± 17.0 g) [18] or HFHF diet (20% fructose and 34% saturated fat; n = 20; initial body weight: 156.5 ± 17.9 g) [19], for eight weeks, to induce metabolic disorders. Then, the phase II (ten weeks), the AIN-93M group (n=10) was kept, and the HFHF group (n = 20) was subdivided into two: HFHF (n=10), which the diet was maintained, and HFHF plus chia oil (HFHF+CO) group, which the animals (n=10) were fed HFHF diet plus 4% of chia oil (soy oil was replaced by 4% chia oil) (**Table 1**).

Table 1. Experimental diets (g/kg)

Ingredients	**AIN-93M**	**HFHF**	**HFHF + CO**
Albumin*	136.40	136.40	136.40
Maize starch	463.50	135.00	135.00
Dethroned Starch	155.00	45.00	45.00
Sucrose	100.00	28.60	28.60
Lard	-	310.00	310.00
Soy oil	40.00	40.00	-
Chia oil	-	-	40.00
Fructose	-	200.00	200.00
Cellulose	55.80	55.80	55.80
Mineral Mix	35.00	35.00	35.00
Vitamin Mix	10.00	10.00	10.00
L-cystine	1.80	1.80	1.80
Choline Bitartrate	2.50	2.50	2.50
Macronutrients			
Carbohydrate (%)	77.40	30.10	30.10
Protein (%)	12.90	9.10	9.10
Lipid (%)	9.70	59.80	59.80

Caloric density (kcal/g)	3.70	5.30	5.30
	Fatty acids (g/kg)		
ω-3**	3.30	10.20#	31.80#
ω-6**	20.20	58.80#	46.50#
Ratio ω-6:ω-3	6.12:1	5.77:1#	1.46:1#

* The diets were calculated to provide 12 g of protein (88%). 100 g-1 of diet [10]. ** The chia oil ω-3 and ω-6 were determined by gas chromatography [10]. # [47] AIN-93M [18]: standard diet; HFHF [19]: high-fat and high-fructose diet; HFHF+CO: HFHF diet plus 4% of chia oil (soy oil was replaced by 4% chia oil).

Food intake and body weight were measured weekly. The weekly ω-3 and ω-6 consumption was multiplied by the ω content in 1 kg diet and divided by 1000. The animals were anesthetized with isoflurane 5% (Isoforine, Cristália®) and euthanized by cardiac puncture. The cecum was weighed, and the ascending large colon and cecal content were collected, weighed, and frozen (-80 °C) with liquid nitrogen. The colon was stored in a formalin solution for histological analysis. The epididymal, abdominal, and retroperitoneal adipose tissues were collected to calculate the total body adiposity (%) [20]. Body mass index (BMI) was obtained as body weight/height² [21].

2.3. Gut Health

2.3.1 Intestinal Permeability, Cecal pH, and Immunoglobulin A (IgA)

Intestinal permeability was performed in animals after 12 h of fasting, received a solution with 0.2 g - lactulose and 0.1g – mannitol (2 mL) (Sigma-Aldrich, São Paulo/SP, Brazil) by gavage. The rats were kept in metabolic cages and fasted for 5 h, and the urine was collected after 24-h and stored at -80°C [22]. This urine was filtered (0.45 mm Millipore), and the analyses were performed at 55°C, a 1920 psi pressure under isocratic conditions, by high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan). The mobile phase was 0.005 mM sulfuric acid in the water, with a flow of 0.6 ml/min, and an aliquot of 20 µL of the sample was injected [23]. The lactulose and mannitol levels were converted into g/L, to obtain the lactulose/mannitol ratio.

Cecal faeces (0.4 g) were homogenized into 4 mL of distilled water, and the pH was measured using a glass electrode [24].

The IgA levels were analyzed by an enzyme-linked immunosorbent kit (ELISA - BT LAB, China). Cecal faeces (0.2 g) were resuspended into 800 µl phosphate buffer and homogenized. The results were read in an ELISA reader (Multiskan Microplate Photometer, Thermo Fisher Scientific, MA, USA) at 450 nm, and expressed in ng/ml [25].

2.3.2 Concentration of Short Chain Fatty Acids (SCFAs)

Cecal faeces (0.4 g) were added into 1 mL Milli-Q water, homogenized, and centrifuged at 12,000 x g for 10 min. The supernatant was collected using the Siegfried, Ruckemann, and Stumpf methodology (1984) [26]. The analysis was carried out by HPLC, Shodex RI-101 refractive index (IR) detector, coupled to Dual Dionex Ultimate 3000 chromatograph and ROA Rezex Phenomenex exclusion column ion, 300 × 7.8 mm at 40°C. Sulfuric acid (5 mM) with 0.7 mL/min and flow rate was used in the mobile phase. Acetic, propionic, and butyric acids were used as standards for the calibration curve, and the results were expressed as mM.

2.3.3 Histological Analyses

Crypt thickness, crypt height, circular muscle layer width, and longitudinal muscle layer width were quantified by Image Pro-Plus® (version 4.5, Media Cybernetics, Rockville, USA); and the goblet cell number, by the ImageJ® software (National Institutes of Health, USA). An automatic microtome (Reichert-Jung®, Germany) was used to semi-serial histological sections of 3 µm thick colon fragments, according to the hematoxylin-eosin technique. The light microscope (Olympus AX 70 TRF, Tokyo, Japan) was used to analyze the pictures, and 20 random fields per animal were selected and measured and quantified [27].

2.3.4 DNA Extraction and Sequencing

The cecal feaces were lysed by mechanical disruption using glass beads and phenol-chloroform and subsequent treatment with RNAse [28]. The samples were loaded onto an Illumina flow cell by Illumina MiSeq platform for paired-end sequencing reactions in Argonne National Laboratory (Lemont, Illinois, USA). The PCR amplicon libraries made by 16S rRNA gene, targeting the hypervariable V4-region, with primers, 515F (5'GTGYCAGCMGCCGCGGTAA3') and 806R (GGACTACNVGGGTWTCTAAT3') and barcoded primer set adapted for Illumina MiSeq platform (Illumina, San Diego, California, USA) [29]. Determined 151bp x 12bp x 151bp in MiSeq run to sequencing amplicons [30].

The raw data were deposited in Sequence Read Archive (SRA) on the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sra) by accession number PRJNA926959. The data were analyzed by the Mothur software (v. 1.47.0) [31]. The R1 and R2 paired-end reads were joined, and sequences smaller than 150 or larger than 300 bp were deleted. The Chimera sequences were removed by UCHIME [32]. SILVA database

v.138.1 was used to align the sequences with the 16S rRNA, and the taxonomic classification was conducted [33]. Sample coverage was assessed by the Good's coverage estimator (Bacteria > 97%). The Operational Taxonomic Units (OTUs) were grouped with a 97% sequence similarity cut-off. Sample normalization was carried out to correct sampling bias [32]. α and β - diversity and the relative abundance of OTUs were standardized [34].

2.5. Statistical Analyses

Multiple comparisons for false discovery rate (FDR) were used to normalize the gut microbiota data using Metagenomic Profiles (STAMP) software (version 2.1.3). IBM SPSS Statistics software (version 20.0) was used to detect the significant differences among the groups; parametric were run by One-way analysis of variance (ANOVA) followed by Duncan *post-hoc*, or non-parametric data by Kruskal-Wallis followed by Bonferroni correction.

The PCoA used the Jaccard dissimilarity index, carried out by the Past 4.12 software. The β-diversity values were performed by Pairwise Permutational by Multivariate Analysis of Variance (PERMANOVA). Graphics of Phylum; Genera; Firmicute/Bacteriodetes ratio; Chao, Shannon, and Simpson indexes; and Pearson's Heatmap were performed by GraphPad Prism (version 9.0).

Kolmogorov-Smirnov was used to test the data normality of intestinal permeability, morphologic markers, murinometric parameters, food intake, cecal pH, IgA, and SCFAs concentrations (GraphPad Prism, version 9.0). ANOVA and *post-hoc* Newman-Keuls test were used to parametric data, and non-parametric data were performed by Kruskal-Wallis and *post-hoc* of Dunn's tests. Pearson was used to correlate intestinal health markers with bacteria genera presenting a high relative abundance in HFHF+CO. All analyses were performed at a significant level of 5%.

3. RESULTS

The food intake decreased in the HFHF and HFHF+CO groups and increased caloric density compared to the AIN-93M group. The treatment HFHF+CO reduced body weight, adiposity percentage, and BMI compared to the HFHF group. Adding chia oil into HFHF increased the ω-3 intake compared to the groups AIN-93M and HFHF and reduced ω-6 intake (HFHF+CO group) compared to the HFHF group (**Table 2**).

Table 2. Effect of the chia oil on murinometric variables and food consumption.

Variables	AIN-93M	HFHF	HFHF + CO
Final body weight (g)	405.50 ± 23.61^{b}	487.10 ± 62.16^{a}	387.50 ± 37.56^{b}
Body mass index (kg/m^2)	0.61 ± 0.05^{b}	0.71 ± 0.07^{a}	0.63 ± 0.05^{b}
Total Adiposity (%)	4.17 ± 1.61^{b}	7.78 ± 1.92^{a}	5.14 ± 0.98^{b}
Total Food Intake (g)	1367.00 ± 69.07^{a}	1046.00 ± 130.20^{b}	1011.00 ± 64.26^{b}
Caloric intake ($kcal.g^{-1}$)	4.85 ± 0.24^{b}	5.50 ± 0.68^{a}	5.26 ± 0.33^{a}
Weekly ω-3 (g)	0.45 ± 0.02^{c}	1.05 ± 0.10^{b}	3.17 ± 0.13^{a}
Weekly ω-6 (g)	2.77 ± 0.11^{c}	6.06 ± 0.63^{a}	4.63 ± 0.20^{b}

AIN-93M: control diet; HFHF: high-fat and high-fructose diet; HFHF+CO: HFHF diet plus 4% chia oil (soy oil was replaced by 4% chia oil). Newman-Keuls and Dunn's tests. n=10 animals/group. Different letters indicate a significant difference ($p < 0.05$), where the letter 'a' indicates the highest value.

Chia oil intake increased propionic acid and IgA levels, which were higher when compared to the other groups. Mannitol and lactulose ratio and butyric acid concentrations were similar among the groups. The HFHF+CO and HFHF groups presented the lowest acetic acid levels (**Table 3**).

Table 3. Chia oil activity on intestinal health variables.

Variables	AIN-93M	HFHF	HFHF + CO
SCFAs (mM)			
Acetic acid	21.36 ± 5.11^{a}	13.71 ± 2.90^{b}	12.22 ± 1.78^{b}
Propionic acid	4.58 ± 1.13^{b}	4.36 ± 1.55^{b}	13.61 ± 2.50^{a}
Butyric acid	2.51 ± 0.77^{a}	3.43 ± 0.96^{a}	2.86 ± 0.50^{a}
Cecum weight (g)	4.66 ± 0.83^{a}	3.58 ± 0.60^{b}	4.38 ± 0.65^{a}
Faeces pH	9.01 ± 0.40^{a}	9.17 ± 0.25^{a}	7.96 ± 0.94^{b}
IgA (ng/ml)	851.50 ± 106.20^{b}	786.50 ± 89.47^{b}	1084.00 ± 212.90^{a}

Goblet cells number	24.80 ± 0.88[a]	19.55 ± 2.66[b]	24.01 ± 1.56[a]
Crypts thickness (µM)	38.62 ± 3.15[a]	22.94 ± 3.22[b]	37.79 ± 4.40[a]
Crypts depth (µM)	201.20 ± 16.27[a]	167.10 ± 4.52[b]	200.70 ± 9.32[a]
Circular muscle layer width (µM)	125.70 ± 38.28[b]	80.97 ± 15.67[b]	231.80 ± 23.38[a]
Longitudinal muscle layer width (µM)	49.61 ± 7.86[b]	33.88 ± 4.02[c]	58.60 ± 5.09[a]
Mannitol/Lactulose urinary excretion (%)	1.79 ± 0.24[a]	1.85 ± 0.68[a]	1.65 ± 0.38[a]

AIN-93M: control diet; HFHF: high-fat and high-fructose diet; HFHF+CO: HFHF diet plus 4% chia oil (soy oil was replaced by 4% chia oil). Newman-Keuls and Dunn's tests. n=10 animals/group. Different letters indicate a significant difference ($p < 0.05$), where the letter 'a' indicates the highest value.

Regarding intestinal morphology, the HFHF+CO group increased crypt thickness, crypt depth, circular muscle layer width, longitudinal muscle layer width, goblet cell number, and cecum weight compared to the HFHF group. In addition, the HFHF+CO group increased circular and longitudinal muscle layers and decreased cecal pH compared to the other groups (**Table 3**; **Fig. 1**).

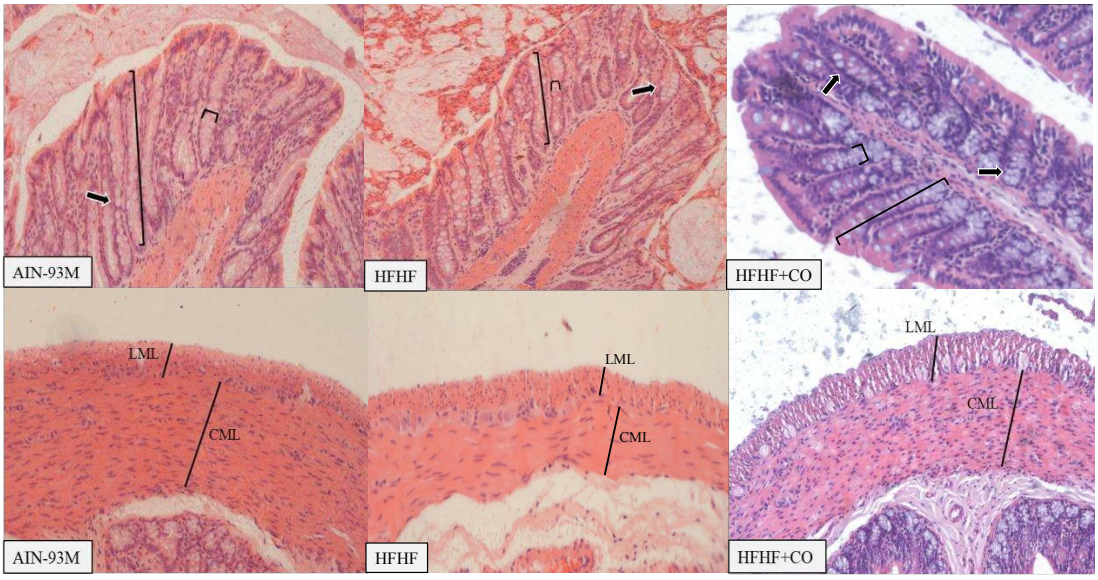

Figure 1. Chia oil effect on colonic morphology characteristics. n=10 animals/group. AIN-93M: standard diet; HFHF: high-fat and high-fructose diet; HFHF+CO: HFHF diet plus 4% chia oil (soy oil was replaced by 4% chia oil); CML, circular muscle layer; LML, longitudinal muscle layer. Black arrows refer to goblet cells in the crypt. Black brackets refer to the crypt's depth and width. Staining was carried out with hematoxylin and eosin. Objective: 10 × (100 µm).

The 16S rRNA gene generated 823.765 raw sequences, and 627.428 good-quality sequences were obtained after filtering and cleaning. The samples presented > 99% of coverage, which indicates good sequencing quality (**Supplementary Table S1**).

The scatterplot analysis (PCoA) accounted for approximately 51.01% of the dissimilarity in bacterial species composition (**Fig. 2a**). The groups showed differences in the distance metrics by PERMANOVA. The groups HFHF+CO and HFHF showed no difference in the bacterial community clustering compared to AIN-93M. The HFHF group did not differ from the HFHF+CO group.

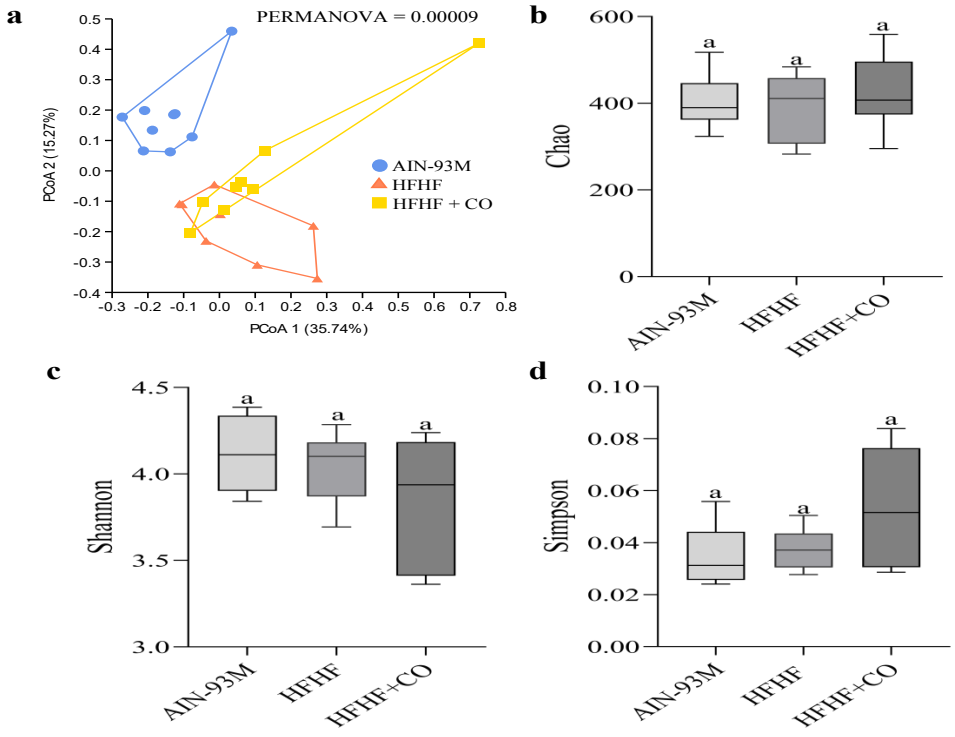

Figure 2. Chia oil consumption effect on beta and alpha diversity. (**a**) Beta diversity was estimated by PCoA based on Jaccard similarity distance of cecal microbial communities in adult *Wistar* rats fed a high-fat and high-fructose diet. Each dot refers to one animal, and the colors refer to the groups. The *p*-value among the groups was obtained by PERMANOVA; Alpha-diversity was estimated by the indices: (**b**) Chao, (**c**) Shannon, and (**d**) Simpson, ANOVA followed by *post-hoc* Ducan ($p<0.05$). n=10 animals/group. AIN-93M: standard diet; HFHF: high-fat and high-fructose diet; HFHF+CO: HFHF diet plus 4% of chia oil (soy oil was replaced by 4% chia oil).

In the α-diversity analyses, the Chao, Shannon, and Simpson indexes did not present any difference among the experimental groups (**Fig. 2b, 2c and 2d**). The taxonomic analyses revealed the occurrence of 18 phyla, 28 classes, 72 orders, 108 families, and 211 genera. The Firmicutes/Bacteroidetes ratio was higher in the HFHF+CO group than in other groups (**Fig. 3a**). All groups presented nine predominant phyla, such as Firmicutes (HFHF+CO: 73%; AIN-93M and HFHF: 69%), Bacteroidetes (HFHF+CO: 11%; AIN-93M: 15%; HFHF: 17%), Actinobacteria (HFHF+CO: 5.4%; AIN-93M: 3.7%; HFHF: 3.5%), Desulfobacterium (HFHF+CO: 3.5%; AIN-93M: 4.3%; HFHF: 4.7%) and Proteobacteria (HFHF+CO: 1.9%; AIN-93M: 2.7%; HFHF: 2.4%) (**Fig. 3b**).

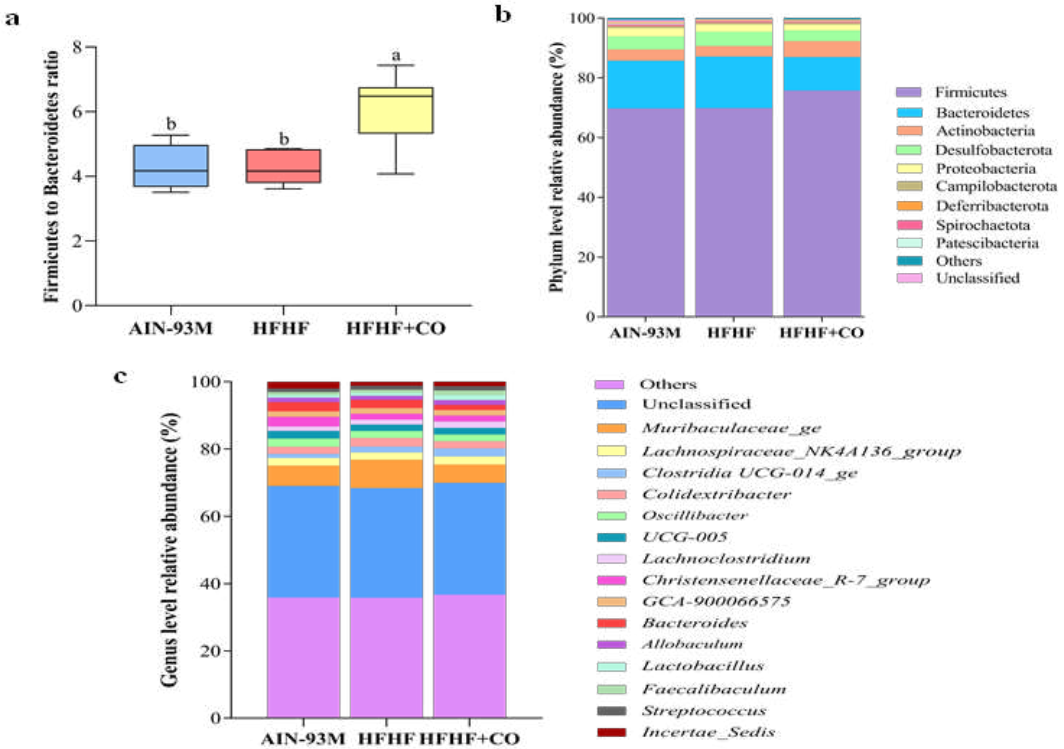

Figure 3. Chia oil consumption effect of phylum and genera relative abundance in microbiota. (**a**) Firmicutes/Bacteroidetes ratio; (**b**) Bacterial composition at phylum level; (**c**) Bacterial composition at the genera level. n=10 animals/group. Phyla with abundance >0.3% and genera with abundance >1.3%. Data were analyzed by Dunn's with FDR and Bonferroni corrections. AIN-93M: standard diet; HFHF: high-fat and high-fructose diet; HFHF+CO: HFHF diet plus 4% of chia oil (soy oil was replaced by 4% chia oil).

The groups comprised 15 predominant genera, such as *Muribaculaceae_ge sp.* (HFHF+CO: 5.4%; AIN-93M: 6%; HFHF: 8%) (**Fig. 3c**). *Lachnospiraceae NK4A136_ge sp.* (HFHF+CO: 2.4%; AIN-93M: 2.3%; HFHF: 2.1%), *Clostridia_UCG-014 sp.* (HFHF+CO: 2.4%; AIN-93M: 1.2%; and HFHF: 1.7%), *Colidextribacter sp.* (HFHF: 2.5%; AIN-93M and HFHF+CO: 2.1%) and *Oscillibacter sp.* (AIN-93M: 2.4; HFHF+CO and HFHF: 2.0%) (**Fig. 3c**).

The HFHF+CO group showed a higher abundance of Firmicutes, *Lactobacillus sp.,* and *Faecalibacterium sp.,* than the other groups, while *Erysipelatoclostridium sp*., compared to the AIN-93M group. Furthermore, the HFHF+CO group presented a lower abundance of Bacteroidetes and *Rikenellaceae RC9 sp.,* compared to HFHF and AIN-93M, and a lower abundance of *Atopobiaceae sp.* and *Alphaproteobacteria sp.,* compared to the AIN-93M group.

The Pearson correlation analyses revealed that the propionic acid was positively correlated with Firmicutes*, Lactobacillus sp., Erysipelatoclostridium sp.,* and IgA, while this acid was inversely correlated with pH and goblet cell number. Firmicutes was positively correlated with *Lactobacillus sp.* and IgA; and inversely correlated with pH. *Lactobacillus sp*. was correlated with Firmicutes, longitudinal muscle layer width, and circular muscle layer width. The goblet cell number was positively correlated with crypt thickness, crypt depth, and circular muscle layer width; and negatively correlated with adiposity. A positive correlation was observed between Crypt depth and crypt thickness. Crypt thickness positively correlated with circular muscle layer width, whereas both negatively correlated with BMI. The longitudinal muscle layer width positively correlated with the circular muscle layer width. The circular muscle layer width positively correlated with IgA concentrations. Finally, BMI positively correlated with total weight, and the last positively correlated with adiposity (**Fig. 4**).

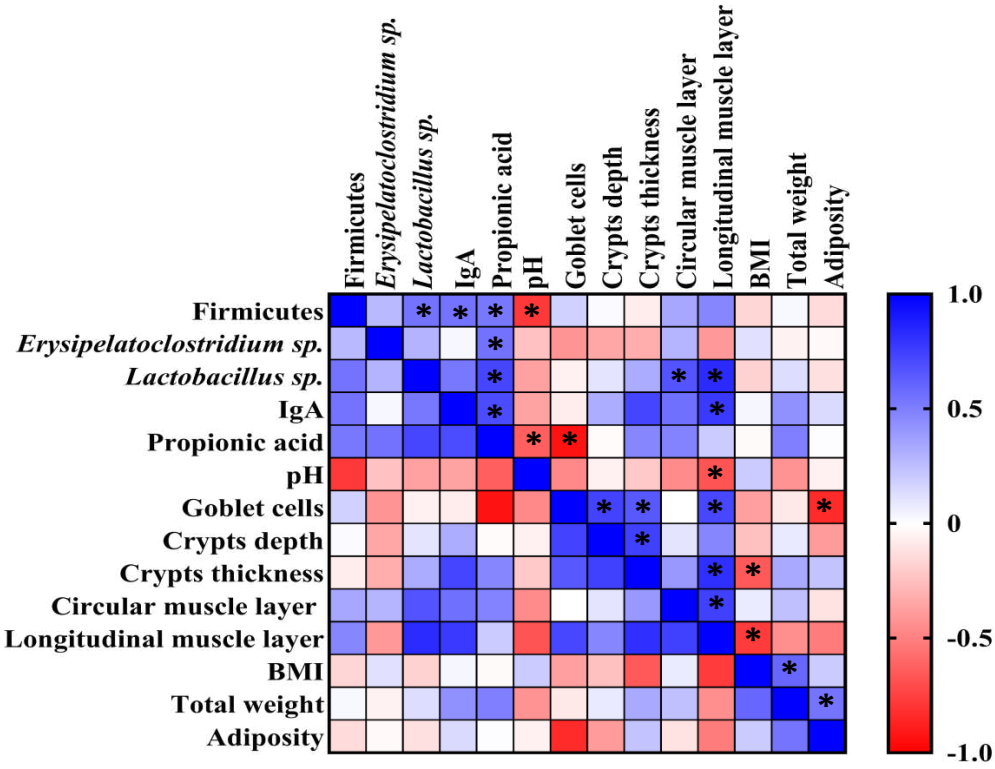

Figure 4. Pearson's correlation among murinometric markers, microorganisms (gender level), and intestinal health variables. Blue and red colors indicate positive and negative correlations, respectively. Correlation matrix *($p < 0.05$). n=10 animals/group. BMI, body mass index; IgA, immunoglobulin A. Specific bacteria, such as Firmicutes, *Lactobacillus sp*., and *Erysipelatoclostridium sp.,* were selected due to their increased abundance in the chia oil group.

4. DISCUSSION

This study sheds light on chia oil properties beneficial to the intestinal health of animals with metabolic disorders caused by the consumption of a HFHF diet. In this research, chia oil, rich in ω-3 [9, 10], increased the propionic acid concentrations and decreased cecal pH. Probably chia oil provided energy to the colonocytes, thus enhancing IgA production, and promoting the proliferation of beneficial bacterial strains, such as Firmicutes phylum, *Lactobacillus sp*. *Faecalibacterium sp*. and *Erysipelatoclostridium sp.* genera. Chia oil did not promote changes in the β and α diversity. However, chia intake increased the relative abundance of Firmicutes/Bacteroidetes ratio, and reduced pathogenic bacteria such as *Rikenellaceae RC9 sp.*, *Atopobiaceae sp*., and *Alphaproteobacteria sp.* Besides, chia oil improved intestinal morphology and decreased BMI, body weight, and total body adiposity percentage.

The HFHF+CO and HFHF groups showed the highest energy consumption and the lowest food intake. This could be justified by the high energy density of the fat in the HFHF diet, which delays the digestion process and prolongs satiety [9, 20]. The animals that received HFHF+CO consumed higher ω-3 content than those that received only the HFHF diet. It could be attributed to the chia oil composition, which has higher ω-3 and lower ω-6 concentrations than soy oil [9, 10]. HFHF+CO diet changed the body composition by decreasing adiposity tissue, body weight, and BMI. This may be explained by a high amount of ω-3 in the diet, which modulates the expression of genes involved in lipid metabolism and the synthesis of anti-inflammatory molecules [35, 36]. Moreover, ω-3 intake could modulate leptin levels, an adipokine that regulates hunger and energy expenditure, reducing food intake [37, 38]. Another explanation is that chia oil ingestion could improve intestinal morphology. It is known that colonocytes act as a functional intestinal barrier, preventing the passage of pathogens and lipopolysaccharides and protecting against a pro-inflammatory environment. Pearson's correlation evidenced that the goblet cell number, crypt thickness, and longitudinal muscle layer were inversely correlated with murinometric variables, corroborating the proposed justification.

The groups demonstrated similar species richness and diversity, and dominance of gut microbiota after ten weeks. The β-diversity analysis revealed that only animals from the AIN-93M group presented differences in the gut bacterial community clustering. However, the HFHF+CO group exhibited a lower abundance of Bacteroidetes and a higher abundance of Firmicutes phylum, which led to a higher Firmicutes/Bacteroidetes ratio than the control groups. Some studies have demonstrated that the increased Firmicutes/Bacteroidetes ratio is correlated with obesity [39–41]. However, this can be explained by the increased *Lactobacillus sp*. relative abundance in the HFHF+CO group, which belongs to the firmicutes phylum. Nevertheless, it is a probiotic bacteria that acts in anti-inflammatory pathways, including suppressing the TLR4/NF-κB signaling, and anti-obesogenic, which could promote lipolysis and activate the brown adipose cell [42, 43]. Therefore, it could explain the loss of body weight, adiposity, and BMI in the HFHF+CO group.

Animals fed HFHF+CO decreased fecal pH and increased cecal weight and propionic acid concentration, which indicates a proliferation of probiotic strains, such as *Lactobacillus sp., Faecalibacterium sp.* And *Erysipelatoclostridium sp.* (belonging to the Firmicutes phylum). In addition, chia oil consumption increased the goblet cell number, crypt thickness, crypt depth, circular muscle layer, and longitudinal muscle layer. The high amount of ω-3 in the HFHF+CO could be considered a substrate for probiotic bacteria, and their propionic acid synthesis. This acid can be used as an energetic substrate by colonocytes, decreasing cecal pH and contributing

to intestinal homeostasis. The correlation analyses demonstrated that *Lactobacillus sp*. and *Erysipelatoclostridium sp.* were positively correlated with propionic acid, which confirms a probiotic action of these bacteria. Although the intestinal permeability remained constant in all groups, the HFHF+CO group increased propionic acid, IgA, and the goblet cell number, since IgA protects the gut microbiota against pathogenic bacteria and their toxins [44, 45], while goblet cells increase mucus production [46]. In addition, the increased IgA levels probably reduced *Rikenellaceae RC9 sp.* (Bacteroidetes phylum), *Atopobiaceae sp.* (Actinobacteria phylum), and *Alphaproteobacteria sp.* (Proteobacteria phylum). The Person's correlation exhibited the highest Firmicutes abundance, propionic acid levels, and circular muscle layer width, increased IgA content, which could improve the immune system.

5. CONCLUSION

The consumption of chia oil associated with a HFHF diet promoted intestinal health in the animals, mainly by increasing the propionic acid levels, correlated with the IgA levels and cecal pH. These modifications, in turn, modulated the intestinal microbiota. All these alterations lead to an improvement in intestinal morphology and murinometric variables. Therefore, it is possible to conclude that chia oil, rich in ω-3, shows a great potential as a functional food and food product. Our results encourage further research to evaluate the effects of chia oil on intestinal health, aiming to optimize chia oil doses and their impacts on human consumption (**Fig. 5**).

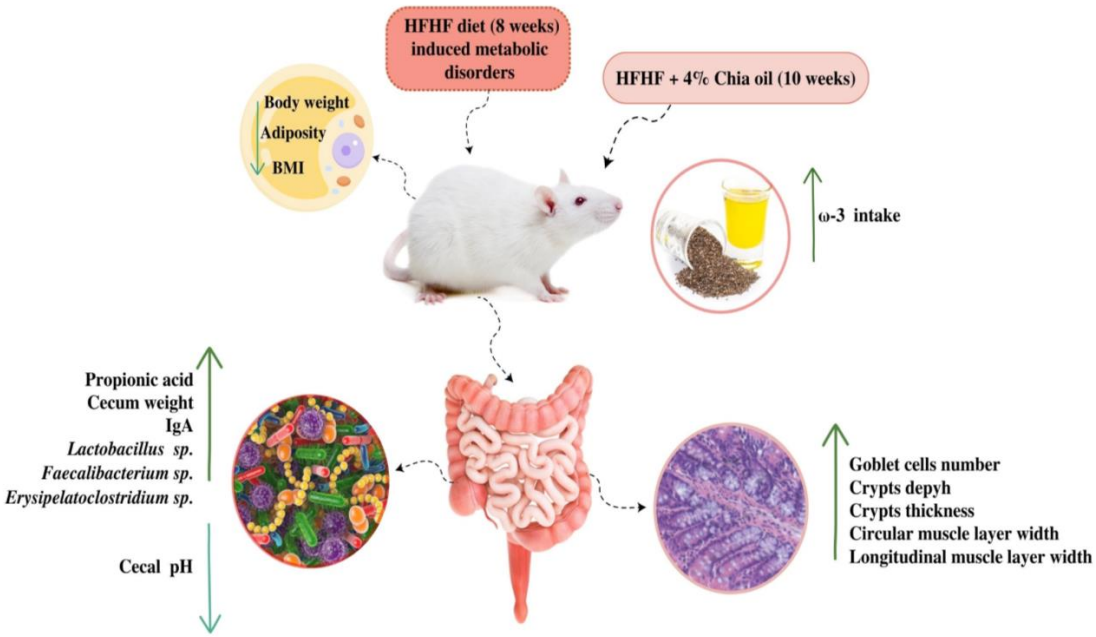

Figure. 5 Chia oil intake on the intestinal health of *Wistar* rats with metabolic disorders. The consumption of HFHF promotes metabolic changes. Nonetheless, chia oil modulated the bacterial community and improved intestinal morphology. AIN-93M: standard diet; HFHF: high-fat and high-fructose diet; HFHF+CO: HFHF diet plus 4% of chia oil (soy oil was replaced by 4% chia oil); BMI: body mass index; BW: body weight; IgA: immunoglobulin A; SCFAs: short-chain fatty acids.

Supplementary Material

Table S1. Sequencing data in baseline at the end of treatment.

Treatment	Good's coverage	Raw Sequences	After Filtering and Cleaning		After Normalization	
		Reads	Reads	OTUs	Reads	OTUs
AIN-93M	0.99 ± 0.00	27923 ± 4549	21346± 3395	350 ± 44	17572 ± 14	356 ± 43
HFHF	0.99 ± 0.00	26414 ± 5420	20322 ± 3843	332 ± 53	17568 ± 10	342 ± 55
HFHF+CO	0.99± 0.00	28038 ± 7379	21073 ± 5511	295 ± 88	17568 ± 12	315 ± 80

Values were represented by average ± standard deviation. AIN-93M: standard diet group; HFHF: high-fat and high-fructose diet; HFHF+CO: HFHF diet plus 4% chia oil (soy oil was replaced by 4% chia oil); OTUs: Operational Taxonomic Units.

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7 CONCLUSÃO GERAL

O consumo da farinha e do óleo de chia aumentaram a ingestão de ω-3 e reduziram o consumo de ω-6 em ratos alimentados com dieta HFHF. Entretanto, a permeabilidade intestinal não apresentou alterações significativas entre os grupos experimentais.

O consumo da farinha de chia reduziu o peso corporal, o índice de massa corporal, o índice de Simpson e o pH das fezes do ceco, aumentando o peso do ceco, o teor de imunoglobulina A (IgA), o índice de Shannon, a concentração de ácido acético e butírico, além de aumentar a espessura da camada muscular longitudinal, o número de células caliciformes, assim como altura e espessura das criptas intestinais. Além disto, promoveu maior abundância relativa de *Monoglobus sp., Lachnospiraceae sp.* e *Prevotellaceae sp.,* e menor abundância de Spirochaetota, Patescibacteria, *Alphaproteobacteria sp.*, *Treponema sp.* e *Erysipelatoclostridium sp*. Dessa forma, a farinha de chia demonstrou ser um potencial modulador intestinal, visto que os animais alimentados com a farinha de chia apresentaram dissimilaridade da comunidade bacteriana (β diversidade) quando comparado com os grupos controles.

O consumo de óleo de chia promoveu a redução de peso, índice de massa corporal, adiposidade corporal e pH das fezes do ceco, enquanto aumentou a produção de ácido graxo propiônico, de IgA e do peso do ceco. Além disso, o consumo de óleo de chia foi capaz de aumentar a espessura da camada muscular longitudinal e circular, o número de células caliciformes, a altura e espessura das criptas intestinais. Além disso, observou-se que a relação Firmicutes/Bacteroidetes aumentou frente ao consumo dessa matriz alimentar. As bactérias mais abundantes foram *Lactobacillus sp.* e *Erysipelatoclostridium sp.,* enquanto as menos abundantes foram *Rikenellaceae RC9 sp.*, *Atopobiaceae sp.*, e *Alphaproteobacteria sp.*

Diante disso, concluímos que a farinha e óleo de chia modulam o microbioma intestinal, atuando de maneira distinta na microbiota intestinal, mas de forma positiva, melhorando as variáveis murinométricas (peso corporal, índice de massa corporal, adiposidade), morfológicas (altura e espessura da criptas, largura do tecido muscular circular e longitudinal, número de células caliciformes, e peso do ceco) e funcionais (IgA, ácidos graxos de cadeia curta, pH do ceco e comunidade bacteriana) em ratos *Wistar* machos adultos submetidos a alterações metabólicas por meio do consumo de dieta rica em gordura saturada e frutose.

8 CONSIDERAÇÕES FINAIS

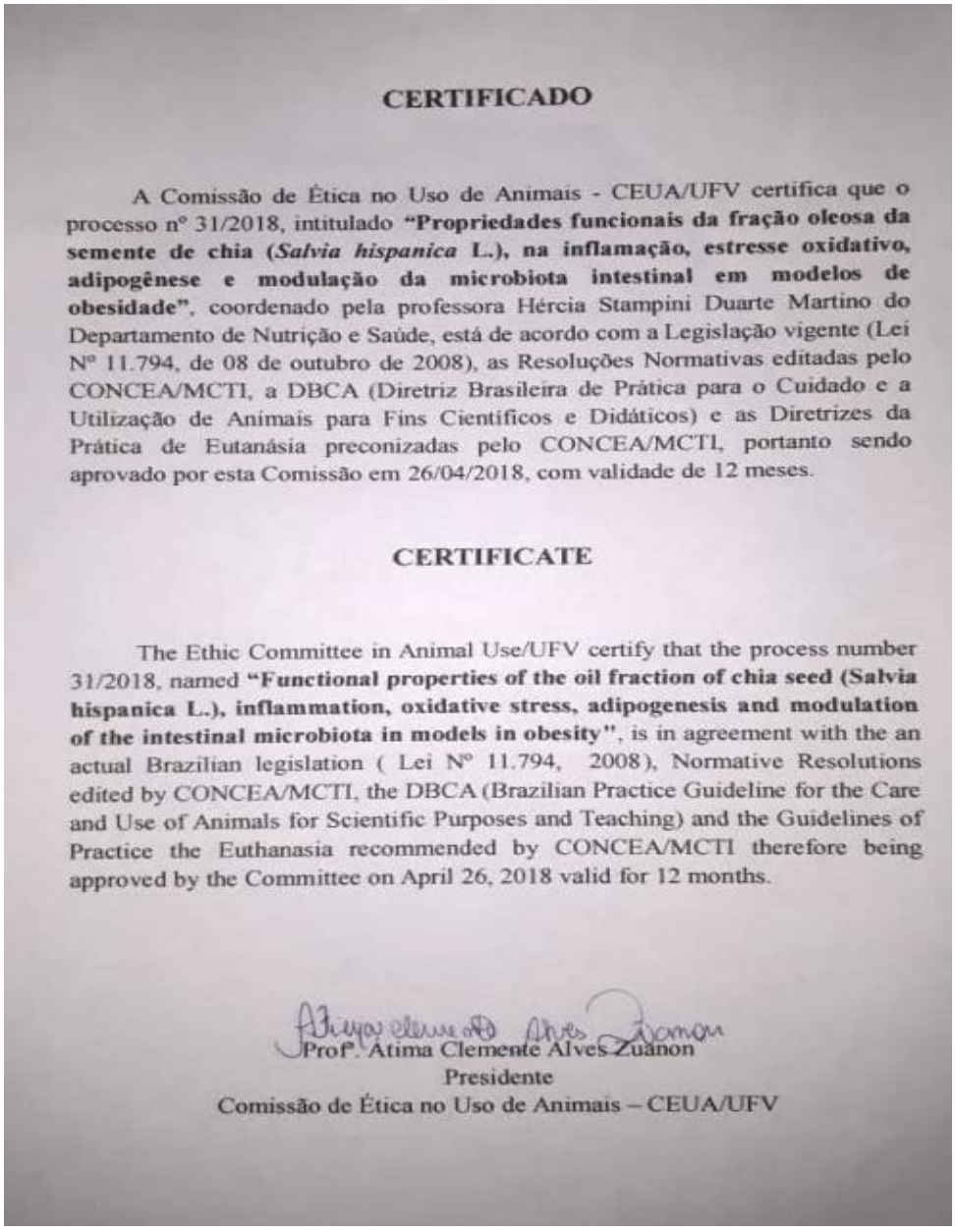
No intuito de avaliar a eficiência do óleo e da farinha como alimento funcional em condições saudáveis, o óleo e a farinha de chia além de serem ofertados na dieta HFHF, poderiam ter sido ofertados em animais alimentados com dieta saudável, AIN-93M.

Sugere-se a possibilidade de continuidade da pesquisa, no entanto, voltada para a ação da farinha e do óleo de chia na função neural, interligando a mecanismos relacionados ao eixo intestino-cérebro-tecido adiposo, visto à inexistência de estudos que abordam essa temática.

Mostra-se promissor a elaboração de estudos futuros voltados para investigação da ação do consumo de farinha e óleo de chia na saúde intestinal de humanos saudáveis e com disfunções metabólicas.

ANEXOS

ANEXO A – Certificado da Comissão de Ética no Uso de Animais – CEUA/UFV (1°Artigo)



CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 31/2018, intitulado **"Propriedades funcionais da fração oleosa da semente de chia (*Salvia hispanica* L.), na inflamação, estresse oxidativo, adipogênese e modulação da microbiota intestinal em modelos de obesidade"**, 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 26/04/2018, com validade de 12 meses.

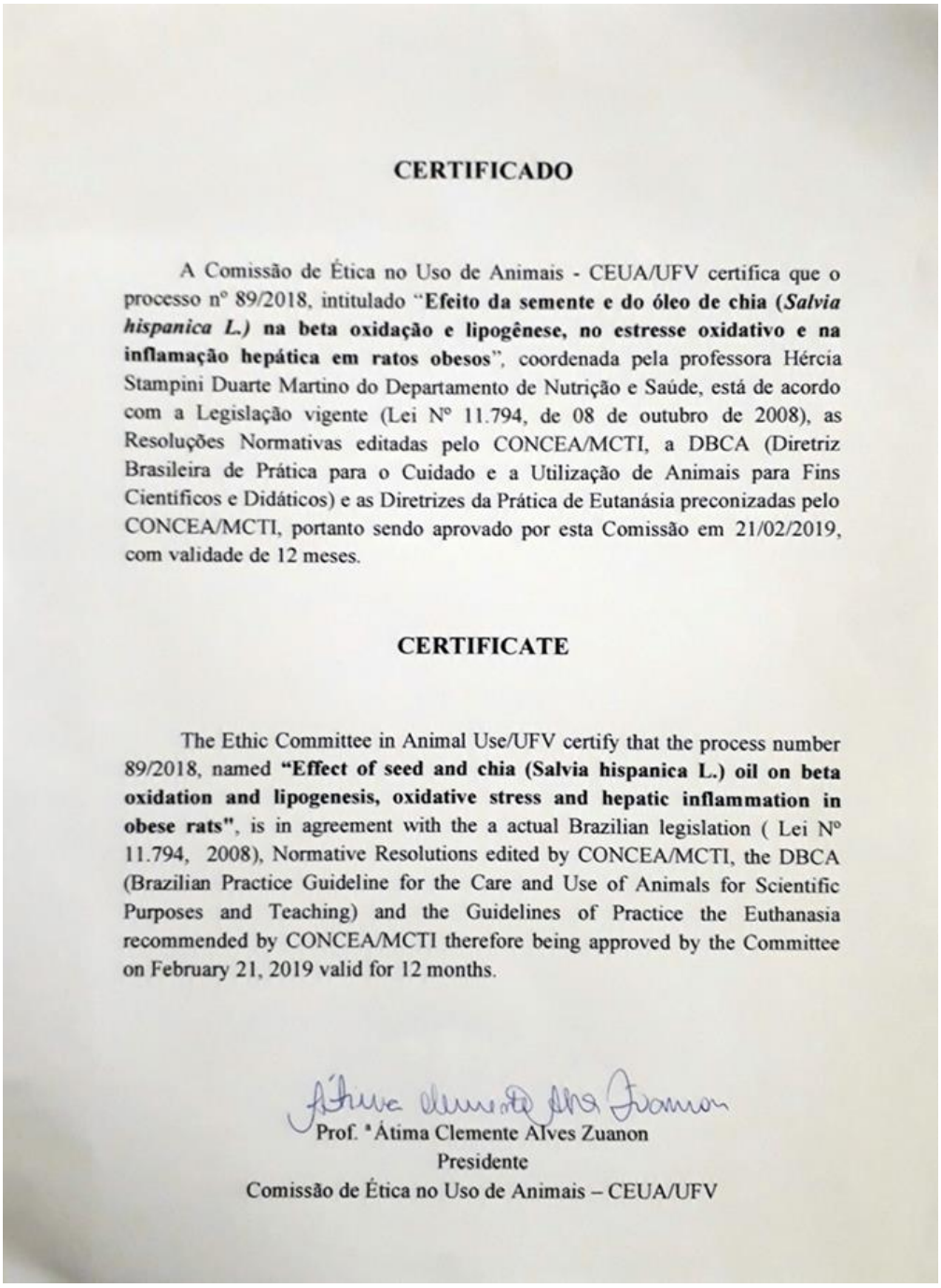
CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 31/2018, named **"Functional properties of the oil fraction of chia seed (Salvia hispanica L.), inflammation, oxidative stress, adipogenesis and modulation of the intestinal microbiota in models in obesity"**, is in agreement with the an 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 April 26, 2018 valid for 12 months.

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

Fonte: ENES, 2020.

ANEXO B – Certificado da Comissão de Ética no Uso de Animais – CEUA/UFV (2°Artigo)



CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 89/2018, intitulado "**Efeito da semente e do óleo de chia (*Salvia hispanica L.)* na beta oxidação e lipogênese, no estresse oxidativo e na inflamação hepática em ratos obesos**", coordenada 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 89/2018, named **"Effect of seed and chia (Salvia hispanica L.) oil on beta oxidation and lipogenesis, oxidative stress and hepatic inflammation in obese rats"**, 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

Fonte: MOREIRA, 2019.