UNIVERSIDADE FEDERAL DE VIÇOSA

MARCELLA DUARTE VILLAS MISHIMA

FUNCTIONAL PROPERTIES OF CHIA (*Salvia hispanica* L.) FLOUR, ITS HYDROLYZED PROTEIN AND PHENOLIC EXTRACT ON MICROBIOTA AND GUT HEALTH *IN VIVO*

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Thesis submitted to the Science of Nutrition Graduate Program of the Universidade Federal de Viçosa, in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Profª Hércia Stampini Duarte Martino

Co-advisers: Bárbara Pereira da Silva
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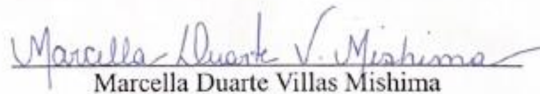

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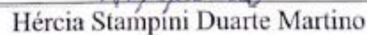

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ABSTRACT

MISHIMA, Marcella Duarte Villas; D.Sc., Universidade Federal de Viçosa, December, 2023; **Functional properties of chia (*Salvia hispanica* L.) flour, its hydrolyzed protein and phenolic extract on microbiota and gut health *in vivo***; Adviser: Hércia Stampini Duarte Martino. Co-advisers: Barbara Pereira da Silva, Mariana Grancieri and Elad Tako.

Chia has a composition with potential to promote changes in the intestinal tissue that favor its functionality, morphology, and microbiota. **Objective**: evaluate the functional properties of chia flour, its hydrolyzed protein and phenolics on intestinal microbiota and gut health *in vivo*. **Methodology**: four experimental trials were conducted. *First experiment*: 32 male *Wistar* rats received the following diets (5 weeks): standard (SD), SD+chia, high-fat diet (HFD), or HFD+chia. *Second experiment*: 64 female *Wistar* rats fed either SD or HFD (7 weeks). Then, 32 rats underwent ovariectomy (OVX) while 32 rats underwent surgery without removal of the ovary (SHAM). After a 3-week recovery period, the rats were divided into eight groups and received the following diets (8 weeks): SD, SD+chia, HFD, or HFD+chia, for both OVX and SHAM groups. Intestinal microbiota, short-chain fatty acids (SCFA), IgA production, intestinal pH, histomorphometry, and brush border membrane functionality were analyzed. Then, a systematic review was conducted according to PRISMA guidelines, to answer the question: "How does food derived bioactive peptides can impact on gut health and inflammatory mediators *in vivo*?". The third and fourth experiments were conducted *in ovo*. *Third experiment*: 45 fertile eggs (*Gallus gallus*) were divided into five groups receiving different treatments: not injected; 18MΩH_20; 10mg/mL of hydrolyzed chia protein (1%); 10mg/mL hydrolyzed chia protein+10^6 CFU *Lacticaseibacillus paracasei* or 10^6 CFU *Lacticaseibacillus paracasei. Fourth experiment*: 27 eggs divided into three groups: not injected; 18MΩH_20; 10mg/mL (1%) of chia phenolic extract. Upon hatching, the animals were euthanized for analysis of intestinal microbiota composition, morphology, and gene expression related to functionality, inflammation, and intestinal barrier proteins. **Results:** *Paper 1*: chia flour consumption in male rats increased SCFA production and improved the circular muscle layer. Diversity and abundance of intestinal bacteria were not affected, but richness increased. Proteins associated with intestinal functionality were downregulated. *Paper 2:* In OVX female rats, chia flour intake increased production of acetic and butyric acids and decreased the cecum content pH, improved muscle layers and crypt thickness, improved richness and decreased microbiota diversity, and improved the expression of *Ap* and *Si*. *Paper 3*: In SHAM female rats, chia consumption increased the production of acetic and butyric acids in the SD group and propionic

acid in the HFD group and decreased the pH of cecal content, while reducing IgA concentration in the HFD+chia group. Nevertheless, it increased microbial richness and diversity. The SD+chia group increased *Si* and *Ap* gene expression and decreased *Sglt1* and *Pept1*. *Paper* 4: The systematic review highlighted the potential positive effects of bioactive peptides on inflammation and gut health. *Paper 5:* The hydrolyzed chia protein downregulated the gene expression of *Tnf-α*, increased *Ocln*, *Muc2*, and *Ap*, reduced *Bifidobacterium*, increased *Lactobacillus* and improved the intestinal morphology. *Paper 6*: Chia phenolic extract reduced *Tnf-α* and increased *Si* gene expression, reduced the *Bifidobacterium*, *E. coli* populations and Paneth cell diameter, increased depth crypt, and maintained villus height. **Conclusion**: chia can be considered a food with biological potential to improve intestinal health. The effects were demonstrated in different fractions of the seed.

Key words: Intestinal Microbiota; Intestinal Morphology; Short Chain Fatty Acids; Intestinal Functionality; Intra-Amniotic Administration; *Lacticaseibacillus paracasei*; Brush Border Membrane; Ovariectomy.

RESUMO

MISHIMA, Marcella Duarte Villas; D.Sc., Universidade Federal de Viçosa, dezembro de 2023; **Propriedades funcionais da farinha de chia (*Salvia hispanica* L.), sua proteína hidrolisada e extrato fenólico na microbiota e na saúde intestinal *in vivo***; Orientadora: Hércia Stampini Duarte Martino. Coorientadores: Barbara Pereira da Silva, Mariana Grancieri e Elad Tako.

A chia possui uma composição com potencial para promover alterações no intestino que favorecem sua funcionalidade, morfologia e microbiota. **Objetivo**: avaliar as propriedades funcionais da farinha de chia, sua proteína hidrolisada e fenólicos na microbiota intestinal e na saúde intestinal *in vivo*. **Metodologia**: foram realizados quatro experimentos. *Primeiro*: 32 ratos *Wistar* machos receberam as seguintes dietas (5 semanas): padrão (SD), SD+chia, dieta hiperlipídica (HFD) ou HFD+chia. *Segundo*: 64 ratas *Wistar* fêmeas receberam dieta SD ou HFD (7 semanas). 32 ratas foram ovariectomizadas (OVX) enquanto 32 ratas foram submetidas à cirurgia sem remoção do ovário (SHAM). Após 3 semanas de recuperação, as ratas foram divididas e receberam as seguintes dietas (8 semanas): SD, SD+chia, HFD ou HFD+chia, para os grupos OVX e SHAM. Microbiota intestinal, ácidos graxos de cadeia curta (AGCC), produção de IgA, pH intestinal, histomorfometria e funcionalidade da membrana da borda em escova foram analisados. Foi realizada uma revisão sistemática de acordo com as diretrizes PRISMA, para responder à pergunta: "Como os peptídeos bioativos derivados de alimentos podem impactar na saúde intestinal e nos mediadores inflamatórios *in vivo*?". Terceiro e quarto experimentos foram conduzidos *in ovo*. *Terceiro*: 45 ovos férteis (*Gallus gallus*) divididos em: não injetados; 18MΩH20; 10mg/mL de proteína de chia hidrolisada (1%); 10mg/mL de proteína de chia hidrolisada+10^6 UFC *Lacticaseibacillus paracasei* ou 10^6 UFC *Lacticaseibacillus paracasei*. *Quarto*: 27 ovos férteis divididos em: não injetados; 18MΩH20; 10mg/mL (1%) de extrato fenólico de chia. Após a eclosão, os animais foram eutanasiados para análise da composição da microbiota intestinal, morfologia e expressão gênica relacionada à funcionalidade, inflamação e barreira intestinal. **Resultados**: *Artigo 1*: A farinha de chia em ratos machos aumentou AGCC, melhorou a camada muscular circular. Diversidade e abundância de bactérias não foram afetadas, mas a riqueza aumentou. As proteínas associadas à funcionalidade intestinal foram reguladas negativamente. *Artigo 2*: Em ratas OVX, a farinha de chia aumentou ácidos acético e butírico e diminuiu o pH do conteúdo cecal, aumentou camadas musculares, espessura das criptas, riqueza da microbiota, diminuiu a diversidade, e melhorou a expressão de *Ap* e *Si*. *Artigo 3*: Em ratas SHAM, a farinha de chia aumentou ácidos acético e butírico (grupo SD) e ácido propiônico (grupo HFD), diminuiu o pH do conteúdo

cecal, reduziu a concentração de IgA (grupo HFD+chia), aumentou a riqueza e a diversidade microbiana. No grupo SD+chia, aumentou a expressão dos genes *Si* e *Ap* e diminuiu *Sglt1* e *Pept1*. *Artigo 4*: A revisão sistemática destacou os potenciais efeitos positivos dos peptídeos bioativos na inflamação e saúde intestinal. *Artigo 5*: A proteína hidrolisada regulou negativamente a expressão gênica de *Tnf-α*, aumentou *Ocln*, *Muc2* e *Ap*, reduziu *Bifidobacterium*, aumentou *Lactobacillus* e melhorou a morfologia intestinal. *Artigo 6*: O extrato fenólico reduziu *Tnf-α* e aumentou a expressão do gene *Si*, a profundidade da cripta, manteve a altura das vilosidades e reduziu *Bifidobacterium*, *E. coli* e o diâmetro das células de Paneth. **Conclusão:** a chia pode ser considerada um alimento com potencial biológico para melhorar a saúde intestinal. Os efeitos foram demonstrados em diferentes frações da semente.

Palavras-chave: Microbiota Intestinal; Morfologia Intestinal; Ácidos Graxos de Cadeia Curta; Funcionalidade Intestinal; Administração Intra-Amniótica; *Lacticaseibacillus paracasei*; Membrana da Borda em Escova; Ovariectomia.

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LIST OF ACRONYMS AND ABBREVIATIONS

18 MΩ H_2O	ultrapure water
µL	microliter
µm	micrometers
µmol	micromole
5-LOX	5-lipoxygenase
AAR	antiradical activity
AIN-93G	standard diet for growing rodents
AIN-93M	standard diet for adult rodents' maintenance
ANOSIM	analysis of similarities
ANOVA	analysis of variance
AP	aminopeptidase
Ap	representation of gene expression of AP in animal model
AP-1	activator protein 1
BBM	brush border membrane
BMI	body mass index
cDNA	complementary DNA (deoxyribonucleic acid)
CFU	colony-forming units
COX-1/2	Cyclooxygenase-1/2
Cp	Cycle threshold
Da	dalton
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphate
DPPH	1,1-diphenyl-2-picrylhydrazyl

EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immuno Sorbent Assay
FDR	false discovery rate
g	gram
G6Pase	glucose 6-phosphatase
GAE	gallic acid equivalents
h	hour
HFD	high-fat diet
HPLC	high-performance liquid chromatography
hsCRP	highly sensitive C-reactive protein
Ig	Immunoglobulins
IgA	immunoglobulin A
IgG	immunoglobulin G
IL	interleukin
IL-10	interleukin-10
IL-12	interleukin-12
IL-13	interleukin-13
IL-1β	interleukin-1β
IL-6	interleukin-6
IL-8	interleukin-8
iNOS	inducible nitric oxide synthase
kDa	kilodalton
kg	kilograms
L	liter

LDL	low-density lipoprotein
LPS	lipopolysaccharides
mg	milligram
min	minutes
mL	milliliter
mRNA	messenger RNA (ribonucleic acid)
MRS	De Man Rogosa Sharpe
MUC2	mucin 2
Muc2	representation of gene expression of MUC2 in animal model
n	number of animals per group
NF-κB	nuclear factor-Kb
Nf-κb	representation of gene expression of NF-κB in animal model
nm	nanometer
ºC	Celsius degree
OCLN	occludin
Ocln	representation of gene expression of OCLN in animal model
OSM	osmolarity
OTUs	Operational Taxonomic Units
OVX	ovariectomized
PAI-1	inhibitor 1 of plasminogen activator
PBS	phosphate-buffered saline
PCoA	principal coordinate analysis
PCR	polymerase chain reaction
PEPCK	phosphoenolpyruvate carboxykinase

PepT1	Peptide transporter 1
Pept1	representation of gene expression of PepT1 in animal model
PFK	phosphofructokinase
p-JNK	c-Jun N-terminal cinase
PK	pyruvate kinase
PPARγ	peroxisome proliferator-activated receptor gamma
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribossomic RNA (ribonucleic acid)
RT-qPCR	real time quantitative reverse transcription polymerase chain reaction
SCFA	short chain fatty acid
SD	standard deviation
SGLT1	sodium-dependent glucose transporter protein-1
Sglt1	representation of gene expression of SGLT1 in animal model
SHAM	non-ovariectommized
SI	sucrase isomaltase
Si	representation of gene expression of SI in animal model
sIgA	secretory IgA
SREBP1	sterol regulatory element-binding transcription
Srebp1	representation of gene expression of SREBP1 in animal model
TLRs	toll like receptors
TNF-α	tumor necrosis factor-alpha
Tnf-α	representation of gene expression of TNF-α in animal model

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1 INTRODUCTION

Chia is a food with properties that can reduce the risk of metabolic changes as it has a series of health benefits. Among these effects, it is highlighted improvement of inflammation, antidiabetic effects, antioxidant protection, cardiovascular and hepatic protection, (Enes *et al*., 2020a; Melo; Machado; Oliveira, 2019; Mishima *et al*., 2021; Silva *et al*., 2021) and reduction of fat content deposited in the heart (Mishima *et al*., 2021). Those benefits are related to the composition of chia seeds, which have elevated nutritional value, with high concentrations of lipids (30.17 $g.100g^{-1}$), proteins (19.72 g. $100g^{-1}$) with amino acid such as glutamine, aspartate and arginine, and total dietary fiber (37.18 g. $100g^{-1}$) with the majority being insoluble fiber (33.30 g. $100g^{-1}$). Furthermore, this food is rich in minerals and antioxidant compounds beneficial to health, including phenolic compounds such as rosmarinic acid, danshensu glycoside, ferulic acid, caffeic acid, rosmarinyl glucoside and protocatechuic acid, tocopherols and tocotrienols (Da Silva *et al*., 2017, 2019a; Enes *et al*., 2020a).

Our previous studies have demonstrated that the consumption of chia flour reduced inflammatory processes and improved the lipid profile in male rats and ovariectomized female rats (Da Silva *et al*., 2019b, 2019c), in addition to improving antioxidant activity when associated with the consumption of a high-fat diet (HFD) (Da Silva *et al*., 2019c; Mishima *et al*., 2021). Chia is a good source of dietary fiber, with potential to promote changes in intestinal tissue that favor its functionality (Da Silva *et al*., 2016, 2017). The intra-aminiotic (*in ovo*) administration of a soluble extract from chia improved intestinal morphology and increased the relative abundance of *Bifidobacterium* and *Lactobacillus* in the cecum content (Da Silva *et al*., 2019a). The chia seed also contains a high concentration of bioactive peptides with promising composition and sequence (Grancieri *et al*., 2019a). In the *in vitro* studies, hydrolyzed chia seed protein, serving as a source of bioactive peptides, exhibited beneficial biological potential by preventing adipogenesis, atherosclerosis, and inflammation (Grancieri; Martino; De Mejia, 2021; Grancieri; Martino; De Mejia, 2019). Furthermore, an *in vitro* study has shown that the phenolic extract of chia also has beneficial effects by decreasing the activity of gluconeogenic and glycolysis enzymes (Enes *et al.*, 2020). Besides that, polyphenols can also exert beneficial effects as a prebiotic substrate, increasing the growth and establishment of probiotic bacteria, and reducing the number of potentially pathogenic bacteria (Dingeo *et al.*, 2020).

On the other hand, the excessive consumption of a western diet, which is rich in fat and low in vegetables and dietary fiber, is one of the main risk factors for several chronic metabolic and inflammatory disorders. In addition to obesity and metabolic syndrome, many diseases that

affect multiple organs are induced or aggravated by the chronic consumption of a high-fat diet (Sferra *et al.*, 2021; Tessitore *et al.*, 2017; Wali *et al.*, 2020). These metabolic changes, caused by excessive high-fat diet consumption, result from the hypertrophy of adipocytes; as they increase, the adipose tissue undergoes molecular and cellular changes that affect systemic metabolism (Greenberg and Obin, 2006). Adipocytes are active endocrine units that perform multiple functions in the body, and their metabolic role changes as they enlarge. One of the changes that occurs in this situation is the increase in the secretion of inflammatory cytokines, which initiate and maintain the inflammatory process (Greenberg and Obin, 2006; Da Silva, Toledo, Grancieri, *et al.*, 2019; Da Silva, Toledo, Mishima, *et al.*, 2019). In this way, low-grade chronic inflammation begins in adipose tissue, which can then reach the circulation, increasing the plasma concentration of inflammatory biomarkers and the number of activated inflammatory cells. This inflammation is a crucial factor in the etiology of several pathologies, such as insulin resistance, cardiovascular diseases and an imbalance in the intestinal microbiota (Akash, Rehman and Liaqat, 2018; Gérard, 2016; Gomes, Hoffmann and Mota, 2018; Maruvada *et al.*, 2017). Furthermore, there is a direct correlation between excessive fat intake and the development and progression of various enteropathies, with changes in the intestinal microbiota and changes in the permeability and integrity of the intestinal mucosal barrier (Araújo *et al.*, 2017; Rohr *et al.*, 2020; Sferra *et al.*, 2021).

In addition to diet, factors such as gender, fat distribution, waist and hip circumferences have also been linked to inflammation and changes in the intestinal microbiota. Growing interest in the dynamic role of microbiome disorders in health has shown that the intestinal microbiota is also linked to estrogen metabolism and menopause in women (Schreurs, Romano e Werner, 2021; Shieh *et al.*, 2020). Menopause is correlated with the cessation of ovarian production of oocytes and a decline in estrogen levels (Takahashi and Johnson, 2015). This reduction in estrogen levels in post-menopause is associated with several changes in the body, such as sleep and mood disorders, vasomotor symptoms, metabolic disorders and obesity (Lobo *et al.*, 2014) and the development of chronic diseases, such as osteoporosis (Cano *et al.*, 2018) and cardiovascular diseases (Bittner, 2009; Murphy, 2012). The postmenopausal period is also capable of affecting the abundance of some genera, microbial diversity, permeability and intestinal motility in humans (Schreurs, Romano and Werner, 2021; Shieh *et al.*, 2020). Estrogen and its receptors, in addition to reproductive functions and their role in regulating various biological functions, play a multifactorial role in inflammation, autoimmunity and the physiology of the gastrointestinal tract, contributing to the prevention and/or progression of

various conditions, such as inflammatory bowel diseases. At low estrogen levels (as seen after menopause), pro-inflammatory pathways are stimulated. Reduced levels of estrogen receptors in the colon increase inflammatory signaling and cell proliferation, which is generally associated with an increased risk of colorectal cancer (Garcia-Villatoro and Allred, 2021). These hormonal changes are also related to greater body adiposity, which can be aggravated by the consumption of a high-fat diet and be an aggravating factor in inflammation. Thus, a relationship is suggested between the intestinal health and systemic health, which can be negatively affected by changes in menopause and inflammation (Schreurs, Romano and Werner, 2021; Shieh *et al.*, 2020).

The intestine is the largest vital epithelial organ. Its main functions are to absorb nutrients from food and establish selectively permeable barriers against the external environment. To perform these functions, the intestinal epithelium needs to form a barrier, which depends on a well-balanced cellular homeostasis, orchestrated by the interaction and balance between differentiation, renewal, proliferation, and the intestinal ecosystem. Under healthy conditions, there is a balance among all microorganisms present in the intestine. However, some factors such as a lifestyle characterized by an unbalanced diet, sedentary lifestyle, chronic drug intake, lack or reduced number of hours of sleep and physiological or psychological stress can lead to an imbalance in the microbiota with a predominance of potentially pathogenic bacteria. A disruption of the balance in intestinal homeostasis alters the barrier function exerted by intestinal epithelial cells and immune components, allowing the passage of components with inflammatory potential, such as lipopolysaccharides (LPS), endotoxins produced by the microbiota. Thus, LPS reaches the circulation and induces the expression of chemokines, activate the immune response and increases the inflammation process (Gomes, Hoffmann and Mota, 2018), which is associated with pathological changes, including metabolic disorders, inflammatory and autoimmune diseases (Redondo-Useros *et al.*, 2020; Sun and Zhu, 2017).

The plasticity of the microbiota, that is, its ability to be molded, means that, by manipulating external factors, it is possible to remodel the architecture and products of intestinal microorganisms to improve the health. Regarding diet, there are several nutrients and bioactive compounds that can affect the microbiota and intestinal health. Foods of plant origin may contain bioactive compounds and non-digestible dietary fiber, with potential effects on the bacterial population and improvement in gastrointestinal motility, functionality and intestinal morphology, with increased mucus production, number and diameter of goblet cells, surface

area of villi and crypt depth (Martino, Kolba and Tako, 2020; Da Silva, Kolba, *et al.*, 2019; Slavin, 2013). Furthermore, probiotics, which are live microorganisms beneficial to health, have been identified as an option to modulate the intestinal microbiota and to improve the immune system, thus allowing prevention and/or reducing the risk of immunological/inflammatory diseases (Mazziotta *et al.*, 2023). Strains belonging to *Lactobacilli* have been shown to exhibit probiotic properties, particularly within *Lacticaseibacillus spp* (Duan *et al.*, 2021; Duar *et al.*, 2017; Zheng *et al.*, 2020). *Lacticaseibacillus paracasei* positively regulated the expression of tight junction proteins, negatively regulated the production of pro-inflammatory cytokines and altered the structure of the intestinal microbiota (Ren *et al.*, 2022).

Therefore, given the beneficial biological potential of chia, we proposed in this work to investigate functional properties of chia flour, its hydrolyzed protein and phenolic extract on microbiota and gut health, *in vivo*. The potential effect of chia flour on intestinal health has been previously observed and we now seek to confirm these effects in a model of male *Wistar* rats, evaluating the effects of chia in association with a high-fat diet, and also in ovariectomized female *Wistar* rats, in order to induce metabolic changes related to the post-menopause period. Furthermore, regarding the chia protein fraction, we evaluated the effects of the hydrolyzed protein on intestinal health, mainly due to its anti-inflammatory effect, previous demonstrated by our research group, and a possible enhancement of the effects when associating it with the probiotic *Lacticaseibacillus paracasei.* The *Lacticaseibacillus paracasei* used in this study is an innovative culture to be tested in plant products, without fermentation, with a promising probiotic effect. Finally, we evaluated the effects of chia phenolic extract, as a potential prebiotic, on intestinal health.

2 JUSTIFICATION

The present study continues the investigation of existing results that analyze the nutritional composition of chia and its effects on health. Chia is rich in dietary fiber, linolenic acid, protein, and bioactive compounds (such as phenolics), which are some of the main components with nutritional and functional activities in the seed.

It was demonstrated in *in vivo* studies with *Wistar* rat model, that chia consumption reduced inflammatory processes and improved the lipid profile of the animals. In the model of ovariectomized (OVX) female *Wistar* rats, chia consumption associated with a standard diet improved the lipid profile and antioxidant activity, and when associated with a high-fat diet reduced the levels of inflammatory markers. The consumption of chia associated with the high-fat diet provided to OVX rats improved antioxidant activity in the heart, indicating the potential to inhibit and/or reduce the damage caused by the action of reactive oxygen species, increasing superoxide dismutase and reducing nitric oxide. Chia, when consumed by SHAM animals, led to a lower fat content in the heart, and the content of minerals and ATPase enzymes in the heart was maintained with the consumption of chia in OVX animals, demonstrating a cardioprotective action, but without reversing the deleterious effects of ovariectomy. Besides that, chia also has positive effects in relation to its mineral composition and maintained bone health when offered as part of a diet that met 100% of the calcium recommendation for rodents. Regarding to intestinal health, a soluble extract of chia, rich in dietary fiber promoted the improvement of intestinal morphology and increased the relative abundance of *Bifidobacterium* and *Lactobacillus* in the cecum contents, suggesting the beneficial effect of the dietary fiber fraction of chia on intestinal health in the intra-amniotic administration (*in ovo*) model.

In *in vitro* studies, total protein and peptides from chia showed good results against adipogenesis and inflammation in adipocytes through inhibition of PPARγ and NF-κB expression and atherosclerosis pathways in macrophages, reducing p-NF-κB, inducible nitric oxide synthase (iNOS), c-Jun N-terminal cinase phosporilated (p-JNK), and activator protein 1 (AP-1). Peptides derived from glutelin demonstrated beneficial anti-adipogenic results both, in *in vitro* and *in silico* analysis. Also, digested total protein of chia and fractions showed scavenging capacity for superoxide, hydrogen peroxide, nitric oxide and 1,1-diphenyl-2-picrylhydrazyl (DPPH), and inhibition of 5-lipoxygenase (5-LOX), cyclooxygenase-1/2 (COX-1-2), and iNOS. In a *in vivo* study with animals fed a high-fat diet, the digested protein of chia was able to improve the body composition, murinometric and biochemical parameters. Furthermore, it reduced adipocytes area, foci of inflammation, levels of p-NF-κB p65, PPARγ,

mRNA *Srebp1* (sterol regulatory element-binding transcription) and *Tnf-α*, also, increased mRNA adiponectin on adipose tissue. *In vitro*, a phenolic extract of chia decreased the expression of gluconeogenesis and glycolytic enzymes (PEPCK and G6PASE, and PFK and PK, respectively) on HepG2 insulin resistant cells.

Then, due to the rich nutritional composition of chia, it is important that studies are carried out continuing these investigations with the chia flour and evaluating the isolated effect of its components, to better understand the role of these fractions. Therefore, this study aims to continue the investigations, advancing and deepening knowledge about the effect of chia and its fractions on intestinal health.

3 HYPOTHESIS

The chia flour consumption improves the composition and functionality of the microbiota, improves intestinal morphology, intestinal immunology, and the functionality of the brush border membrane in male and female (ovariectomized or not) *Wistar* rats fed a diet rich in saturated fat.

The intra-amniotic administration of digested total chia protein together with the probiotic *Lacticaseibacillus paracasei*, as well as the intra-amniotic administration of chia phenolics, promotes an improvement in the composition of the microbiota, with an increase in the abundance of potentially beneficial bacteria and a reduction in potentially pathogenic, improves functionality, barrier function, inflammation, and intestinal morphology in an *in ovo* experimental model (*Gallus gallus*).

4 OBJECTIVES

4.1 General objective

Evaluate the functional properties of chia (*Salvia hispanica* L.) flour, its hydrolyzed protein and phenolic extract on microbiota and gut health *in vivo*.

4.2 Specific objectives

- Determine the effects of chia flour on murinometric variables, food consumption, composition and functionality of the microbiota, intestinal morphology and immunity, and on the functionality of the brush border membrane in young male *Wistar* rats fed a standard or high-fat diet;
- Determine the effects of chia flour on murinometric variables, food consumption, composition and functionality of the microbiota, intestinal morphology and immunity, and on the functionality of the brush border membrane in female *Wistar* rats, ovariectomized or not, fed a standard or high-fat diet;
- Review the literature about the effects of food derived bioactive peptides on intestinal health;
- Investigate the effects of intra-amniotic administration of hydrolyzed protein of chia, associated or not with the probiotic *Lacticaseibacillus paracasei* on the microbiota, morphology, functionality, barrier and intestinal inflammation, *in ovo*;
- Investigate the effects of intra-amniotic administration of chia phenolic extract on microbiota, morphology, functionality, barrier, and intestinal inflammation, *in ovo*.

5 LITERATURE REVIEW

5.1 Chia

5.1.1 General information, nutritional composition, and functional properties

Chia (*Salvia hispanica* L.) is an herbaceous plant that belongs to the Lamiaceae family, native to southern Mexico and northern Guatemala (Capitani *et al.*, 2012). Chia plant is well-adapted to arid environment but is not frost-resistant and is sensitive to day length. In nature, it grows mainly in mountainous regions (Kaur e Bains, 2020; Kulczynski *et al.*, 2019). Initially, chia was grown in tropical and subtropical climates. At present, it is grown worldwide, particularly in Argentina, Peru, Paraguay, Ecuador, Mexico, Nicaragua, Bolivia, Guatemala and Australia, and in Europe, it is grown in greenhouses (Busilacchi *et al.*, 2013; Kulczynski *et al.*, 2019).

Chia seeds have been consumed for many years and were used by Mayan and Aztec civilizations for artistic and therapeutic purposes and as a staple food, combined with amaranth, beans and corn (Marcinek and Krejpcio, 2017; De Souza Ferreira *et al.*, 2015). However, chia consumption has increased over the years due to its health benefits, resulting from its nutritional composition, being considered a complete and functional food (Lara *et al.*, 2021). Chia seed is small and oval shaped, with size ranging from 1-2 mm in length and 1 mm in width, varies from black, gray and black spotted to white color, and the seed surface is smooth and shiny (Kaur and Bains, 2020; Kulczynski *et al.*, 2019; Muñoz *et al.*, 2013). It is consumed as the whole seed, in the form of flour or oil, alone (*in natura*) or added to other foods, such as yogurts, salads and fruits, in preparations such as breads, cakes, food bars, drinks, among others.

The incorporation of chia into foods improves their physical-chemical, sensorial characteristics and mainly their nutritional properties, increasing the concentrations of proteins, unsaturated fatty acids, antioxidant compounds, and dietary fiber (Grancieri, Stampini, Martino and Mejia, 2019; Da Silva *et al.*, 2017). In addition to the nutritional and functional benefits, chia seeds do not contain other components that could have harmful effects in specific physiological or pathological situations, such as gluten and other food allergens, or mycotoxins, thus enabling their inclusion as an ingredient or complement in different foods and preparations. In the food industry, fibrous gums extracted from chia seeds are used as an additive to control the viscosity, stability, texture and consistency of foods (Lara *et al.*, 2021).

Chia has different components that result in different actions, and its health benefits are mainly due to its high nutritional value. Chia has high concentrations of total dietary fiber in its

composition, the amount of this compound varies from around 33% to 42% of the seed composition (Lara *et al.*, 2021; Malik and Riar, 2022; Da Silva *et al.*, 2017) and most of the dietary fiber is insoluble (30% to 39%) (Malik and Riar, 2022; Da Silva *et al.*, 2017). The concentration of dietary fiber in chia is higher than in other cereals and grains such as corn (13.40%), soy (15%), wheat (12.60%), flaxseed (22.30%) and sesame (7.79%) (Da Silva *et al.*, 2017). Chia also has a high concentration of lipids (studies report variations from 18% to 40%) (Lara *et al.*, 2021; Malik and Riar, 2022; Salgado *et al.*, 2023; Da Silva *et al.*, 2017). The main type of fatty acid found in chia is polyunsaturated, mainly n-3 fatty acids (Lara *et al.*, 2021; Da Silva *et al.*, 2017). The ratio of α-linoleic acid (omega-6)/α-linolenic acid (omega-3) in chia seeds grown in Brazil was reported to be 1:3 (Da Silva *et al.*, 2017), both of which are nutrients essential to the human organism. Chia seeds are also considered a good source of vegetable protein, containing around 18% to 21% protein (Lara *et al.*, 2021; Malik and Riar, 2022; Salgado *et al.*, 2023; Da Silva *et al.*, 2017), an amount higher than that found in cereals, such as wheat (14%), barley (9.2%), oats (15.3%), corn (14%) and rice (8.5%).

A study evaluated the nutritional, functional and structural characteristics of the oil and protein isolates from two different chia seed genotypes (black and white). The fatty acid profile indicated the presence of 18 different fatty acids with linolenic acid dominance (~65%), followed by linoleic acid (~19%) and oleic acid (~6%), contributing to polyunsaturated fatty acids up to ~85% in both genotypes. Saturated fatty acids contributed less than 10% of the total fatty acids present (Malik and Riar, 2022). Regarding the general composition of amino acids in chia seeds, the presence of all nine essential amino acids for human nutrition (leucine, isoleucine, lysine, phenylalanine, methionine, tryptophan, threonine, histidine and valine) was revealed (Sandoval-Oliveros and Paredes-López, 2013). Since chia contains a large amount of protein, it contains a high concentration of bioactive peptides with promising composition and sequence, including antioxidant, antidiabetic, anti-inflammatory and hypotensive actions (Grancieri, Stampini and Mejia, 2019; Rabail *et al.*, 2021). The composition of chia can vary according to environmental factors related to seed cultivation, such as geographic location, soil composition and temperature changes. For example, low temperatures generally increased the level of unsaturation of chia fatty acids (Lara *et al.*, 2021; Da Silva *et al.*, 2017). The protein content of the seed tends to decrease with increasing temperature at the place of cultivation (Ayerzah and Coates, 2011). It can be observed that the main protein fractions presented in chia seeds may vary based on different extrinsic factors, besides the growing conditions, the protein

fractions obtained will be highly affected by the method/conditions of extraction used (Wang, Yan *et al.*, 2023).

Regarding the mineral content of the seed, significant concentrations of calcium, potassium, magnesium, iron and zinc can be mentioned, with emphasis on the amount of calcium present in chia, around 430 mg of the mineral in 100g of seed (Da Silva *et al.*, 2017). Chia seeds are a good source of phytochemicals, such as phenolic compounds, and their high antioxidant activity can be attributed to the presence of these compounds (Enes *et al.*, 2020; Da Silva *et al.*, 2017; Da Silva *et al.*, 2019). According to some studies, the main phenolic compounds identified in chia were: rosmarinic, salvianolic, caffeic, gallic, protocatechuic, daidzin, ferulic acid, glycitin, genistin, glycitein and genistein acids (Enes *et al.*, 2020; Lara *et al.*, 2021; Martínez-Cruz and Paredes-López, 2014; Da Silva *et al.*, 2019).

The consumption of chia seeds, flour, and oil has presented a series of health benefits. In the study conducted by Enes (2020), the consumption of chia flour and oil decreased adiposity in *Wistar* rats fed a diet high in fat and fructose, and the chia oil was able to improve glucose tolerance (Enes *et al.*, 2020). Also, in *Wistar* rats, the consumption of chia flour showed a hypoglycemic effect, reduced liver fat deposition (Da Silva *et al.*, 2016), improved the lipid profile in a standard or a high-fat diet (Da Silva *et al.*, 2019; Da Silva *et al.*, 2016; Silva *et al.*, 2021) and reduced inflammatory processes (Da Silva *et al.*, 2019; Da Silva, Kolba, *et al.*, 2019). The consumption of chia by *Wistar* rats, as a therapeutic strategy after the changes caused by the consumption of a diet rich in sucrose, improved the expression of interleukin-1β (IL-1β), interleukin-10 (IL-10), inhibitor 1 of plasminogen activator (PAI-1), recognized as a factor that exacerbates systemic inflammation, while reducing the expression of nuclear factor-κB (NF-κB) in the liver (Vega Joubert *et al.*, 2022). *In vitro* studies also reported a reduction in the levels of markers related to atherosclerosis, suggesting a promising effect of the chia protein fraction in the risk reduction of cardiovascular diseases by modulating inflammatory and atherosclerotic processes (Grancieri, Stampini and Gonzalez, *et al.*, 2019). Furthermore, chia has also been shown to promote antioxidant protection in male and female *Wistar* rats (Mishima *et al.*, 2021; Da Silva *et al.*, 2019) and reduce heart fat content in female rats fed a high-fat diet (Mishima *et al.*, 2021). In a study in which chia was offered as part of a diet that met 100% of the calcium recommendation for rodents, chia consumption maintained the animals' bone health (Mishima *et al.*, 2020). Besides that, intra-aminiotic (*in ovo*) administration of a soluble chia extract positively regulated the gene expression of proteins related to zinc and iron metabolism (Da Silva, Kolba, *et al.*, 2019). The consumption of chia by *Wistar* rats also showed the potential to

promote changes in intestinal tissue that favor its functionality (Da Silva *et al.*, 2016) and the intra-amniotic administration (*in ovo*) of a soluble extract of chia increased the intestinal villus surface area, villi leght, villi width, goblet cell number and diameter, and increased relative abundance of *Bifidobacterium* and *Lactobacillus* in the contents of the cecum (Da Silva, Kolba, *et al.*, 2019).

Therefore, studies consider chia as a potential bioactive food and suggest it as a functional food, as the realistic consumption of chia and oil can be a dietary strategy to reduce the risk of metabolic changes (Enes, *et al.*, 2020; Lara *et al.*, 2021; Vega Joubert *et al.*, 2022).

5.1.2 Bioactive peptides from chia

Research consistently supports the notion that increased consumption of plant-based functional foods can play a pivotal role in reducing the risk of chronic diseases (Lj, Veronese and Barbagallo, 2023; Shang *et al.*, 2023; Wang, Yeli *et al.*, 2023). Notably, these foods also serve as a vital protein source in developing countries, emphasizing the importance of a well-rounded and adequate nutritional intake (Rabail *et al.*, 2021). Plant-derived proteins, readily available in cereals, nuts, fruits, and other natural sources, have been linked to a spectrum of health benefits, such as anti-inflammatory (He *et al.*, 2022; Liu, W. *et al.*, 2022; Zhu, Xu e Li, 2022), antibacterial (Ezeorba *et al.*, 2024; León Madrazo and Segura Campos, 2022; Wang *et al.*, 2022) and antioxidant properties (Chai *et al.*, 2020; Zhao *et al.*, 2022), validated both *in vivo* and *in vitro* studies.

Dietary proteins serve as sources of exogenous peptides that can perform regulatory activities comparable to those of endogenous peptides in our body. These exogenous peptides, called "bioactive peptides" for their physiological relevance and nutritional significance, emerge through the hydrolysis of proteins and typically comprise 2 to 20 amino acid residues (Rabail *et al.*, 2021). They have the ability to regulate physiological processes, alter cellular metabolism by acting as signaling agents through hormone-receptor interactions and signaling cascades, causing effects locally or in various organs (Chauhan and Kanwar, 2019; Valenzuela Zamudio and Segura Campos, 2022). Bioactive peptides remain inactive when bound within the parent protein sequence, and they need to be activated by hydrolysis/digestion using enzymes, fermentation, chemical or gastrointestinal digestion. Thus, the human body can produce these bioactive peptides from dietary proteins (Rabail *et al.*, 2021).

Bioactive peptides are characterized by their low molecular weights and easy digestion and absorption and can be quickly absorbed by the body through a specific mode of transport

in the intestine, unlike large protein molecules and even small amino acid molecules. The functions of plant-derived bioactive peptides are related to their structures, molecular weight, amino acid composition and location are the main factors that affect the functions of these peptides (Liu *et al.*, 2022; Monteiro *et al.*, 2016; Ren *et al.*, 2023).

Chia seeds is an alternative gluten-free protein source, and there are some researches into the development of recipes for gluten-free bread containing chia seeds, for example. Researchers developed gluten-free bread by adding chia seed flour (Zhang *et al.*, 2023) or bioactive peptides derived from chia (Ozón *et al.*, 2023) and found that the addition of chia seeds improved the nutritional value of the product. Furthermore, the chia seed components can be linked to other components to enhance the viscoelasticity of the dough by simulating the gluten network and forming a larger bread volume (Zhang *et al.*, 2023). In a study by Wang *et al.* (2023a), two chia seeds grown in different locations were evaluated, revealing that the essential amino acid index ranged in 189.40–496.73% in contrast to standard (white egg protein), which means that protein chia samples have almost or more than 2-fold essential amino acid index than the reference protein source. The biological value indicates the potential biological value of chia, due to its complete amino acid profile. Like pseudo-cereals, most of the protein in chia seeds is stored in the albumins and globulins portion of the seed (Valenzuela Zamudio and Segura Campos, 2022; Wang, Yan *et al.*, 2023). These fractions present high denaturation temperatures, indicating an excellent thermal stability of the seed, suggesting the presence of hydrophobic bonds between amino acids. Furthermore, chia protein isolates show good water-holding capacity (amount of water withheld by the hydrated protein after having applied an external force) and oil-holding capacity (union of fat by means of the lateral nonpolar protein chains), and both characteristics indicate the presence of hydrophobic amino acids (Grancieri, Stampini and Mejia, 2019; Valenzuela Zamudio and Segura Campos, 2022).

The anti-inflammatory functions of bioactive peptides have garnered significant attention, with studies demonstrating their ability to counter excessive inflammatory responses, modulate inflammatory signaling pathways, and inhibit the secretion of inflammatory factors. The molecular weight of bioactive peptides influences their anti-inflammatory activity, with lower molecular weight peptides (less than 1kDa) exhibiting heightened efficacy (Liu *et al.*, 2022). Furthermore, the use of bioactive peptides is an alternative to using leftovers at an industrial level. Some industrial processes, such as the extrusion of seed oils, discard a relevant amount of proteins that can serve as a source for bioactive peptides, and generate value-added products in the food and medicinal industries (Rabail *et al.*, 2021).

Chia seeds, owing to their high protein concentration and essential amino acids, emerge as a promising source of bioactive peptides. A review highlights the beneficial biological potential of peptide sequences identified in chia seeds, emphasizing antioxidant, antihypertensive, and hypoglycemic properties (Grancieri, Stampini and Mejia, 2019). A study on RAW264.7 macrophage-like cells demonstrate the protective effects of digested total chia protein against inflammation, atherosclerosis, and oxidative stress markers. The bioactive potential extends to angiotensin-converting enzyme inhibition, dipeptidyl peptidase-IV inhibition, and antioxidant capacity, further substantiating the health benefits of chia (Grancieri, Stampini and Gonzalez, *et al.*, 2019; Segura Campos *et al.*, 2013). Furthermore, the ability of proteins and peptides present in chia to block peroxisome proliferator-activated receptor gamma (PPARγ) was suggested, causing a regulation of adipogenesis and inflammation. Glutelin was pointed as one of the main proteins responsible for the effects observed with digested total protein, however the best results were observed in set of proteins than their isolated (Grancieri, Martino and Mejia, 2021).

Exploring the antibacterial potential, Coelho *et al.* (2018) reveal that chia peptides inhibit cholesterol homeostasis *in vitro* and exhibit activity against gram-positive and gram-negative bacteria. The effect of the peptides on cholesterol homeostasis can be explained by the conformational structure of the peptides, in addition the hydrophobicity of antibacterial peptides has been described as a major factor in peptide interactions with bacterial cytoplasmic membranes. The intensity of the bioactivity varied with the method for obtaining the protein and with the enzyme used in the hydrolysis process. San Pablo-Osorio *et al.*, (2019) hydrolyzed chia seed protein isolates using various proteases and highlighted the antihypertensive potential of chia seed pepsin protein hydrolysate, suggesting the utility of chia seed oil industry meal by products as economical sources of protein and bioactive peptides.

The diverse functions of bioactive peptides also include the modulation of intestinal homeostasis. The bioactive peptides can produce anti-inflammatory effects via immune system and then modulate the intestinal microbiota and homeostasis (Ren *et al.*, 2023), affecting the barrier function, villous surface area, mucosal immune responses, inflammation and intestinal microbiota (Hou, Kolba, Glahn *et al.*, 2017; Jiao *et al.*, 2019; Ma *et al.*, 2019; Requena *et al.*, 2017a). In addition to the fact that these peptides are formed by amino acids, therefore, according to their primary construction function, they can contribute to the formation, including of intestinal tissue. An intra-amniotic administration model was used to evaluate the effects of prebiotics and duck egg white peptides in promoting calcium absorption. Prebiotics and

peptides were found to increase the relative abundance of probiotics, the surface area of intestinal villi, the diameters of goblet cells, and regulate calcium-related gene expressions (Hou, Kolba, Glahn *et al.*, 2017). Bioactive peptides are known to have low bioavailability and little evidence supports that they can cross the intestinal epithelium intact (Bao and Wu, 2021; Miner-Williams, Stevens and Moughan, 2014). Therefore, additional studies are still needed to understand the effects of the intestinal environment on the bioavailability of bioactive peptides and to address fundamental questions regarding the effectiveness of peptides on intestinal health and their interactions between intestinal barrier function, intestinal microbiota, immune system and the host.

Most of the peptides identified from chia seeds so far have been shown to exert their active potential against various disease markers such as antioxidant, hypoglycemic, immunomodulatory, anti-inflammatory, hypocholesterolemic, and antihypertensive potential (Rabail *et al.*, 2021). When these bioactive peptides are consumed daily, whether through direct or indirect consumption in the form of supplemented products, it is assumed that nutraceutical benefits can be achieved for various disease biomarkers. Thus, the functional properties of chia combined with the high nutritional value, good lipid profile, dietary fiber and the benefit of using protein, make the seed an excellent source of raw material for food formulations and dietary supplements (Malik and Riar, 2022).

5.1.3 Phenolics from chia

Chia seed is rich in phenolic compounds, which are some of the main components with nutritional and functional activities of the seed, with high antioxidant capacity (Lara *et al.*, 2021; Martínez-Cruz and Paredes-López, 2014). According to some studies, the main phenolic compounds identified in chia were rosmarinic, salvianolic, caffeic, gallic, protocatechuic, daidzin, ferulic acid, glycitin, genistin, glycitein and genistein acids (Enes *et al.*, 2020; Lara *et al.*, 2021; Martínez-Cruz and Paredes-López, 2014; Da Silva, Kolba, *et al.*, 2019). A study evaluated the phytochemical profiles of the seeds, sprouts, leaves, flowers, roots and herb of chia and observed that rosmarinic acid was the quantitatively dominant compound found in all tested extracts and was identified as the major compound responsible for antioxidant activity (Motyka *et al.*, 2023). Rosmarinic acid is a derivative of caffeic acid and is one of the most abundant compounds in chia seeds (Enes *et al.*, 2020; Lara *et al.*, 2021; Oliveira-alves *et al.*, 2017; Salgado *et al.*, 2023). This acid is involved in biological activities as it has antioxidant, astringent, anti-inflammatory, antithrombotic, antimutagenic, antibacterial, and antiviral properties (Lara *et al.*, 2021).

Regarding some of the other phenolic compounds identified, salvianolic acid stands out with beneficial properties such as anti-inflammatory and antioxidant effects. Studies show that salvianolic acids have promising effects in some chronic fibrotic diseases (Lara *et al.*, 2021). Caffeic acid also acts as an antioxidant and enzyme inhibitor and has even been reported to inhibit LDL (low-density lipoprotein) oxidation *in vitro* and may therefore protect against cardiovascular disease. Gallic acid shows antioxidative and anti-inflammation properties, and also a selective cytotoxicity against a variety of tumor cells with greater activity than that shown against normal cells being an important compound in the carcinogenic process (Martínez-Cruz and Paredes-López, 2014; Xu *et al.*, 2021).

An *in vitro* study that evaluated the influence of chia hydrolyzed phenolics extract on glucose metabolism of HepG2 cells observed that phenolics downregulated mRNA of enzymes involved in gluconeogenesis, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase), and glycolysis as phosphofructokinase (PFK) and pyruvate kinase (PK), respectively (Enes *et al.*, 2020). Phenolic compounds ingested as part of a regular diet pass through the gastrointestinal tract to reach the large intestine, where they can exert health-promoting effects. They favor a diverse bacterial profile of the resident microbiota and promote colon health. The mechanisms of action of phenolic compounds in exerting these effects are varied, but include prebiotic-type actions, regulation of gene expression associated with inflammatory processes and preservation of an adequate intestinal barrier (Domínguez-Avila *et al.*, 2021). A study evaluated the antioxidant effects of polyphenols in plants (gallic acid proanthocyanidins, ellagic acid and tannic acid) on ulcerative colitis. The authors found that the polyphenols can alleviate ulcerative colitis, including decreasing disease activity index and improving histological alteration. The mechanism involves regulation of inflammatory factors, intestinal barrier repair, regulation of oxidative stress, and regulation of intestinal microbiota (Chen *et al.*, 2023).

5.2 Intestinal health

5.2.1 Microbiota and microbiome

The human microbiota is a complex ecosystem that colonizes the gastrointestinal tract. This ecosystem is formed by a high number of bacteria (more than 1000 identified species), archaea, fungi and viruses, which add up to 1.3 times the number of eukaryotic cells in the entire human organism (Sender, Fuchs and Milo, 2016). Microbial colonization in the human body begins immediately after birth and the composition of the microbial community is shaped, highly dependent and subject to change, by several environmental factors, such as genotype,

host ethnicity, birth canal, breastfeeding, age, sex, diet, hormonal cycles, illness, medications and travel history (Ghosh and Pramanik, 2021). The portion of the intestine most densely populated by bacteria is the large intestine, which is colonized mainly by the phyla Firmicutes and Bacteroidetes, representing 90% of the total community, as well as the phyla Proteobacteria, Actinobacteria and Verrucomicrobia, which are less dominant. Some of the 200 different genera belonging to the Firmicutes phylum are *Lactobacillus*, *Bacillus*, *Clostridium*, *Enterococcus* and *Ruminococcus*, while the most predominant genera belonging to the Bacteroidetes phylum are *Bacteroides* and *Prevotella* (Abokor *et al.*, 2021; Rinninella *et al.*, 2019). Microbiota describes the living microorganisms found in a defined environment, such in the intestine. The concept of gut microbiome not only includes the gut microbiota, but also the collection of genomes from all the microorganisms in the environment, the microbial structural elements, metabolites, mobile genetic elements and the environmental conditions. In this regard, microbiome encompasses a broader spectrum than that of microbiota. The gut microbiome is involved in many life activities, including the metabolism of dietary and endogenous substances and immune regulation (Berg *et al.*, 2020; Han, Min and Pang, 2023; Hou *et al.*, 2022).

The intestinal microbiota plays fundamental roles in the digestion of nutrients and is widely related to the health of the host, regulating several metabolic processes, such as fermentation and digestion of biomolecules, maturation and functioning of innate and adaptive immunity, production of various metabolites, energy production and homeostasis epithelial, in addition to regulating the release of intestinal hormones and peptides (Bliss and Whiteside, 2018; Covasa *et al.*, 2019; Ghosh and Pramanik, 2021). The microbiota also plays an important role in the host's defense against pathogens by repairing damage to the intestinal mucosa, producing several antimicrobial peptides and inducing the secretion of interleukins (IL) such as IL-10, IL-17 and IL-22 by host immune cells. The degradation of dietary carbohydrates, lipids and proteins by the intestinal microbiota gives rise to several biochemical metabolites, with local and systemic action. The production of short-chain fatty acids (SCFA) upon degradation of pyruvate are the most abundant fecal metabolites (Ghosh and Pramanik, 2021), such as butyrate which promotes colonic health, and propionate which promotes the release of glucagon like peptide-1 and peptide YY and improves insulin sensitivity and weight. Also, some unfavorable metabolites are produced by the gut microbiome, such as trimethylamine, the most prominent, which is oxidized to trimethylamine N-oxide and works by increasing low-density lipoprotein uptake in cells, reducing cholesterol excretion, and promoting recruitment of activated leukocytes and platelet aggregation (Antony *et al.*, 2023).

The small intestine is divided into three sections: the duodenum, with pH 5–7 and bacterial load of $10^{3–4}$ cells/mL where gram positive aerobic bacteria predominate, followed by the jejunum and ileum with pH 7–9 and cell density of $10^{4–8}$ cells/mL, comprising strict and facultative anaerobic gram-positive and gram-negative bacteria. The composition of the intestinal lumen microbiome is highly diverse. The large intestine or colon is strictly anaerobic with a pH ranging from 5 to 7. This part of the intestine is the fermentation center where various amino acids and the short-chain fatty acid butyrate are fermented, and the production of various phenolic and indolic compounds occurs. This compartment of the intestine houses the most complex bacterial diversity, where cell density reaches 10^{11} cells/mL. The high bacterial diversity and abundance in the large intestine is due to several factors such as pH, low concentration of bile salts and longer retention time due to relatively slower peristalsis (Dabke, Hendrick and Devkota, 2019; Ghosh and Pramanik, 2021).

The diversity of the microbiota is classified into alpha and beta, and it is measured using indices, in addition to metrics such as the Firmicutes/Bacteroidetes ratio and the abundance of bacteria at different levels such as phylum, genus and species. Alpha diversity is about the local diversity and corresponds to diversity within a habitat or community. Each community can be evaluated individually, independently of the community evaluated together. Diversity (distribution of species) is related to both richness (number of different species in the community) and abundance (distribution of the number of different individuals in that community). The high richness of the gut microbiota is associated with healthy host metabolism. The alpha diversity indices are:

- Chao-1: Estimates species richness (number of different species).
- Shannon: Indicates how different species are within a community. If the number of species increases, diversity can increase. If each microbe belongs to a different species, the diversity index is higher, if each microbe belongs to the same species, the diversity index is the lowest.
- Simpson: Estimates species dominance, the number of species within the community. It varies from 0 to 1, the higher it is, the greater the probability that individuals are of the same species.

Beta diversity corresponds to diversity between habitats or communities. It is related to the similarity between communities. In this index, communities are not evaluated individually, but together. The dissimilarity or correction indices (Pearson correlation coefficient, Bray

Curtis, Jaccard, Bonferroni correction, multivariate analysis with the formation of clusters, PCoA...) show the degree of association or similarity between communities (Pinart *et al.*, 2022).

The gut microbiome has been implicated in the etiopathogenesis of multiple diseases ranging from the intestinal (inflammatory bowel diseases, colon cancer) to the neurological diseases (Parkinson's disease, autism). However, the definition of the microbiome's role in metabolic diseases has remained elusive because the same factors believed to be the central drivers of dysmetabolism are also believed to be the main drivers of the composition of the gut microbiome: diet and lifestyle (Dabke, Hendrick and Devkota, 2019). Diet is one of the main factors capable of modulating the intestinal microbiota and different dietary patterns are associated with qualitative and quantitative changes in the microbiota. Intestinal microorganisms, in turn, influence the absorption, metabolism and storage of nutrients, with effects on the host's physiology, using the ingested nutrients as fuel for their fundamental biological processes (Gentile and Weir, 2018; Ley *et al.*, 2008; Li *et al.*, 2009). Studies have investigated how different aspects of the intestinal microbiota can be influenced by changes in diet and show that the human intestinal microbiota responds quickly to changes in diet. Despite these rapid dynamics, long-term eating habits are determinant in the composition of an individual's gut microbiota, and a specific change in diet can have a highly variable effect on different people due to the individualized nature of the microbiota (David *et al.*, 2014; Gentile and Weir, 2018; Sonnenburg and Bäckhed, 2016).

The imbalance in microbial composition on or inside the body relative to healthy state is associated with chronic inflammation and several autoimmune and inflammatory pathological conditions including allergies, central nervous system disorders, cancers, insulin resistance, metabolic syndrome, diabetes mellitus, obesity, polycystic ovarian syndrome and cardiovascular disease (Antony *et al.*, 2023; Kant *et al.*, 2022). This imbalance can influence local or systemic immunity and inflammation by regulating intestinal barrier permeability or by triggering the innate immune system (Antony *et al.*, 2023).

The term "leaky gut" is used to indicate abnormal translocation of large molecules from the lumen to the villi or excessive absorption of such molecules from the lumen into the systemic circulation, which in turn induces various disorders in the body (Usuda, Okamoto and Wada, 2021). The increase in the population of pathogenic bacteria alters the barrier function exerted by intestinal epithelial cells, allowing the passage of components with inflammatory potential such as lipopolysaccharides (LPS) which are endotoxins produced by the microbiota, thus causing endotoxemia (when LPS reaches the circulation), and the induction of damage to

epithelial cells, which is one of the possible mechanisms for increasing intestinal permeability (Belancic, 2020; Salguero *et al.*, 2019; Xi *et al.*, 2019). Thus, the disturbed ecology of the gut microbiome has been associated with several human diseases, such as obesity and metabolic syndromes, non-alcoholic fatty liver disease, coronary heart disease, irritable bowel syndrome, inflammatory bowel disorders, allergy, asthma, among others (Ghosh and Pramanik, 2021). Current research is directed towards finding treatment options for improving the number of beneficial gut microbiota and to reduce the harmful ones with the use of prebiotics, probiotics, synbiotics, and postbiotics (Antony *et al.*, 2023).

5.2.2 Short-chain fatty acids (SCFA)

Dietary fibers in the colon provide a source of energy for intestinal bacteria and some are nutritionally specialized in degrading this type of carbohydrate, which are anaerobically fermented and produce SCFA. The SCFAs that occur in greatest quantities in the intestine are acetate, propionate and butyrate, which are the most abundant fecal metabolites (Hee, Van Der and Wells, 2021).

These SCFAs play a role in intestinal homeostasis and have effects on tissues and organs beyond the intestine through their circulation in the blood. The production of these metabolites benefits the host, serving both as energy recovered from inaccessible carbohydrates, as well as regulatory molecules with various physiological effects, including energy homeostasis, lipid and carbohydrate metabolism, and suppression of inflammatory signals (Fu, Lyu and Wang, 2023; Ghosh and Pramanik, 2021; Hee, Van Der and Wells, 2021). SCFAs can suppress intestinal inflammation by inhibiting the excessive signaling of toll like receptors (TLRs) (Zhang *et al.*, 2022). Butyrate and propionate also act as histone deacetylase inhibitors, suggesting that they can epigenetically influence host gene expression (Hee, Van Der and Wells, 2021). Butyrate is the primary fuel source for the colonocyte, where almost 90% of the butyrate generated is metabolized locally. Furthermore, it improves the integrity of intestinal epithelial cells, promoting tight junctions and cell proliferation, increases mucin production by goblet cells, inhibits the secretion of pro-inflammatory cytokines while increasing the secretion of anti-inflammatory cytokines and modulates the function of T cells. Furthermore, by increasing the immunoglobulin-A (IgA) and immunoglobulin-G (IgG) antibody response of B cells, butyrate increases specific immunity (Siddiqui and Cresci, 2021). Both acetate and propionate also help in the anti-inflammatory process and the production of cytokines. While acetate is readily absorbed by circulatory tissue for peripheral distribution, propionate is metabolized by hepatocytes. The proportion of SCFA (acetate, propionate and butyrate) produced by intestinal

microorganisms in the degradation of dietary fiber and resistant starch, although it depends on the diet, microbial composition and intestinal transit time, generally maintains a ratio of 3:1:1 in the intestinal lumen (Gentile and Weir, 2018; Ghosh and Pramanik, 2021; Hee, Van Der and Wells, 2021).

Higher levels of production of various types of SCFA can act to increase the diversity of the intestinal microbiota. The level of propionate production, for example, is correlated with the abundance of *Bacteroides* species in the intestine, which is consistent with the involvement of these bacteria in propionate production (Salonen *et al.*, 2014). *Bacteroides* growth is reduced relatively to Firmicutes and Actinobacteria because SCFAs negatively impact Bacteroides at mild acid pH. Thus, SCFAs production by Firmicutes and *Bacteroides* may be regulated by pH variations, with more Firmicutes fermentation in proximal colon (pH≈5.6) and conditions favoring *Bacteroides* fermentation in the distal colon with a more neutral pH (pH≈6.3). This selective gradient is limiting the propionate production and promoting butyrate formation in the most proximal part of the colon (Martin-gallausiaux *et al.*, 2021). Short-chain fatty acids play a significant role in reducing the pH of the intestines, suppressing the growth of harmful pathogens, and facilitating optimal absorption of minerals and vitamins (Wang and Hu, 2023). A decreased pH of the colon contents and inhibits the growth of potentially pathogenic bacteria and may reduce the complexation of minerals like calcium, resulting in more calcium being available for uptake in the colon. However, the decreased colonic pH might not be the only mechanism by which SCFA enhance calcium absorption. In the *in vitro* and *in vivo* experiments, SCFA increased mRNA expression of proteins involved in paracellular calcium uptake, calbindin-D9k, and increased transepithelial calcium transport. These findings support the concept of additional stimulatory effects of SCFA on mineral, particularly calcium, uptake (Blaak *et al.*, 2020; Hou and Tako, 2018).

5.2.3 Morphometric and intestinal functionality parameters

The small intestine is highly specialized in the hydrolysis and absorption of nutrients and constitutes the barrier between the host's external and internal environment, where most enzymatic digestion and absorption occurs. Its wall is made up of four layers: mucous, submucosal, muscular layer and serous. The mucous layer is made up of finger-shaped protrusions called villi. The structure of the villi is capable of increasing the surface area, and increasing the length of the villi leads to greater efficiency in the process of digestion and absorption of nutrients. These villi are made up of many columnar epithelial cells called enterocytes, and all enterocytes contain many microvilli that form the brush border membrane.

The brush border membrane is a unique morphological structure between the enterocyte and the intestinal lumen. In this membrane, there are functional transporters and enzymes that are used as biomarkers of the digestive and absorption capacity of the brush border membrane and the general functionality of the tissue. The submucosal is a layer of connective tissue that supports the mucosa. The muscular layer is composed of smooth muscle fibers, maintaining the mucosal surface and underlying glands in a constant state of gentle agitation to expel the contents of the glandular crypts and increase contact between the epithelium and the contents of the lumen. Finally, the serous layer consists of a thin layer of connective tissue covered by mesothelium (Da Silva, Martino and Tako, 2021; Sobolewska *et al.*, 2017; Yamauchi, 2002).

Histological parameters of the small intestine, such as villus measurements and crypt depth, are used as indicators of intestinal development and functionality. Deeper crypts lead to increased secretion of digestive enzymes. Thus, the surface area of the villi, the depth of the crypts, and the relationship between the height of the villi and the depth of the crypts are indicators of intestinal development and functional status. Therefore, an increase in any of these morphometric parameters is expected to be capable of improving the digestive and absorption capabilities of the brush border membrane, as well as increasing the gene expression of membrane proteins (Hou and Tako, 2018; Liu *et al.*, 2023).

The mucus layers and epithelium are the most important and main structures that form the intestinal barrier. The epithelium is located just below the mucous layer and is composed of normal epithelial cells and several types of cells that have specific functions, such as Paneth cells that secrete antibacterial peptides, enterocytes that produce chloride in response to noxious stimuli, and goblet cells that contribute to the maintenance of a mucous layer by secreting mucin. Epithelial cells are connected by tight junctions and adherens junctions. Tight junctions are composed of claudin, occludin, junctional adhesion protein molecule-A, zonula occludens and cingulin. Large molecules (> 600 Da) can be absorbed when tight junctions become loose or collapse due to harmful stimuli (Usuda, Okamoto and Wada, 2021). Increased intestinal permeability is determined by the disruption of tight junctions, which leads to the release of pro-inflammatory agents, including reactive oxygen species, which contribute to the progression of a pathological cascade (Blagov *et al.*, 2023). The paracellular absorption pathway can be regulated by inflammatory cytokines such as interleukin-13 (IL-13) and tumor necrosis factor-alpha (TNF-α). Therefore, it is likely that this permeability pathway, when increased, is associated with inflammation, as it allows the passage of macromolecules, bacterial products and food antigens (Usuda, Okamoto and Wada, 2021).

The functionality of brush border membrane is commonly assessed by the expression of proteins involved in the digestive and absorptive process, such as:

- Aminopeptidase (AP): exopeptidase that cleaves amino acids from the N-terminal position of peptides.
- Sucrase isomaltase (SI): disaccharidase that hydrolyzes disaccharides or oligosaccharides into monosaccharides for absorption.
- Sodium-glucose transport protein 1 (SGLT1): highly expressed in the brush border membrane of villous enterocytes in the proximal part of the small intestine and is responsible for the absorption of dietary glucose.
- Peptide transporter 1 (Pept1): expressed abundantly in the duodenum, jejunum and ileum, and its function is to move peptides from the lumen of the small intestine to the enterocyte (Chen *et al.*, 2002; Dias *et al.*, 2019; Diaz-Sotomayor *et al.*, 2013; Hou and Tako, 2018; Knez *et al.*, 2018; Oguma *et al.*, 2015).

5.2.4 Immunoglobulin-A (IgA)

Immunoglobulins (Ig) are involved in eliminating and neutralizing foreign particles in the body by identifying, binding and eliminating specific bacterial, fungal and viral antigens. Among the five Igs in the body, IgA is the second most abundant antibody found in the circulation and the predominant antibody generated in mucosal secretions, whose primary function is to defend mucosal surfaces. IgA provides dual humoral responses that create a symbiotic environment for the resident gut microbiota and prevent the invasion of enteric pathogens (Abokor *et al.*, 2021; Takeuchi and Ohno, 2022). The review by Abokor (2021) includes studies that emphasize that IgA insufficiency can result in the proliferation of opportunistic pathogens that invade the intestinal epithelium and disseminate in the circulation. Secretory IgA (sIgA), the predominant form of IgA, is widely influential in maintaining intestinal homeostasis, remodeling the composition of the intestinal microbiota to promote intestinal symbiotic growth and suppress the proliferation of pathogenic bacteria (Abokor *et al.*, 2021; Pracht *et al.*, 2023).

The primary task of the intestinal immune system is to eradicate pathogens, as the intestine is a gateway for them, while tolerating or even promoting colonization by commensals and mutualists. Thus, adaptive immunity plays a role in maintaining homeostasis of the host microbiota. In the intestinal lumen, secretory IgA antibodies bind to the surface of commensal bacteria to maintain their homeostatic existence with intestinal tissues (Pracht *et al.*, 2023; Yang

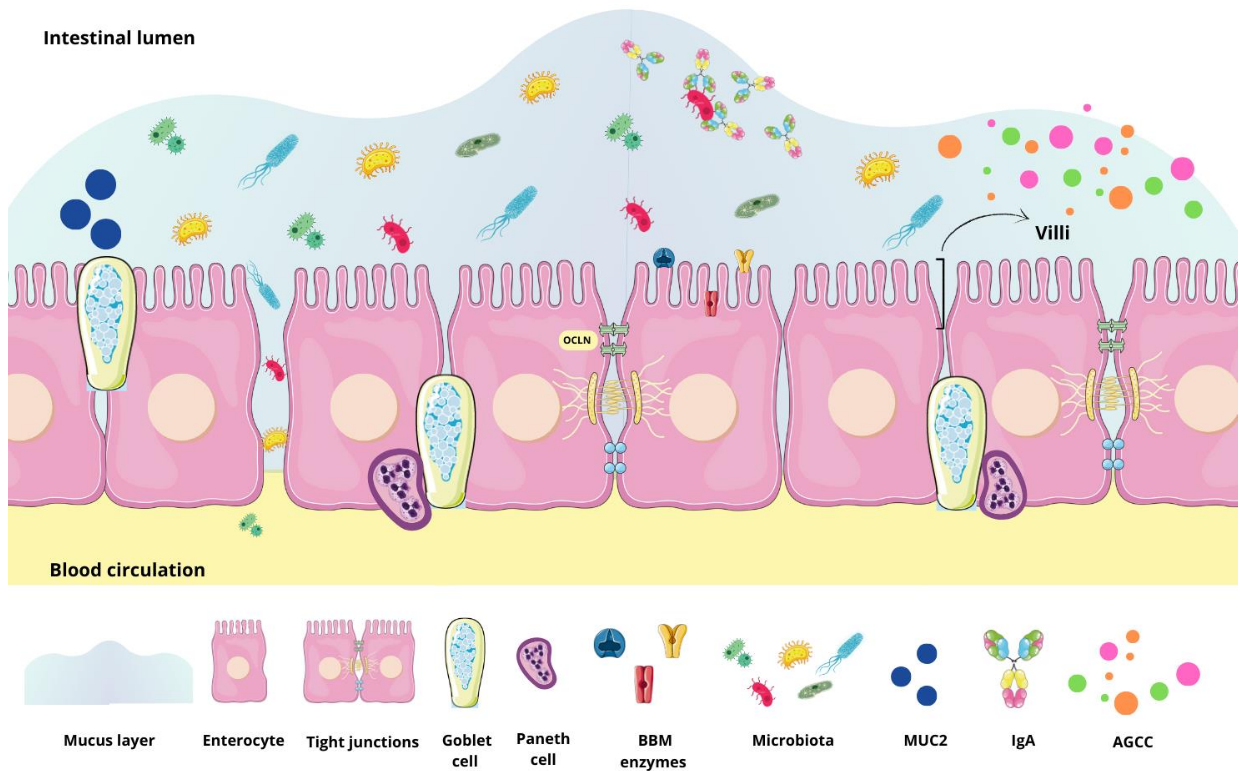
and Palm, 2020). It was demonstrated that mice lacking IgA or with defective mucosal antibody responses exhibit reduced overall microbial diversity, altered bacterial composition, increased susceptibility to intestinal inflammation, and increased bacterial translocation (Yang and Palm, 2020).

IgA targets foreign antigens such as microorganisms and dietary components that are deeply involved in the induction of intestinal IgA. Regarding the relationship between IgA and the intestinal microbiota, what has been found is that specific intestinal microorganisms are capable of inducing intestinal IgA more efficiently than others. Therefore, the need to explore compounds derived from the microbiota, such as metabolites and cellular components that may be determinants of IgA levels, is justified (Geva-zatorsky *et al.*, 2017; Takeuchi and Ohno, 2021). Dietary fiber can be fermented by the microbiota, leading to the production of SCFAs that act in the production of IgA, which can also be modulated by microbial components such as lipopolysaccharides and flagellin. Thus, both the diet and the microbiota work together to regulate the production of IgA in the intestine (Kim, Kim and Park, 2016; Takeuchi and Ohno, 2021).

IgA can bind to and coat microorganisms by several mechanisms. IgA-coated bacteria include both beneficial and potentially pathogenic species. In this way, IgA can act both in the elimination of pathogens, restricting the colonization or expansion of intestinal microorganisms, and in reinforcing the beneficial microbiota, increasing colonization, stability and resilience (Yang and Palm, 2020). Specific IgAs affect the intestinal microbiota, in particular pathobionts, affecting their motility to exclude them from the intestinal surface or inhibiting their growth. On the other hand, IgA can also bind to beneficial microorganisms and anchor them in the mucus layer. IgA can facilitate intestinal colonization by certain microorganisms, such as *Bacteroides*, which are associated with SCFA production and beneficial effects on the intestine. Both types of IgA work together to balance intestinal homeostasis (Rios-Covian *et al.*, 2017; Takeuchi and Ohno, 2021). Although it is not known exactly how IgA discriminates between different bacterial species, it is believed that the carbohydrate moieties of the bacterial surface play a significant role in the selectivity of IgA between taxonomic species (Sterlin *et al.*, 2020). Certain intestinal environments can also affect gene expression and functions of microorganisms and thus affect the relationship between IgA and microbiota. Microorganisms and their metabolites can determine the balance between IgA types, for example, acetate preferentially increases T cell-dependent IgA compared to microbial components in the colon (Takeuchi and Ohno, 2021).

In Figure 1 there is a graphic representation of the intestinal environment and the aforementioned biomarkers.

Figure 1 - Representation of the intestinal lumen and biomarkers of intestinal microbiome, representing an environment with altered intestinal permeability.



Enterocytes are joined by tight junctions (represented by occludin) and there are some regions with reduced tight junctions, which increases intestinal permeability. It is possible to observe Paneth cells, goblet cells and mucus production. Furthermore, the representation of the intestinal microbiota is noted, interacting with immunoglobulin A and short-chain fatty acids as metabolites of the microbial community. In the villi on the brush border membrane, there are transporters and enzymes, indicators of intestinal functionality. IgA: immunoglobulin A; SCFA: short-chain fatty acids; MUC2: mucin 2. Source: The author.

5.3 Inflammation

Inflammation acts as a physiological defense response to maintain homeostasis in the body. However, if the inflammatory defense persists for a long time, it may have destructive roles and cause various chronic diseases, such as diabetes, rheumatoid arthritis, cancer, atherosclerosis, and some other diseases (Li *et al.*, 2023). Intestinal inflammation is often associated with intestinal barrier alterations due to decreased mucin gene expression and/or altered tight junction, resulting in increased permeability. The altered mucus layer and increased

intestinal permeability favors microbial antigens recognition by innate immune receptors which further trigger inflammation. Microbial sensing plays a key role in cytokine production by both the immune and intestinal cells. Following their activation, a large amount of pro-inflammatory cytokines is produced (Padoan, Musso and Contran, 2023).

The transcription factor nuclear factor-kappa B (NF-κB) plays a vital role in the development of inflammation. NF-κB transmits external signals to nuclear and plays a pivotal role in the cell signal transduction. In the inflammatory process, NF-κB pathway could be highly stimulated by pro-inflammatory cytokines, antigens, microbial or viral infections, cellular stresses, viral proteins, tumor necrosis factor receptors, oxidative stress, and so on and then promotes the expression of inflammatory genes by their NF-κB binding sites. Then the transcription factor NF-κB enters into the nucleus and initiates the target gene expression. These target genes include, but are not limited to inflammatory cytokines, such as IL-1β and TNF-α, proliferation, differentiation, and anti-apoptosis. When getting stimulated by inflammatory stimuli, NF-κB transcriptionally regulates the levels of pro-inflammatory cytokines (Li *et al.*, 2023).

Intestinal homeostasis is maintained through the strict balance between cell death and proliferation to prevent intestinal inflammation. In the regulation of this balance, TNF-α is a central inflammatory cytokine that plays a crucial role (Lima *et al.*, 2022; Ruder, Atreya and Becker, 2019). TNF-α, produced by several cell types, such as macrophages, adipocytes, fibroblasts and T cells, determine inflammation or cell death depending on the alternative engagement of its putative receptors followed by NF-kB or the apoptosis pathways activation, respectively. A dysregulation in its levels, together with the increase of some interleukins, can lead to inflammatory bowel disease (Padoan, Musso and Contran, 2023). TNFα plays a pivotal role during steady state or pathologic conditions, for example, infections, injury, inflammation and tumor development. Once released from macrophages, which constitute the first line of defense, TNFα activates other immune cells and mediates production of additional proinflammatory cytokines during inflammatory responses, therefore TNFα is mainly described to function as a proinflammatory cytokine which plays a critical role for maintaining the intestinal integrity but conversely also for the pathogenesis of intestinal inflammation (Ruder, Atreya and Becker, 2019).

Some conditions can increase the inflammation markers in the body, as explained below:

5.3.1 High-fat diet

Excessive consumption of high-fat diets can cause adipocyte hypertrophy. As adipocytes enlarge, adipose tissue undergoes molecular and cellular changes that affect systemic metabolism. It is well established that adipocytes are not only a fat storage reservoir, but are active endocrine units that perform various functions in the body, and their metabolic role changes as they enlarge. One change that occurs in this situation is the increase in the secretion of inflammatory cytokines, such as TNF-α, interleukin 6 (IL-6), interleukin 1 beta (IL-1β), interleukin 8 (IL-8) and interleukin 12 (IL- 12) (Akash, Rehman and Liaqat, 2018; Greenberg and Obin, 2006; Da Silva, Toledo, Grancieri, *et al.*, 2019; Singh and Rai, 2019).

The process of low-grade chronic inflammation, associated with an increase in adipose tissue, originates in adipose tissue, and then reaches the circulation, increasing the plasma concentration of inflammatory biomarkers and the number of activated inflammatory cells. Thus, this inflammatory response is a crucial factor in the etiology of other important conditions such as resistance to insulin action, cardiovascular diseases and alteration of intestinal microbiota (Akash, Rehman and Liaqat, 2018; Gérard, 2016; Gomes, Hoffmann and Mota, 2018; Maruvada *et al.*, 2017). Another factor closely linked to the onset of this type of chronic inflammation is oxidative stress, which occurs as a result of an imbalance between the endogenous production of reactive oxygen species (ROS) and the natural antioxidant system. A crosstalk between adipocytes and resident macrophages reinforces oxidative stress through the generation of ROS mediated by TNF-α within adipose tissue cells (Nani *et al.*, 2021).

Chronic inflammation due to increased adipose tissue has been associated with altered intestinal microbiota, with a reduction in the abundance of *Bifidobacteria*, such as Bacteroidetes, and a proportional increase in Firmicutes, such as *Clostridium* (Gérard, 2016; Maruvada *et al.*, 2017). The increase in *Bifidobacteria* inhibits the growth of potentially pathogenic microorganisms by producing bacteriocins and creating a more acidic luminal environment, which is hostile to the growth of pro-inflammatory bacteria (Quigley, 2019). The increase of potentially pathogenic microorganisms and inflammation alters the barrier function of intestinal epithelial cells, LPS reach the circulation, and these higher levels of endotoxins activate receptors in adipose tissue which, in turn, induce the expression of chemokines and activate the immune response, which further increases the inflammation process (Gomes, Hoffmann and Mota, 2018). A meta-analysis of clinical studies (Pinart *et al.*, 2022) found differences in microbiota (beta diversity) between body mass index (BMI) categories in the majority of included studies, confirming that microbial population abundances in the gut of

obese and non-obese groups were distinct from each other. The same study found evidence that the composition of the gut microbiome of obese people differs from that of non-obese people at both the phylum and gender levels.

The effect of fats on the microbiota differs according to the type of fatty acid, whether saturated, monounsaturated or polyunsaturated fatty acids (Ceballos, Hernández-Camba and Ramos, 2021). Studies have shown that mice fed saturated fatty acids exhibit increased levels of endotoxins in the circulation compared to mice consuming polyunsaturated fatty acids. Also, high intake of fats and saturated fatty acids had a negative impact on microbiota diversity associated with an increase in anaerobic microbiota and *Bacteroides* (Cani *et al.*, 2007; Ceballos, Hernández-Camba and Ramos, 2021). The source of dietary fat may therefore have specific interactions with the microbiota that lead to changes in the relationship with the innate immune system and contribute to metabolic diseases (Sonnenburg and Bäckhed, 2016). In general, priority should be given to oils rich in polyunsaturated fatty acids (Ceballos, Hernández-Camba and Ramos, 2021).

5.3.2 Changes related to reduced function of the female reproductive system

Ovariectomy is consolidated as a good model to simulate the postmenopausal changes in animals, inducing alterations in the body related to osteoporosis, cardiovascular system and inflammation, which shows the impact of low estrogen observed in postmenopausal women. Confirming ovariectomy as a good model to simulate postmenopausal changes (Kalu, 1991; Mishima *et al.*, 2020, 2021).

The menopause transition is a biological event in women, with the average age of onset corresponding to 47 years and the average age for the final menstrual period of 51.4 years. This transition is initially characterized by changes in the menstrual cycle, changes in estrogen and progesterone levels and, finally, by the cessation of menstruation (Khoudary, El *et al.*, 2019; Yang, Heitkemper and Kamp, 2021). This reduction in estrogen levels that occurs in post-menopause is associated with sleep and mood disorders, vasomotor symptoms, urogenital atrophy, skin lesions, metabolic disorders, obesity and the development of chronic diseases (Lobo *et al.*, 2014). Post-menopause has been associated with a change in fat distribution, increased central fat accumulation, dyslipidemia and inflammation (Ambikairajah *et al.*, 2019; Taleb-belkadi *et al.*, 2016), weight gain, in addition to greater waist circumference, BMI and adiposity in an animal model (Mishima *et al.*, 2020).

Estrogen modulates the concentration of reactive oxygen species through a mechanism that involves interaction with its nuclear receptors, decreasing oxidative proteins and/or increasing the expression of antioxidant enzymes (Novella *et al.*, 2012). Sex hormones also alter the production of inflammatory cytokines; therefore, estrogen can also act on the inflammatory response (Corcoran *et al.*, 2011). Sex hormones were also related to gastrointestinal functions and a higher prevalence of symptoms of irritable bowel syndrome or functional dyspepsia and/or a greater severity of symptoms was found in women compared to men (Yang, Heitkemper e Kamp, 2021). The Across the Nation Women's Health Study report showed that intestinal permeability increased from pre-menopause (at an average age of 49.9 years) to post-menopause (at an average age of 57.5 years) (El Khoudary *et al.*, 2019). The study by Shieh (2020) supports the hypothesis that, in humans, intestinal permeability increases from pre- to post-menopause, and suggests that greater intestinal permeability is associated with greater inflammation, and greater intestinal permeability. In a rodent model, increased intestinal permeability is linked to increased inflammation related to the menopausal transition. Menopause (chemical or surgical) led to decreased expression of epithelial junction proteins (claudins 1, 2 and 3) and increased intestinal permeability (Li *et al.*, 2016). The increased permeability allows the translocation of microorganisms from the intestinal lumen to the subepithelial space, inducing immune cells to produce pro-inflammatory cytokines (Shieh *et al.*, 2020).

There is growing interest in the dynamic role of microbiome disorders in health and disease, and the gut microbiome is intrinsically linked to estrogen metabolism, menopausal status, and systemic inflammation in women (Schreurs, Romano and Werner, 2021). Studies that compared the intestinal microbiota of men and women found differences in the relative abundance of several genera (Haro *et al.*, 2016; Min *et al.*, 2019), and alpha diversity was lower in the postmenopausal compared to the premenopausal state (Santos-marcos *et al.*, 2018; Shin *et al.*, 2019; Zhao *et al.*, 2019). The study by Min and colleagues (2019) hypothesized that the gut microbiome is not only associated with general obesity, but also with fat distribution. As men and women differ in both the proportion and distribution of total body fat, there would be a specific, sex-related microbiome associated with fat distribution. Thus, the researchers found that as the abundance and diversity of the microbiome increased, the proportion of android fat (accumulation of fat in the abdominal region) decreased in both sexes and suggested a potential negative association between the proportion of gynoid fat (fat that is distributed in the lower half of the body, particularly in the region around the hips and thighs) and the abundance of the

microbiome in both sexes. The sex-induced difference in regional adiposity could lead to differences in microbiome species that, in turn, modulate fat distribution.

Data on a higher waist/hip circumference ratio were related to a greater presence of the Firmicutes phylum, obesity and inflammatory markers. Women with high circumferences (such as high intra-abdominal fat) presented increased systemic inflammatory markers, such as highly sensitive C-reactive protein (hsCRP), TNF-α and leptin (Miranda *et al.*, 2019). The review by Schreurs (2021) suggests that menopausal changes and female obesity are correlated with intestinal microbiome imbalance, including studies that indicate the relative abundance of Firmicutes increased and that of Bacteroidetes decreased, resulting in a higher Firmicutes to Bacteroidetes ratio in post-menopause (Santos-marcos *et al.*, 2018; Shin *et al.*, 2019; Zhu *et al.*, 2018). At the family and genus levels present in the gut microbiome, the most relevant findings concerned a change, predominantly reduction, in SCFA-producing organisms at menopause (Santos-marcos *et al.*, 2018; Shin *et al.*, 2019). The studies included showed that, in general, with increasing weight, the diversity of the intestinal microbiome reduced (Chávez-Carbajal *et al.*, 2019; Min *et al.*, 2019; Miranda *et al.*, 2019) and that this correlation was more pronounced in postmenopausal individuals. Obesity and menopause can lead to a shared alteration in gut microbiome, which can be recognized by an altered gut microbial diversity, as well as the ratio of Firmicutes to Bacteroidetes. Thus, a relationship is highlighted between the intestinal microbiome and systemic health, negatively affected by menopausal changes.

5.4 Prebiotics

Prebiotic is defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson *et al.*, 2017). Most prebiotic compounds are carbohydrates with a variety of molecular structures that occur naturally in the human diet. Common types of prebiotics include inulin, fructooligosaccharides, galactooligosaccharides, and lactulose. They can be found in various foods such as whole grains, onions, garlic, and bananas (Antony *et al.*, 2023; Ji, 2023). In general, any residue from the upper digestive system that reaches the bacteria in the colon has the potential to modulate the microbiota. Currently established prebiotics are carbohydrate-based, but other substances such as polyphenols, proteins and polyunsaturated fatty acids, converted to respective conjugated fatty acids, might fit this definition (Gibson *et al.*, 2017). An *in vitro* study showed that when fiber and protein were combined, and also when pure protein was used as a substrate, the production of SCFA was maintained or increased. The production of propionate and butyrate increased with increasing content of protein, indicating that indigestible proteins in fiber-rich substrates can promote the production of microbial

SCFAs. Thus, suggesting potential use of slow fermentation fibers mixed with low digestibility proteins to promote SCFA-producing bacteria in the distal colon (Jackson et al., 2024).

Prebiotics will promote bacteria that already exist in the intestine, thus, they can modulate the intestinal microbiota and improving the microbiota is related to health benefits. Prebiotics beneficially affect the host by selectively stimulating the growth and activity of one or several types of bacteria in the colon such as *Bifidobacteria* and *Lactobacilli*, reduce the abundance of pathogenic bacteria, and thus help to maintain a balanced gut microbiota. They essentially serve as "food" for the beneficial bacteria in the gut, helping to increase their numbers and promote a healthier microbiome (Ji, 2023). They are nondigestible by human enzymes, so they reach the colon intact where they are fermented by the gut bacteria. Simultaneously produced metabolites can provide energy to the cells lining the colon, regulate the immune system through increased production of interleukins and immunoglobulins with reduction of pro-inflammatory interleukins, enhance gut barrier function, production of SCFAs, including acetate, propionate, and butyrate (Antony *et al.*, 2023; Ji, 2023).

Gut microbiota is intimately involved in regulating energy metabolism, immune response, and inflammation of the host. Altered gut microbiome has been associated with enhanced energy extraction from the non-digestible dietary carbohydrates, increased gut permeability, increased translocation of LPS resulting in systemic inflammation. Gut microbiota modulation can be achieved by dietary manipulation using nutritional interventions including prebiotics, probiotics, and synbiotics, and is suggested to have a major positive impact on obesity-associated imbalance in gut microbiota (Balas *et al.*, 2023; Sankararaman *et al.*, 2023).

5.4.1 Dietary fiber as a prebiotic

Dietary fiber is composed of carbohydrate polymers, which are neither digested nor absorbed in the human intestine and pass into the large bowel, where they are partially or completely fermented by the colonic microbiota (Fuller *et al.*, 2016; Vitetta *et al.*, 2023). Not all dietary fibers can be classified as prebiotic. Currently, only the resistant oligosaccharides, fructans and galactans, are well documented in literature as prebiotics. Other fibers are candidates to prebiotics or have prebiotic potential, but more studies are warranted to prove such effects, and there are fibers that seem to have no prebiotic effect (Rezende, Lima and Naves, 2021; Vitetta *et al.*, 2023).

In addition to the benefits associated with a reduced risk of developing chronic diseases, dietary fibers may affect the composition and metabolic activity of intestinal bacterial communities. The chemical structure of prebiotics and the bacterial composition of the intestines determine the fermentation products produced. The biochemical process of the fermentation of fiber-rich compounds by the intestinal microbiota have been shown to increase the colonic luminal concentration of SCFAs (Rezende, Lima and Naves, 2021; Vitetta *et al.*, 2023).

Bacteria degrade specific structures, and all the structural details of the fibers will influence which bacteria ferments the structure (monosaccharide composition, polymer size, distribution of branches, types of bonds...). Then, the impact of fiber consumption on the gastrointestinal microbiota will vary based on the type of fiber consumed (Cantu-jungles and Hamaker, 2023; Holscher, 2017; Markowiak and Ślizewska, 2017). The speed and degree of fermentation of prebiotic dietary fibers by the intestinal microbiota depend on factors such as solubility, chain size, porosity, total surface area of the particles, and the structure and organization of the fiber cell wall. Generally, soluble fibers are fermented more quickly and to a greater extent than insoluble fibers, while oligosaccharides are fermented faster than polysaccharides. More complex dietary fiber structures require more enzymes to degrade these structures, so not all bacteria will grow with this type of structure and competitiveness is reduced. In this way, certain fibers stimulate specific bacteria and, knowing the way the bacteria act, it is possible to select the type of prebiotic for it to grow, aligning the physical-chemical structure of the fibers to promote the growth of a certain group of bacteria. Thus, it is expected that the fermentation result of a mixture of fibers will not be equal to the fermentation result of each dietary fiber individually. The dose and the type of the dietary fiber consumed, either isolated, in solution, or inside the original food matrix can influence the fermentability in the intestine (Cantu-jungles and Hamaker, 2023; Fuller *et al.*, 2016; Holscher, 2017; Rezende, Lima and Naves, 2021).

Despite the variety of carbohydrates that exhibit the prebiotic activity, the effect of their administration is an increased count of beneficial bacteria, mostly of the *Lactobacillus* and *Bifidobacterium* genus. These bacterial communities could provide benefits to the host's health, such as an improvement of the intestinal barrier function, the maturation and regulation of the immune system, and the ability to suppress the invasion of pathogens (Markowiak and Ślizewska, 2017; Rezende, Lima and Naves, 2021; Sanders *et al.*, 2019).

5.4.2 Phenolic as a prebiotic

Phenolic compounds are presented as prebiotic substrates, due to the fact that not all of them are absorbed in the small intestine. Thus, most of them reach the colon microbiota, serving as a substrate for this resident microbiota, providing a wide range of effects, mainly modulating the intestinal microbiota and maintaining its homeostasis. Compared to other compounds such as carbohydrates, lipids and proteins, phytochemicals are not necessary for physiological functions but can induce biological effects. For example, after absorption, they can improve the integrity of the intestinal barrier, inducing an increase in the expression of tight junctions (Dingeo *et al.*, 2020; Plamada and Vodnar, 2022).

Polyphenols exert their beneficial effects as a prebiotic substrate, increasing the growth and establishment of families of probiotic bacteria, such as Bifidobacteriaceae and Lactobacillaceae, and, on the other hand, reducing the number of pathogenic bacteria, such as *Escherichia coli*, *Clostridium perfringens* and *Helicobacter pylori*. These impacts on the intestinal microbiota occur through various means, including antioxidant, anti-inflammatory, bactericidal, immunological functions and mucus production and SCFA have been ascribed as main mediators of polyphenols metabolic effects, also the polyphenols modulatory effects on the gut microbiota might be due the synergy between their prebiotic and antimicrobial properties (Dingeo *et al.*, 2020; Freitas *et al.*, 2022).

Due to their bioavailability, the polyphenol effects are closely related to the food matrix, depending on whether they are consumed as isolated compounds or in the whole food (Plamada e Vodnar, 2022). The effects of isolating phenolic extracts on intestinal health, nutrient digestibility, absorption of some vitamins and minerals, and intestinal microbiota may differ. Depending on their dosage, polyphenols can have negative impacts due to interference with nutrient metabolism. Therefore, phenolic compounds and other bioactive compounds from food sources need to have their effects and safety validated *in vivo* (Abdel-Moneim *et al*., 2020).

5.5 Probiotics

Probiotics are defined as "live microorganisms that, when administered in adequate quantities, confer a health benefit on the host". Certain effects can be attributed to probiotics as a general class, considering strains of several well-studied microbial species delivered in a functional dose for use as foods or supplements in the general population (not strains used as drugs). There is a list of bacteria that represent a core group of well-studied species that may bring some general benefits by contributing to a healthy intestinal microbiota, such as bacteria belonging to the genus *Bifidobacterium* (*adolescentis, animalis, bifidum, breve* and *longum*)

and *Lactobacillus* (*acidophilus, casei, fermentum, gasseri, johnsonii, paracasei, plantarum, rhamnosus* and *salivarius*) (Hill *et al.*, 2014).

It is considered that supporting a healthy intestinal microbiota is a central benefit of probiotics, although the current state of science does not allow the clear definition of a healthy intestinal microbiota based only on microbial composition. Probiotics can positively influence the composition of the intestinal microbiota and interact with different immune cells, thus improving immune functions (Ji, 2023; Mazziotta *et al.*, 2023). However, the overall benefit of probiotics on the gut microbiota derives from the creation of a more favorable intestinal environment, through mechanisms shared by most probiotics. The International Scientific Association of Probiotics and Prebiotics also considered two common general benefits often associated with probiotics: supporting a healthy digestive tract and immune system. Some mechanisms are widespread among a diversity of strains, others are less so, others can be observed frequently among most strains of a probiotic species; and yet, others may be rare and present in only a few strains of a given species. Generalized mechanisms may be associated with effects that are observed across taxonomic groups, such as the inhibition of potential pathogens or the production of useful metabolites or enzymes. Other effects at the intestinal or extraintestinal level, including immunological effects, are more likely strain-specific (Hill *et al.*, 2014).

The use of probiotics, specifically *Lactobacillus* and *Bifidobacterium* genera, can improve intestinal alterations in microbiota and mediate the activation/modulation of innate and adaptive immune responses in the intestine, stimulating the production of cytokines and chemokines from dendritic cells, lymphocytes, macrophages, granulocytes, intestinal epithelial cells and IgA-producing cells and consequent secretion of IgA. Probiotics can therefore improve the host's immune system and induce important beneficial effects, allowing the prevention and/or reducing the risk of immunological/inflammatory diseases, including inflammatory bowel disease, irritable bowel syndrome, inflammation, diarrhea, pathogenic infections, improve serum lipid levels, regulate the production of short-chain fatty acids, and improve metabolic parameters (Mazziotta *et al.*, 2023; Mijangos-trejo *et al.*, 2023).

The strain(s), dose, duration of intake and time of ingestion of the probiotic are determining factors to be considered so that the probiotics have the expected effects (Araújo and Botelho, 2022). In many cases, a given probiotic can exert several health-promoting effects. Although multiple mechanisms are often represented in a single strain, no single probiotic is expected to have all of the effects found in the literature. It is possible to support the concept of

central benefits of certain probiotics, while each probiotic strain is different and likely to elicit a different outcome in the host. Thus, there are common health benefits derived from consuming (or delivering) an adequate dose of any safe strain of a species that is known to include an effective probiotic (Hill *et al.*, 2014).

5.5.1 *Lactobacillus*

Lactobacillus are gram-positive, fermentative, facultatively anaerobic and non-spore-forming. Many strains of *Lactobacillus* are used in food fermentation and are typically used in the dairy industry to produce cheese, yogurt, and other fermented dairy products. *Lactobacillus* are found in a variety of habitats, such as human and animal mucous membranes, in plant material and fermented dairy products. They are important for immunity, maintenance of intestinal microbial balance and prevention of gastrointestinal infection (Aprea *et al.*, 2023; Chiang and Pan, 2012).

The genus *Lactobacillus* has received much attention due to its biological activities and intestinal health benefits. *Lactobacillus* species are considered autochthonous resident in the gastrointestinal tract of humans, rodents and chickens. They can promote intestinal health and host immune function in different ways, such as strengthening the epithelial barrier, competitively rejecting pathogenic microorganisms, producing antimicrobial substances, and interacting with immune cells by stimulating pattern recognition receptors (Duan *et al.*, 2021). Studies showed that *Lacticaseibacillus paracasei* positively regulated the expression of tight junction proteins, increased SCFA production, negatively regulated the production of pro-inflammatory cytokines and altered the structure of the intestinal microbiota (Danshiitsoodol *et al.*, 2022; Ren *et al.*, 2022; Zeng *et al.*, 2021). Also, *L. paracasei* was able to regulate the expression of genes related to lipid metabolism, improve dyslipidemia, reduce lipid accumulation, oxidative stress injury and inflammatory response, in addition to improving the intestinal microbiota, reducing the Firmicutes/ Bacteroidetes and increasing the abundance of *Akkermansia* (Liu *et al.*, 2022). *Lactobacillus* are considered probiotics for their immunomodulatory and anti-inflammatory actions, inhibition of bacterial toxins, competition with pathogens and for stimulating the release of antimicrobial substances, such as mucin that activates the mucin 2 gene and prevents the adhesion of pathogens to the epithelial barrier (Yeşilyurt *et al.*, 2021).

The *Lacticaseibacillus paracasei* used in this study is an innovative culture to be tested in vegetable products without fermentation with a promising probiotic effect. It was isolated from *coalho* cheese and cultivated in extruded sorghum flour. It is a potential probiotic and the

first one completely isolated and produced in Brazil. Probiotic food products represent a market trend, associated with growing consumer awareness of the relationship between food and well-being and, above all, intestinal health. In this sense, it is important to determine the possibilities of using the bioactive compounds that we are studying, in order to find better health-promoting results, whether through the consumption of food in its more complex form, or through the use of isolated compounds, or even the combination of bioactive compounds with each other, or with other options such as probiotics.

5.6 *In ovo* nutrients administration

In ovo nutrient administration was first applied in the 1980s for vaccination against Marek's disease (Sharma and Burmester, 1982). Currently, this feeding model, mainly intra-amniotic administration, is widely used as an *in vivo* method to evaluate the effects of bioactive compounds of plant origin on intestinal health, including effects on intestinal brush border membrane (BBM) functionality, microbiota and intestinal morphology, and dietary mineral bioavailability. This type of approach is a useful and cost-effective *in vivo* method for evaluating the prebiotic effects of nutrients (Hou e Tako, 2018; Da Silva, Martino and Tako, 2021).

Two moments are suggested in the literature during embryonic development for the *in ovo* procedure, namely: on the 12th day of embryonic development, when the chorioallantoic membrane is fully developed and vascularized, and the embryo is surrounded by amniotic fluid that remains in contact with the embryonic gastrointestinal tract, which allows the transport of substances from the air chamber to the intestine (Sobolewska *et al.*, 2017). Or, on the 17th day, just before oral consumption by the embryo of the amniotic fluid, which occurs on the 19th day (Uni and Ferket, 2003). It is also important to know that the embryos are transferred from the incubator to the incubation basket on day 17-18, which may be an appropriate time to administer nutrients in a practical way. Thus, the egg compartment targeted for injection is the amniotic fluid, on day 17 of embryonic development, and this was the procedure adopted in this work. Another point to be considered when using this methodology is that the solution that will be injected (with a specific size needle) must maintain an osmolarity value ≤320 OSM to ensure that the embryo is not dehydrated (Hou and Tako, 2018).

The chicken (*Gallus gallus*) animal model contains a complex and dynamic intestinal microbiota, strongly influenced by host genetics, environment and diet (Yegani and Korver, 2008). The intestinal microbiota of these animals is similar to that of humans in terms of the types of bacteria predominant in the gastrointestinal tract. There is considerable similarity at the phylum level with Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria

representing the four dominant bacterial phyla in both (Hou, Kolba, Glahn *et al.*, 2017; Reed *et al.*, 2017).

The use of the *in ovo* feeding model for compounds such as peptides, isoflavones, carbohydrates and synbiotics of plant origin resulted in an indication of the prebiotic effects of these compounds, such as mineral absorption, intestinal microbiota composition, intestinal morphology, short chain fatty acid content and immune system response (Hou and Tako, 2018). It is suggested that following *in ovo* administration, intestinal bacterial populations are affected, particularly as the population of beneficial bacteria increases. The increase in beneficial bacteria (such as *Bifidobacterium* and *Lactobacillus*) promotes the production of short-chain fatty acids. The morphological effects (increased surface area of villi and number of goblet cells) can potentially stimulate the expression of intestinal functional genes, mainly proteins that are necessary for intestinal absorption of minerals. Furthermore, the administration of prebiotics *in ovo* also indicates a positive effect on the immune system (Hou and Tako, 2018; Da Silva, Martino and Tako, 2021; Wang *et al.*, 2019).

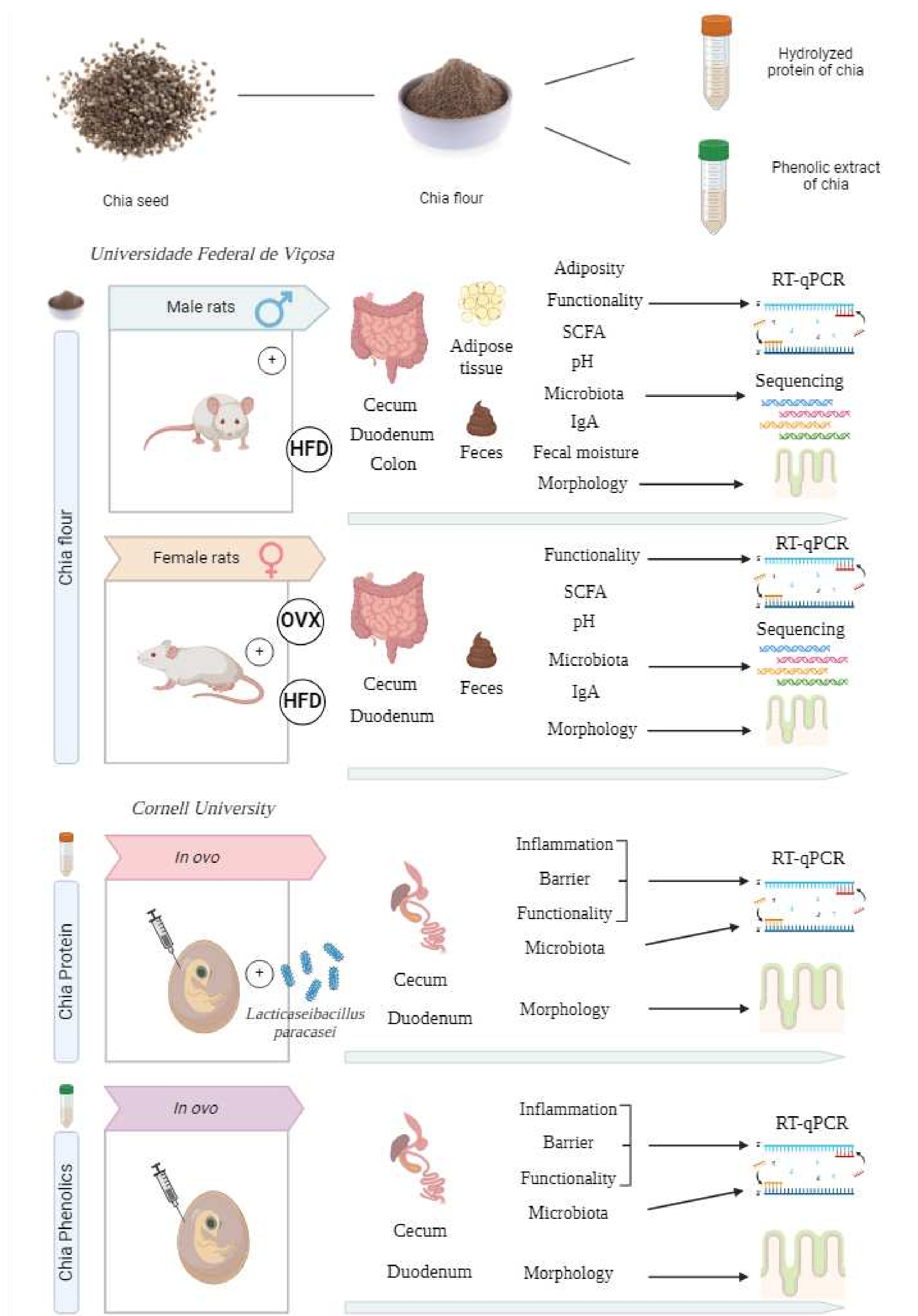
The dietary fiber fraction of chia seeds was tested in an intra-amniotic administration study (*in ovo*), and this extract was able to positively regulate the gene expression of proteins related to mineral metabolism, in addition to affecting the morphology and composition of the intestinal microbiota (Da Silva *et al.*, 2019a). Thus, the investigation of the effects of intra-amniotic administration of the protein fraction and a phenolic extract from chia are innovative studies, which continue the investigation of the effects of bioactive compounds from chia seed *in vivo*.

6 GENERAL METODOLOGY

The experiments with *Wistar* rats were carried out at the Experimental Nutrition Laboratory of the Department of Nutrition and Health at *Universidade Federal de Viçosa* (UFV), as well as their respective analyses. The surgical procedure (ovariectomy) was performed at the Veterinary Hospital in *Universidade Federal de Viçosa* (Department of Veterinary Medicine/UFV). The Laboratory of Metabolism and Fermentation, Laboratory of Biochemistry and Molecular Biology of Infectious and Parasitic Agents (Department of Biochemistry/UFV), Laboratory of Microbiology of Anaerobes and Animal Microbiota (Department of Microbiology/UFV) were used for analysis. The sequencing of samples for intestinal microbiota analysis was carried out at the Environmental Sample Preparation and Sequencing Facility (ESPSF) at Argonne National Laboratory (Lemont, Illinois, IL). The hydrolyzed protein of chia was produced at Department of Food Science and Human Nutrition at University of Illinois, Urbana-Champaign. The *in ovo* experiments and analyzes were carried out at the Food Science Department of the Cornell University, Ithaca, NY, United States.

The thesis was conducted in 3 phases: first the experiments with male and female rats, second a theoretical phase conducting a systematic review and a third phase with *in ovo* experiments (Figure 2).

Figure 2 – Experiments design



The present study investigated the functional properties of chia flour, its hydrolyzed protein and phenolic extract on microbiota and gut health in different conditions and *in vivo* models. The chia flour was

consumed by male rats and female rats, ovariectomized or not. In both studies, the following parameters were evaluated: intestinal functionality by RT-qPCR, morphology by histomorphometry and microbiota by sequencing, the production of short chain fatty acids, IgA and intestinal pH were also evaluated. The hydrolyzed protein (with or without a probiotic) and the phenolic extract were isolated from the chia seeds and administered *in ovo*. The following parameters were evaluated: intestinal functionality, inflammation, barrier and microbiota by RT-qPCR and morphology by histomorphometry. HFD: high-fat diet; SFCA: short-chain fatty acids; IgA: immunoglobulin A; RT-qPCR: real time quantitative reverse transcription polymerase chain reaction; OVX: ovariectomy. Source: The author, created with BioRender.com

6.1. Raw material

6.1.1 Chia seeds

The chia seeds (*Salvia hispanica* L.) used in this study were cultivated in the state of Rio Grande do Sul (Brazil), planted in January 2015 and harvested in June 2015 (Da Silva *et al.*, 2017). The seeds were provided by the company Dubai Alimentos, from Ijuí, Rio Grande do Sul, Brazil. The characterization of the seeds was performed by our research group (Da Silva *et al*., 2017). Chia seeds showed brown pericarp and approximate diameter of 2 mm. The state of Rio Grande do Sul (Brazil) has temperate climate, with relative humidity ranging from 60 to 80%. The soy type is clay soil, and the rainfall is regular. The samples were packaged and transported to the laboratory in cardboard boxes, and then stored in hermetically sealed plastic bags, protected from light, and kept at freezing temperatures (-18 ºC ± 1 ºC) until use.

6.1.2 Chia flour

To obtain the flour, the seeds were ground in a knife mill (Marconi Equipment, Piracicaba, SP, Brazil) until they obtained a particle size of 850 µm.

6.1.3 Hydrolyzed protein

Chia seed samples were prepared according to Orona-Tamayo *et al* (2015) and the total digested protein was produced and analyzed in a laboratory coordinated by Prof. Dr. Elvira Gonzalez de Mejia at the University of Illinois, USA as detailed by Grancieri *et al*. (2019b).

The seeds were immersed in distilled water (1:20 / g: mL) for 1 hour to form mucilage and frozen overnight (-80 °C). Subsequently, the seeds were freeze-dried (Labconco Freeze Dryer 4.5; Kansas, Missouri, MO) and the mucilage was manually removed from the seeds using a sieve (500µm/35 mesh). The mucilage-free seeds were crushed using a coffee grinder (Mr. Coffee®, Cleveland, Ohio, OH) and sieved (500µm/35 mesh) to obtain a uniform flour. To remove lipids, hexane (1:10 g: mL) was added at 60°C for 2h, under constant stirring.

Subsequently, it was centrifuged (6000 g, 15 min, 4 ºC), the supernatant was discarded, and the precipitated flour was left overnight in an air exhaust hood and then stored at 4 ºC until use.

To extract the protein, deionized water was added to the fiber- and lipid-free flour, previously prepared, at a concentration of 1:20 (g: mL), the pH was adjusted to 8.0 and kept under constant stirring at 35 °C for 1 h. Then the mixture was centrifuged (5000 g; 15 min; 25 °C), the precipitate was discarded, and the supernatant (protein concentrate) was collected, freeze dried and stored at -20 °C.

For the digestion of the extracted protein, a previously described technique was used (Megías *et al.*, 2004) in which gastrointestinal digestion is simulated. Briefly, the extracted protein was suspended in deionized water (1:20 g: mL), the pH adjusted to 2.0 and pepsin added at a concentration of 1:20 (enzyme: protein) and kept under stirring for 2h, at 37 °C. Afterwards, the pH was adjusted to 7.5, pancreatin (1:20 enzyme: protein) was added and digestion was then conducted as above. At the end, the simulated digestion was stopped by placing the mixture in a water bath (75°C, 20 min) and then centrifuged twice at 20,000g for 15 min at 4 °C. The supernatant was collected (digested total protein) and dialyzed using a 100-500 Da molecular weight exclusion membrane (Spectra/Por®, Biotech CE Membrane), freeze-dried and stored at -20 °C until use.

6.1.4 Phenolic extract

To extract chia phenolics, 20g of chia flour was mixed with 100 mL of methanol: water (80:20, v/v), then the mixture was placed in an ultrasonic water bath for 60 min at room temperature and then taken to centrifuge at 1792g for 30 min. The supernatant was collected and taken to a rotavapor at 50 °C, thus obtaining the extraction of total phenolics. Next, acid hydrolysis was carried out by dissolution, the proportion of 1 mL of sample to 1 mL of HCl (2M) was taken to a water bath at 80 °C for 60 min. The extracted hydrolyzed phenolics were stored in sealed plastic tubes (Falcon®) and frozen at -80°C until freeze-drying.

6.1.4.1 Determination of total phenolics

The total of phenolic compounds was determined using the Folin-Ciocalteau reagent (Singleton, Orthofer e Lamuela-Raventos, 1999). For analysis, 500 µL of extract prepared for determination of antioxidant activity were added to 500 µL of 20% Folin-Ciocalteau solution and 500 µL of 7.5% sodium carbonate solution. Then, the solution was vortexed and left to rest for 30 minutes at room temperature (22 °C).

Absorbance reading was performed on a spectrophotometer (Thermo Scientific, Evolution 606, Waltham, Massachusetts, MA) at 765 nm. Quantification was carried out using the analytical curve obtained by reading the absorbance of solutions with different concentrations of gallic acid. The results were expressed in milligrams of gallic acid equivalents per gram of sample (mg GAE/g).

6.1.4.2 Radical scavenging activity (DPPH)

In a test tube, properly protected from light, 100µL of the extract was added to 1.5 mL of methanolic DPPH (1,1-diphenyl-2-picrylhydrazyl) solution and vortexed for 60 seconds. After resting for 30 minutes at room temperature, the absorbance of the solution was read using a spectrophotometer (Thermoscientific, Evolution 606) at 517 nm. The analytical curve was constructed using 0, 5, 15, 35, 70 and 100 µmol/L trolox solution (Trolox concentration 1.5mM). Antiradical activity (AAR) was expressed in µmol of trolox equivalent/g of sample (µmol trolox/g) (BLOOR, 2001).

6.1.5 Probiotic *Lacticaseibacillus paracasei*

The freeze-dried probiotic *Lacticaseibacillus paracasei* (strain TRA038563) was supplied by the Brazilian Agricultural Research Corporation (EMBRAPA) in powder form. The viability analysis of the probiotic *Lacticaseibacillus paracasei* was carried out by the same corporation. The probiotic was isolated from *coalho* cheese and cultivated in extruded sorghum flour, making it the first probiotic fully isolated and produced in Brazil. Enumerations of viable *Lacticaseibacillus paracasei* cells were performed by deep plating on De Man Rogosa Sharpe (MRS) agar and incubation in anaerobiosis at 37 °C for 72h (Potz *et al.*, 2017).

6.2. Experiment 1 – Effects of chia flour intake by male *Wistar* rats

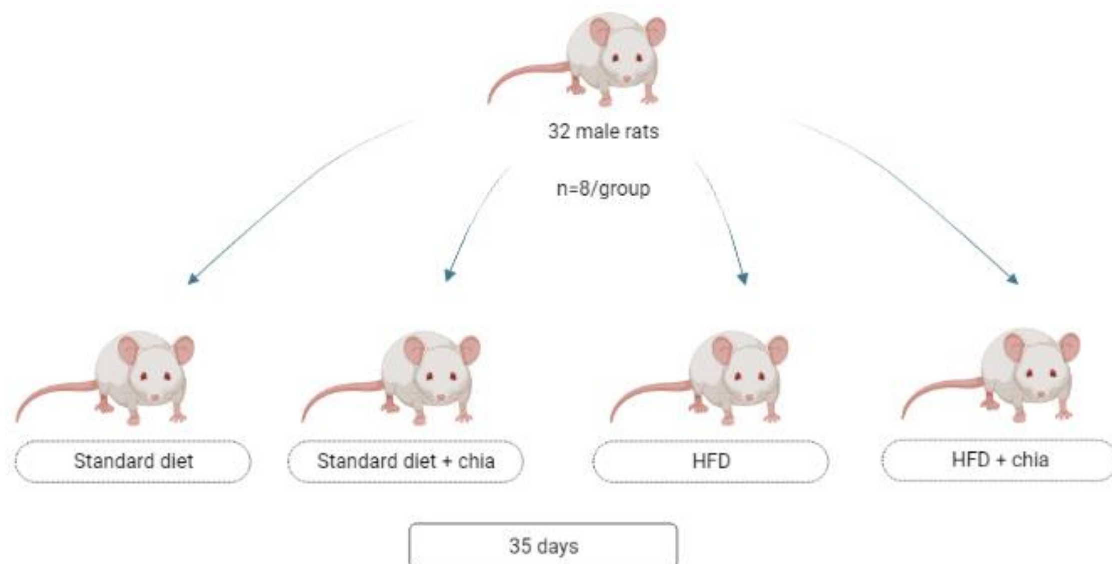
6.2.1 Experimental design

Thirty-two male rats (n=8/group) (*Rattus norvegicus*, *Wistar* lineage, albinus variation), recently weaned, aged 21 days were obtained from the Animal House of the Center for Biological and Health Sciences at *Universidade Federal de Viçosa*, Viçosa (MG). The sample calculation was based on the methodology proposed by Conagin (1959), with each animal considered a replicate. The animal protocols were submitted to the Ethics Committee on the use of Animals at the *Universidade Federal de Viçosa* (Protocol 97/2014) (Appendix A). The animals were systematically divided into 4 groups with 8 animals each, randomized by body weight and distributed in individual stainless-steel cages, in a temperature-controlled

environment (22°C ± 2°C) and automatically controlled 12-hour light and dark cycles. The animals received distilled water and their respective experimental diets *ad libitum*.

The composition of the diets was based on the standard diet for growing rodents (AIN-93G) (Reeves, Nielsen and Fahey, 1993) in powder form (20% proteins, 30% lipids and 50% carbohydrates) or a high-fat diet (64% lipids, 16% proteins and 20% carbohydrates) with modifications depending on which group they were allocated to. The experimental groups received the following diets for five weeks: standard (AIN-93G) (Reeves, Nielsen e Fahey, 1993); standard (AIN-93G) + chia; high-fat diet (HFD) and high-fat diet (HFD) + chia (Figure 3).

Figure 3 – Experimental design 1.



The figure represents the distribution of experimental groups and the chronological order of how the experiment was carried out. Briefly, the animals were divided in four groups receiving a standard diet with or without chia or a high-fat diet with or without chia, for 35 days. n = number of animals per group; HFD: high-fat diet. Source: The author, created with BioRender.com.

At the end of 35 days, after fasting for 12 hours, the animals were anesthetized with isoflurane (3%/body weight, Isoforine, Cristália®, Itapira, São Paulo, Brazil) and euthanized by cardiac puncture. The cecum, cecal contents, duodenum, colon, adipose tissue (visceral, gonadal, retroperitoneal, mesenteric and inguinal) and excreted feces were collected. The weight of the cecum was measured and the cecal content was stored in sterile microtubes, immediately frozen in liquid nitrogen and stored at -80 ºC for analysis of microbiota, pH, short-

chain fatty acids and IgA measurement. The duodenum was also immediately frozen in liquid nitrogen and stored at -80 ºC for analysis of intestinal functionality. The colon was fixed in 10% formaldehyde and kept at room temperature for histological analysis. Adipose tissues (visceral, gonadal, retroperitoneal, mesenteric and inguinal) were weighed individually, and the percentage of adiposity was calculated by summing the weights of visceral, gonadal, retroperitoneal, mesenteric and inguinal adipose tissues, divided by total body weight and multiplied by 100 (Pereira *et al.*, 2012). The excreted feces were collected at the end of the experiment, and the moisture content of the feces, was determined using the gravimetric method. The samples were dried in an oven at 105° C for 24 hours (AOAC, 2016).

6.2.2 Experimental diets

The diets used in the experiment were prepared in the Experimental Nutrition Laboratory. Albumin was used as a protein source and lard as a saturated fat source for the high-fat diet. Chia seeds were ground to obtain flour before preparing the diets. The amount of chia was determined based on the human consumption of 40g of chia/day (2 tablespoons) (Silva *et al.*, 2021). The other ingredients of the diets were added in sufficient quantities to match the composition of lipids, proteins, carbohydrates, fiber and calories of the corresponding diets (Table 1).

Table 1 - Compositions of the experimental diets, experiment 1.

	Experimental Diets			
Ingredients (g/kg of diet)	**AIN-93G**	**AIN-93G +chia**	**HFD**	**HFD+chia**
Albumin *	217.90	117.60	217.92	117.60
Chia flour	0.00	416.80	0.00	416.80
Dextrinized starch	132.00	132.00	132.00	115.10
Sucrose	100.00	100.00	100.00	100.00
Lard	0.00	0.00	200.00	200.00
Soybean oil (mL)	134.20	0.00	134.20	0.00
Microcrystalline cellulose	139.20	0.00	139.20	0.00
Mineral mix	35.00	35.00	35.00	35.00
Vitamin mix	10.00	10.00	10.00	10.00
L-cystine	3.00	3.00	3.00	3.00
Choline bitartrate	2.50	2.50	2.50	2.50
Corn starch	221.20	183.10	21.20	0.00
Total calories (kcal)	3700.42	3624.75	4700.42	4624.11
Caloric density (kcal g^{-1})	3.70	3.62	4.70	4.62

* Considering that albumin is 78% pure. AIN-93G: standard diet for growing rodents, HFD = high fat-diet.

6.3 Experiment 2 – Effects of chia flour intake by female *Wistar* rats

6.3.1 Experimental design

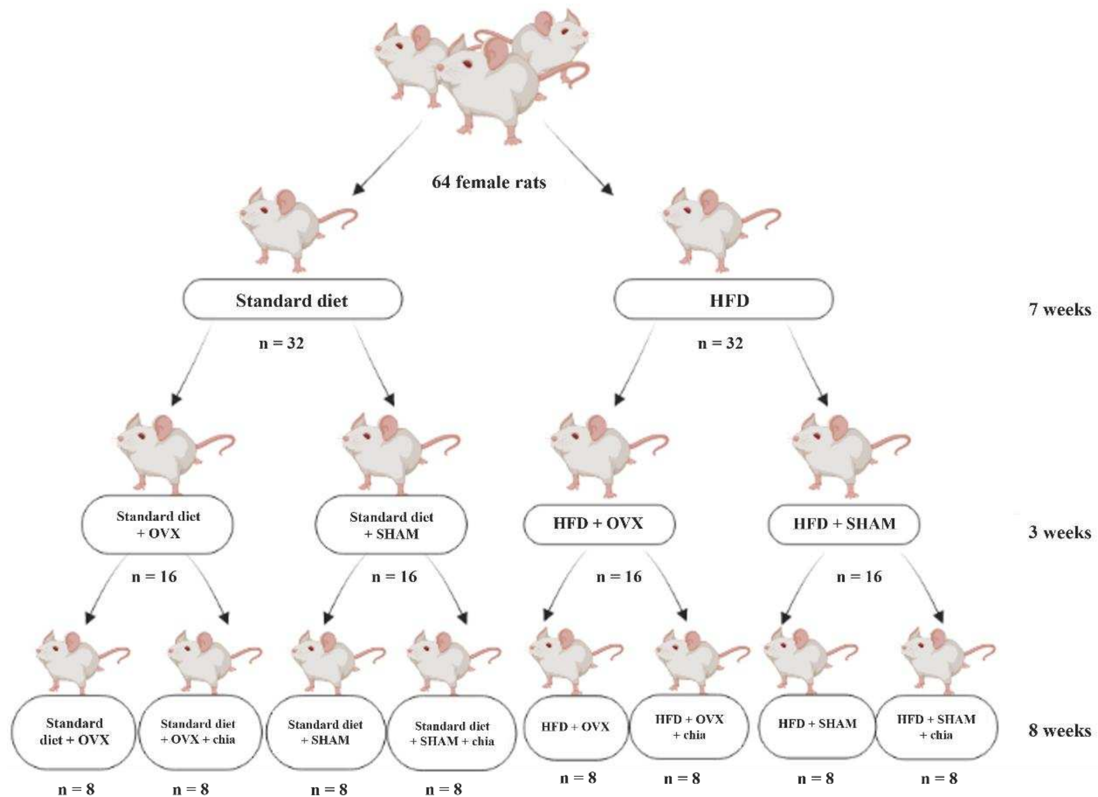
Sixty-four females (n=8/group) (Rattus norvegicus, *Wistar* lineage, albinus variation), recently weaned, aged 21 days were obtained from the Animal Facility of the Center for Biological and Health Sciences at *Universidade Federal de Viçosa*, Viçosa (MG). The sample calculation was based on the methodology proposed by Conagin (1959), with each animal considered a replicate. The animal protocols were submitted to the Ethics Committee on the use of Animals at the Federal University of Viçosa (Protocol 20/2017) (Appendix B). The rats were distributed in individual stainless-steel cages, in a temperature-controlled environment (22 °C ± 2 °C), automatically controlled 12-hour light and dark cycles and received distilled water and their respective experimental diets ad libitum.

The animals were divided into 2 groups with 32 animals each, randomized by body weight, and received standard diets for rodents (Reeves, Nielsen and Fahey, 1993) or a high-fat diet, for 7 weeks. After this period, 32 rats underwent ovariectomy (OVX group) (16 rats from the group received a standard diet and 16 from the group received a high-fat diet) and 32 rats underwent surgery without removing the organ (SHAM group) (16 rats from the group received a standard diet and 16 from the group received a high-fat diet) so that the stress caused by the surgery occurred in both groups. The animals remained on a standard diet for adult rodents' maintenance (AIN-93M) diet in powder form (17% lipid, 19% protein and 64% carbohydrate) or high-fat diet (HFD) (51% lipid, 15% proteins and 34% carbohydrates), for another 3 weeks, during recovery from surgery. After these 10 weeks, the animals were relocated to the following 8 experimental groups, in which they remained for another 8 weeks: (1) standard diet + OVX, (2) standard diet + chia + OVX, (3) standard diet + SHAM, (4) standard diet + chia + SHAM, (5) HFD + OVX, (6) HFD + chia + OVX, (7) HFD + SHAM or (8) HFD + chia + SHAM (Figure 4).

At the end of 126 days, after fasting for 12 hours, the animals were anesthetized with isoflurane (3%/body weight, Isoforine, Cristália®) and euthanized by cardiac puncture. The cecum, cecal contents and duodenum were collected. The weight of the cecum was measured and the cecal content was stored in sterile microtubes, immediately frozen in liquid nitrogen and stored at -80ºC for analysis of microbiota, pH, short-chain fatty acids and immunoglobulin A (IgA) measurement. Part of the duodenum was fixed in 10% formaldehyde and kept at room

temperature for subsequent histological analysis, and the other part was immediately frozen in liquid nitrogen and stored at -80ºC for analysis of intestinal functionality.

Figure 4 - Experimental design 2.



The figure represents the distribution of experimental groups and the chronological order of how the experiment was carried out. Briefly, the animals were divided in two groups receiving a standard diet or a high-fat diet for 7 weeks. After that, the animals in both groups were divided in ovariectomized or non-ovariectomized and remained receiving a standard or high-fat diet to recover from the surgery for 3 weeks. Then, the animals were divided in groups receiving chia flour or not, for 8 weeks. n = number of animals per group; HFD: high-fat diet; OVX: ovariectomized; SHAM: non-ovariectomized. Source: The author, created with BioRender.com.

6.3.2 Ovariectomy procedure

The animals underwent ovariectomy (OVX) with incision and removal of the ovary, or laparotomy (SHAM) with abdominal incision without ovarian removal, to induce surgical stress and its effects. Thirty minutes before the start of surgery, the animals received anti-inflammatory flunixinameglumine (0.68 mg/kg) and the antibiotic enrofloxacin (10 mg/kg) subcutaneously. The animals were anesthetized with isoflurane diluted in oxygen by inhalation through a calibrated vaporizer. Isoflurane (Isoforine, Cristália®) concentration was adjusted to

maintain anesthesia. After anesthetic induction and stabilization, the animals were placed in dorsal decubitus on a heated mattress and the operative field was prepared with 10% povidone-iodine. Analgesic morphine was administered subcutaneously at a dose of 5 mg/kg body weight.

After surgery, the animals remained in a heated chamber to maintain body temperature and then returned to their individual cages. The surgical-anesthetic and post-surgical procedures were performed by veterinarians at the Veterinary Hospital of the Veterinary Department of the *Universidade Federal de Viçosa* (UFV). The surgery went well, without complications and no animals were lost during the procedure.

6.3.3 Experimental diets

All diets used in the experiment were prepared in the Experimental Nutrition Laboratory. Albumin was used as a protein source and lard as a saturated fat source for the high-fat diet. The amount of chia was determined based on human consumption of 25g of chia/day, as used in studies reported in the literature with postmenopausal women (Jin *et al.*, 2012; Nieman *et al.*, 2012; Silva *et al.*, 2021). The other ingredients of the diets were added in sufficient quantities to match the composition of lipids, proteins, carbohydrates, dietary fiber and calories of the corresponding diets (Table 2).

Table 2- Compositions of the experimental diets, experiment 2.

	Induction Diets		**Experimental Diets**			
Ingredients	**ST**	**HFD**	**ST**	**STC**	**HFD**	**HFDC**
Chia (g)	-	-	-	232.60	-	232.60
Albumin (g)*	179.50	179.50	179.50	133.70	179.50	133.70
Dextrinized starch (g)	155.00	155.00	155.00	155.00	155.00	155.00
Sucrose (g)	100.00	100.00	100.00	100.00	100.00	100.00
Soybean oil (mL)	40.00	40.00	70.20	-	70.20	-
Lard (g)	-	240.00	-	-	195.00	195.00
Microcrystalline cellulose (g)	50.00	50.00	86.00	-	86.00	-
Mineral mix (g)	35.00	35.00	35.00	35.00	35.00	35.00
Vitamin mix (g)	10.00	10.00	10.00	10.00	10.00	10.00
L-cystine (g)	1.80	1.80	1.80	1.80	1.80	1.80
Choline bitartrate (g)	2.50	2.50	2.50	2.50	2.50	2.50
Corn starch (g)	420.00	178.45	347.50	319.40	151.00	122.90
Cholesterol	-	1.50	-	-	1.50	1.50
Nutritional composition						
Total calories (kcal)	3778.00	4971.80	3759.80	3717.48	4728.80	4686.48
Caloric density (kcal/g)	3.78	4.97	3.76	3.71	4.73	4.69

* Considering that albumin is 78% pure. ST: standard diet; STC: standard diet + chia; HFD: high fat-diet; HFDC: high-fat diet + chia.

6.4 Analysis of experiments 1 and 2

To calculate the experimental diets, the proximate composition of chia previously obtained by our lab, according to the Association of Official Analytical Chemists (AOAC, 2016) was considered (32.2 g/100g of lipids; 18.2 g /100g of proteins; 33.4 g/100g of dietary fiber, 4.6 g/100g of carbohydrates) (Da Silva *et al.*, 2017). All ingredients were weighed on a semi-analytical scale (Gehaka, BG2000, São Paulo, São Paulo, Brazil), mixed manually, sieved, and homogenized in an industrial mixer for 15 minutes. After preparation, the diets were packaged in polyethylene bags, properly labeled, and stored in a freezer (-18 ºC ± 1 ºC).

The diet offered to the animals was weighed, considering the weight of the feeder. The remains of the diet left in the feeders and the diet wasted in the compartments below the cage were weighed to calculate the animals' food consumption. To manage the deionized water offered, glass bottles of approximately 500 mL were used, adapted with a stopper to maintain the closure and a stainless-steel pipe to provide the necessary opening for the animals to obtain water in the form of a drinking fountain, with verification daily water output.

6.4.1 Food consumption and weight gain

Food consumption and body weight gain were monitored weekly, during the experimental period, using a digital electronic scale (Marte Científica, Santa Rita do Sapucaí, Minas Gerais, Brazil).

6.4.2 pH of cecal content

To analyze the pH of the cecal content, approximately 1g of the cecum content was homogenized in 10 mL of distilled water. Subsequently, the glass electrode of the pHmeter (Bel, pHmetro 966-PLUS, Piracicaba, São Paulo, Brasil) was inserted. Measurements were carried out in duplicate (Grancieri *et al.*, 2017).

6.4.3 Immunoglobulin A (IgA)

For the determination of IgA, a homogenate of cecal contents was prepared at a concentration of 1:5 (weight/volume) according to standard procedures. Samples were thawed on ice and suspensions were made by adding 200 mg of cecal contents to 800 µl of phosphate-buffered saline and homogenized with the aid of vortexing. Mucosal immunity was assessed based on the concentration of IgA in the cecal content using an immunoenzymatic kit (Enzyme Linked Immuno Sorbent Assay - ELISA) (Cat nºEA0032Ra) (Vaz-Tostes *et al.*, 2014).

6.4.4 Analysis of short-chain fatty acids (SCFA)

The evaluation of short-chain fatty acids followed the methodology proposed by Siegfried; Ruckemmann & Stumpf (1984), with modifications. Samples of cecal content were defrozed at room temperature and 500 mg portions were diluted in 1 mL of deionized water and vortexed. Samples were centrifuged at 12,000 × g for 10 minutes. 600µL of the supernatants, in duplicate, were transferred to new tubes, added with 600µL of calcium hydroxide (CHS) and 300µL of cupric sulfate (CSR) and shaken vigorously for 10 seconds. Samples were frozen and then thawed at room temperature for centrifugation at 12,000 × g for 10 minutes. A total of 1.0 mL of the supernatant was transferred to new tubes, which were added with 28 µL of concentrated sulfuric acid (H_2SO_4), followed by freezing. Afterwards, the samples were thawed, refrozen and finally thawed for centrifugation at 12,000 × g for 10 minutes. The supernatant (600µL) was then transferred to vials for analysis by high-performance liquid chromatography (HPLC).

Organic acid concentrations were determined using a Dionex Ultimate 3000 dual detector (Dionex Corporation, Sunnyvale, California, CA) coupled to a Shodex RI-101 refractive index detector, using a Bio-Rad HPX-87H column (Bio-Rad Laboratories, Hercules, California, CA), 300 mm × 4.6 mm which was kept at 45 °C. The mobile phase was composed of 5 mmol/L sulfuric acid, with a flow rate of 0.7 mL/min and an injection volume of 20 µL. Acetic, propionic, and butyric acid standards were prepared at concentrations up to 10 mmol/L.

6.4.5 Histology of the intestine

Semi-serial histological fragments of the intestine (3 µm thick) were obtained using a semi-automatic rotating microtome (Leica, Brazil) and stained using the hematoxylin/eosin technique. The slides were examined using an AX70 photomicroscope (Olympus, Japan). To measure the length and thickness of the crypts, as well as the thickness of the circular and longitudinal muscle layers, 20 random fields (objective x10) were selected per animal. Measurements were carried out with the aid of an optical microscope using ImagePro-Plus® version 4.5 software (Media Cybernetics, Rockville, Maryland, MD) (Hou, Kolba, Glahn *et al.*, 2017)

6.4.6 Intestinal microbiota

Total genomic DNA was extracted from cecal content samples following a mechanical disruption and phenol/chloroform extraction protocol (Stevenson and Weimer, 2007). PCR amplicon libraries targeting the hypervariable V4 region of the 16S rRNA gene were produced using primers 515F (5'GTGYCAGCMGCCGCGGTAA3') and 806R

(GGACTACNVGGGTWTCTAAT3') and a barcoded primer set adapted for the Illumina MiSeq platform (Illumina, San Diego, California, CA) (Caporaso *et al.*, 2011, 2012). Samples were loaded into an Illumina flow cell for paired sequencing reactions using the Illumina MiSeq platform.

Sequences obtained for all samples in the present study were submitted to the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sra).

Data processing and analysis were carried out using the Mothur v.1.44.3 software (Schloss *et al.*, 2009). Briefly, R1 and R2 paired-end reads were joined and sequences shorter than 150 or longer than 300 bp were removed. Sequences that have homopolymers with at least 8 nucleotides or containing ambiguous base pairs were also eliminated. Chimera sequences were detected and removed using UCHIME (Edgar *et al.*, 2011). After cleaning the sequences, they were aligned with the 16S rRNA gene using the *Si*LVA v.138 database (Quast *et al.*, 2013).

Operational Taxonomic Units (OTUs) were grouped according to sequence similarity cutoff and coverage > 97%. A normalized data table was used to calculate alpha and beta diversity, as well as to calculate the relative abundance of OTUs. Alpha diversity was estimated using the Chao1, Shannon and Simpson indices. Beta-diversity between food groups was assessed by Principal Coordinate Analysis (PCoA) based on Jaccard's dissimilarity index (Lozupone and Knight, 2005).

6.4.7 Intestinal functionality

6.4.7.1 Extraction of mRNA from intestinal tissue and cDNA synthesis

Duodenum fragments were macerated in liquid nitrogen under RNAse-free conditions for RNA extraction with TRIzol Reagent (Invitrogen, Carlsbad, California, CA). The extracted RNA was used to synthesize cDNA, using the M-MLV reverse transcription kit (Invitrogen Corp, Grand Island, New York, NY), according to the manufacturer's protocol.

6.4.7.2 Determination of gene expression of proteins involved in intestinal functionality by real-time polymerase chain reaction (RT-qPCR)

The expression of mRNA levels in the duodenum was analyzed using the real-time polymerase chain reaction (RT-qPCR) technique. Fast SYBR green master mix markers from Applied Biosystems (Foster City, California, CA) were used and analyzes were performed on the StepOne™ Real-Time PCR System (Thermo Fisher Scientific, Waltham, Massachusetts,

MA) through the measurement system involving SYBR-Green Fluorescence and Primer Software expressed (Applied Biosystems). cDNA, 1 μL, was used for each 10 μL reaction along with Fast SYBR green master mix (Applied Biosystem) that included buffer, Taq DNA polymerase, dNTPs, and SYBR green dye. Sense and antisense primer sequences were used for genes related to intestinal functionality: sucrose isomaltase (*Si*); sodium-dependent glucose transporter protein (*Sglt1*); aminopeptidase (*Ap*) and peptide transporter 1 (*Pept1*) (Table 3).

The specificity of the products amplified in real-time RT-PCR was verified by dissociation curve analysis. Relative mRNA expression levels were normalized to the reference β-actin control. All steps were performed under RNase-free conditions (Hou, Kolba, Glahn *et al.*, 2017).

Table 3 - Primer sequence for RT-qPCR analysis, experiments 1 and 2.

Gene	Oligonucleotides (5'- 3')	
	Forward	**Reverse**
β-actin	TTCGTTGCCGGTCCACACCC	GCTTTGCACATGCCGGAGCC
Ap	CTCTCTCCTCAAACCACATGAA	AGTTCAGGGCCTTCTCATATTC
Si	CCTCCAGAACACAATCCCTATAC	GGAGAGGTGAGATGGGATTAGA
Pept1	CCTGGTCGTCTGCATCATATT	TTCTTCTCATCCTCATCGAACTG
Sglt1	CATCCAGTCCATCACCAGTTAC	CAATCAGGAAGCCGAGAATCA

β-actin: reference gene; *Ap*: Aminopeptidase; *Sglt1*: Sodium-glucose transport protein 1; *Si*: Sucrase-isomaltase; *Pept1*: Peptide transporter 1.

6.5 Experiment 3 – Effects of intra-amniotic administration of hydrolyzed protein of chia associated with the probiotic *Lacticaseibacillus paracasei*, *in ovo* (*Gallus gallus*)

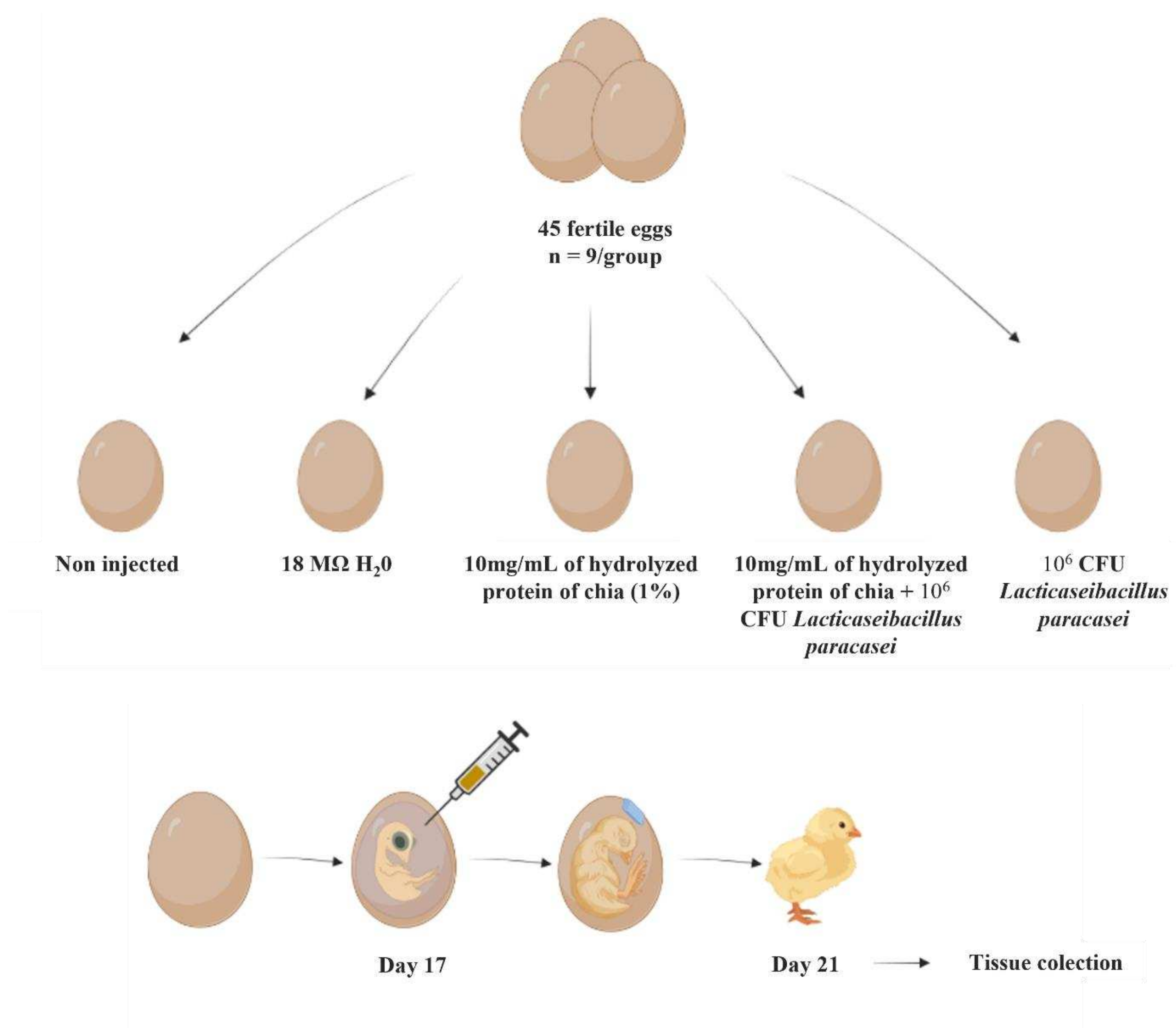
6.5.1 Experimental design

Fertile eggs (n = 45) were obtained from a commercial hatchery (Moyer's chicks, Quakertown, Pennsylvania, PA) and incubated under ideal conditions (37 ± 2º C and 89.6 ± 2% humidity) in the incubator of the poultry farm of the Department of Animal Science at Cornell University. Animal protocols were submitted to the Cornell University Institutional Animal Care and Use Committee (approval code 2020-0077).

Prior to administration, the hydrolyzed protein of chia was diluted in ultrapure water (18 MΩ H_2O) in three different dilution concentrations (1%, 5% and 10%) to determine the concentration that would be administered in order to maintain an osmolarity (OSM) value lower than 320 OSM (Dias *et al.*, 2019) and, in this way, ensure that the chicken embryos were not dehydrated after injecting the solution. It was decided to use a concentration of 1%. The amount of probiotic administered was defined according to values already reported in the literature for experiments similar to ours, in which *in ovo* intra-amniotic administration of a probiotic was performed.

On the 17th day of embryonic incubation, the eggs were evaluated using specific light to discard those that had infertile, cracked, contaminated or prematurely dead embryos. Eggs containing viable embryos were weighed and randomly allocated into 5 groups (n = 9/group) with similar weight distribution. All eggs were disinfected with 70% ethanol, then each group was injected with 1 mL of solution per egg into the amniotic fluid, which was identified with the aid of specific light, with a 19 mm needle, vertically inserted. The 5 groups were distributed as follows: (1) non injected; (2) 18 MΩ H_20; (3) 10 mg/mL of hydrolyzed protein of chia (1%); (4) 10 mg/mL of hydrolyzed protein of chia + 10^6 colony-forming units (CFU) *Lacticaseibacillus paracasei* (800 µL of hydrolyzed chia protein + 200 µL of probiotic/egg); (5) 10^6 CFU *Lacticaseibacillus paracasei* (Figure 5). After injection, the eggs were sealed with cellophane tape and placed in incubation baskets so that each treatment was equally represented in each location of the incubator. Immediately after hatching (day 21), the animals were weighed and then euthanized by exposure to CO_2. The duodenum, pectoral muscle, cecum and contents of the cecum were collected.

Figure 5 - Experimental design, experiment 3.



The figure represents the distribution of experimental groups and the chronological order of how the experiment was carried out. Briefly, the eggs were received on the first day of incubation, on the seventeenth day the solutions were administered into the intra amniotic fluid, the injection hole was sealed and on the twenty-first day the tissues were collected. n = number of animals in the group; CFU: colony forming unit Source: The author, created with BioRender.com

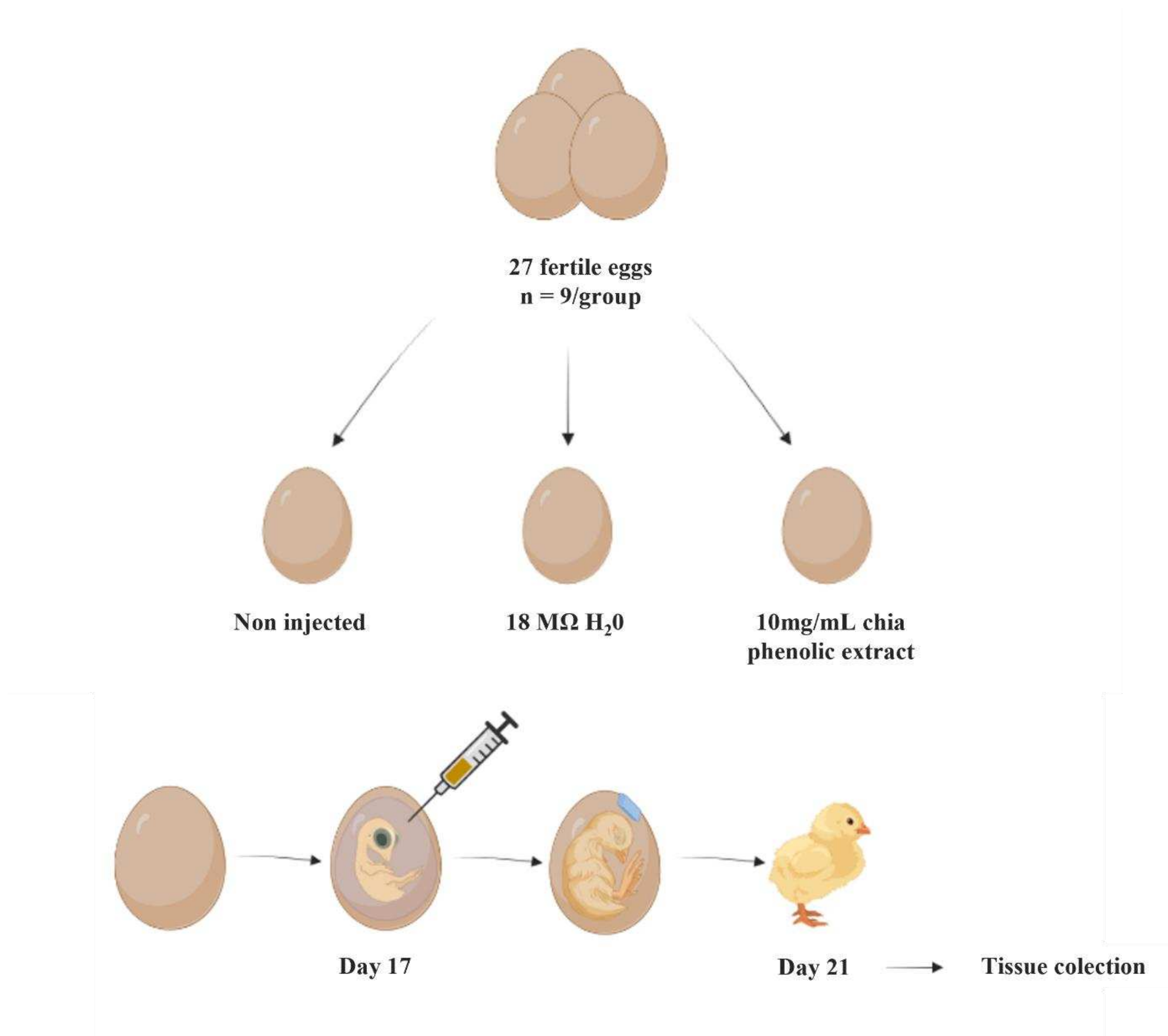
6.6 Experiment 4 – Effects of intra-amniotic administration of chia phenolic extract, *in ovo* (*Gallus gallus*)

6.6.1 Experimental design

Fertile eggs (n = 27) were obtained from a commercial hatchery (Moyer's chicks, Quakertown, PA, USA) and incubated under ideal conditions (37 ± 2º C and 89.6 ± 2% humidity) in the incubator of the poultry farm of the Department of Animal Science at Cornell University. Animal protocols were submitted to the Cornell University Institutional Animal Care and Use Committee (approval code 2020-0077).

The experiment was conducted as described previously in 6.5.1. Eggs containing viable embryos were weighed and randomly allocated into 3 groups (n = 9/group), with similar weight distribution, as follows: (1) non injected; (2) 18 MΩ H_20; (3) 10 mg/mL (1%) of chia phenolic extract (Figure 6).

Figure 6 - Experimental design, experiment 4.



The figure represents the distribution of experimental groups and the chronological order of how the experiment was carried out. Briefly, the eggs were received on the first day of incubation, on the seventeenth day the solutions were administered into the intra amniotic fluid, the injection hole was sealed and on the twenty-first day the tissues were collected. n = number of animals in the group. Source: The author, created with BioRender.com.

6.7 Analysis of experiments 3 and 4

6.7.1 Isolation of total RNA from the duodenum

Total RNA was extracted from 30 mg of proximal duodenal tissue using the Qiagen RNeasy Mini Kit (RNeasy Mini Kit, Qiagen Inc., Valencia, California, CA) according to the kit manufacturer's protocol. The tissues were homogenized, and the tissue lysate was centrifuged for 3 min at 8000 × g in a microcentrifuge. An aliquot of the supernatant was added to 1 volume of 70% ethanol and mixed. Each sample (700 µL) was applied to a RNeasy mini column, centrifuged for 15s at 8000 × g, and the flow through material was discarded. Afterwards, the RNeasy columns were transferred to new collection tubes and 500 µL of RPE® buffer was pipetted into the RNeasy column followed by centrifugation. Total RNA was eluted in RNase-free water. All steps were performed under RNase-free conditions. RNA was quantified by absorbance at A 260/280. The integrity of the 18S ribosomal RNA was verified by electrophoresis in a 1.5% agarose gel followed by staining with ethidium bromide. DNA contamination was removed using the TURBO DNase treatment and removal kit from AMBION (Austin, Texas, TX) (Da Silva, Kolba, *et al.*, 2019).

6.7.2 Real-time polymerase chain reaction (RT-qPCR)

The cDNA was be used for each 10 µL of reaction along with 2× BioRad SSO Advanced Universal SYBR Green Supermix (Bio-Rad Laboratories) which includes buffer, Taq DNA polymerase, dNTPs and SYBR green dye. Sense and antisense primer sequences were used for genes related to BBM functionality, inflammation and intestinal barrier, as described in Table 4, combined with cDNA and added to each PCR reaction. Each race contained seven standard curve points in duplicate. Double-stranded DNA was amplified in the Bio-Rad CFX96 Touch (Bio-Rad Laboratories) using specific PCR conditions. Data on gene expression levels were obtained as Cp values based on the "second maximum derivative" (automated method) calculated by the software. For each gene, reactions were performed in duplicate. All assays were quantified by including a standard curve in the real-time qPCR analysis. The specificity of real-time RT-PCR amplified products were verified by melting curve analysis. All steps were carried out under RNase-free conditions (Hou, Kolba, Glahn *et al.*, 2017).

Table 4 - Primer sequence for RT-qPCR analysis, experiments 3 and 4

Gene	Oligonucleotides (5'- 3')	
	Forward	Reverse

BBM functionality		
Ap	CGTCAGCCAGTTTGACTATGTA	CTCTCAAAGAAGCTGAGGATGG
Si	CCAGCAATGCCAGCATATTG	CGGTTTCTCCTTACCACTTCTT
18S rRNA	GCAAGACGAACTAAAGCGAAAG	TCGGAACTACGACGGTATCT
Inflammation		
Tnf-α	GACAGCCTATGCCAACAAGTA	TTACAGGAAGGGCAACTCATC
Nf-κβ1	CACAGCTGGAGGGAAGTAAAT	TTGAGTAAGGAAGTGAGGTTGAG
Intestinal barrier		
Muc2	CCTGCTGCAAGGAAGTAGAA	GGAAGATCAGAGTGGTGCATAG
Ocln	GTCTGTGGGTTCCTCATCGT	GTTCTTCACCCACTCCTCCA

BBM: brush border membrane; *Ap*: aminopeptidase; *Si*: isomaltase sucrase; *Tnf-α*: tumor necrosis factor alpha; *Nf-κβ1*: nuclear factor kappa beta; *Muc2*: mucin 2; *Ocln*: occludin. 18S rRNA: reference gene

6.7.3 Composition of the intestinal microbiota

6.7.3.1 Collection of microbial samples and isolation of DNA from intestinal contents

The contents of the cecum were removed in a sterile manner and treated as previously described (Hartono *et al.*, 2014). Samples were placed in a sterile 15 mL tube containing 9 mL of sterile Phosphate-buffered saline (PBS) and homogenized by vortexing with glass beads (3 mm in diameter) for 3 min. Debris were removed by centrifugation at 1000×g for 5 min, and the supernatant was collected and centrifuged at 4000×g for 10 min. The pellet was washed twice with PBS and stored at -20ºC until DNA extraction. For DNA purification, the pellet was resuspended in 50 mM ethylenediaminetetraacetic acid (EDTA) and treated with lysozyme (Sigma Aldrich CO., St. Louis, Missouri, MO, final concentration of 10 mg/mL) for 45 minutes at 37 ºC. Bacterial genomic DNA was isolated using a Wizard Genomic DNA purification kit (Promega Corp., Madison, Wisconsin, WI).

6.7.3.2 DNA extraction and sequencing

Primers were used for Lactobacillus, Bifidobacterium, Clostridium and E. coli. The universal primers were designed with the invariant region in the 16S rRNA of bacteria and were

used as internal standards. To estimate the relative proportion of each bacteria studied, each product was expressed in relation to the content of the universal primer product and the proportions of each bacterial group was presented. The PCR products were separated by electrophoresis in a 2% agarose gel, stained with ethidium bromide and quantified using the 1-D Quantity One analysis software (Bio-Rad Laboratories) (Dias et al., 2019).

6.7.4 Histology of the duodenum

Duodenum samples were fixed in fresh 4% (v/v) buffered formalin, dehydrated, clarified and embedded in paraffin. Serial sections were cut at 5 µm and placed on glass slides. The sections were deparaffinized in xylene, rehydrated in a graded alcohol series, stained with Alcian Blue/Periodic Acid-Schiff and examined by light microscopy. The following morphometric measurements were evaluated: villus height (µM) (objective x10), villus surface area (µM) (objective x10), crypt depth (µM) (objective x20), number and diameter of Paneth cells (µM) (objective x40), number and diameter of goblet cells (µM) (objective x40) in villi and crypts and goblet cell type (acidic, neutral and mixed). Four segments for each biological sample (n = 3/treatment group) were performed and ten villi and crypts were randomly selected and analyzed per segment (40 replicates per biological sample), using a light microscope (CellSens Standard software, Olympus, Waltham, Massachusetts, MA). For Alcian Blue/Periodic Acid-Schiff staining, segments were counted only for the type of goblet cells (acidic, neutral or mixed) in the villus epithelium and goblet cells in the crypts. Goblet cells were enumerated in 10 villi/sample, and averages were calculated for statistical analysis. The surface area of the villi was obtained by the equation:

$$Surface\ area\ of\ the\ villi\ = 2\frac{LV}{2}\ X\ CV$$

Where: LV = average of three villus width measurements, and CV = villus length.

6.8 Statistical Analysis

In experiments with rats, data normality was assessed using the Kolmogorov-Smirnov test. The data were submitted to ANOVA followed by the Newman-Keuls mean test at 5% probability. The unpaired t test was used to compare the pairs standard diet x standard diet+chia and high-fat diet x high-fat diet+chia (the same diet with or without chia). Statistical analyzes were performed using the statistical software GraphPad Prism®, version 9 (GraphPad Software Inc., San Diego, California, CA). Data were expressed as mean ± standard deviation (SD) with a significance level set at $p<0.05$. Correlations between biochemical and oxidative stress

parameters and intestinal health markers were assessed using the Pearson or Spearman correlation tests. The biochemical parameters and oxidative stress markers were previously carried out and published (Da Silva *et al*., 2019a).

For the analysis of sequencing data, the Chao1, Shannon and Simpson indices were used to estimate alpha diversity and estimate bacterial richness within the samples. Differences between groups were analyzed by ANOVA. To evaluate the grouping of samples, Principal Coordinate Analysis (PCoA) was performed, based on the Bray-Curtis dissimilarity index and similarity test for non-parametric data (ANOSIM, permutation number = 1000) using the Past software (Hammer, Harper and Ryan, 2001). The data set was tested for homogeneity of variance using the Kolmogorov-Smirnov test and non-parametric and independent data were subjected to the Kruskal-Wallis test followed by Dunn's test for multiple comparisons. The data were corrected using the false discovery rate (FDR) criterion in the STAMP software. Statistical analyzes were performed using SPSS software version 20.0. The significance level established were 5%.

In ovo experiments, data normality was assessed using the Shapiro–Wilk test. The data were submitted to ANOVA followed by the Duncan test of means at 5% probability. The means without normal distribution were analyzed using Kruskal–Wallis and a post hoc Dunn’s test. The statistical analyzes were performed using the statistical software IBM SPSS Statistics®, version 25. Data were expressed as mean ± standard error deviation (SED) with a significance level set at $p<0.05$. Correlations between intestinal health markers were assessed using the Spearman’s rank correlation coefficient test. GraphPad Prism® version 9.0 software packages (GraphPad Software Inc., San Diego, California, CA) were used for graphics.

7. ETHICAL ASPECTS

The experiments with rats were approved by the Ethics Committee on Animal Use at Universidade Federal de Viçosa (CEUA/UFV), protocols 97/2014 (males) and 20/2017 (females). The experiments were carried out in accordance with the Ethical Principles in Animal Experimentation, approved by the National Council for the Control of Animal Experimentation (CONCEA) (Appendix A and B).

The *in ovo* study was submitted to and approved by the Cornell University Institutional Animal Care and Use Committee under ethical approval code 2020-0077.

All experimental procedures were carried out in accordance with ethical principles in animal experimentation.

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8. THESYS PUBLICATION

The experiments performed in the present thesis and the review were published between 2022 and 2023, as one systematic review and five original papers (Table 5).

Table 5 - Description of papers published in the thesis.

Paper	Title	Journal	Impact Factor	Year of publication
Original paper 1	Effect of Chia (*Salvia hispanica* L.) Associated with High-Fat Diet on the Intestinal Health of *Wistar* Rats	Nutrients	5.9	2022
Original paper 2	Effect of chia four associated with high fat diet on intestinal health in female ovariectomized *Wistar* rats	European Journal of Nutrition	5.0	2022
Original paper 3	Chia flour combined with a high fat diet increases propionic acid production and improves the microbial richness and diversity in female *Wistar* rats	Food & Function	6.1	2023
Systematic review	Effect of food derived bioactive peptides on gut health and inflammatory mediators *in vivo*: a systematic review	Critical Reviews in Food Science and Nutrition	10.2	2023
Original paper 4	Effects of Intra-Amniotic Administration of the Hydrolyzed Protein of Chia (*Salvia hispanica* L.) and *Lacticaseibacillus paracasei* on Intestinal Functionality, Morphology, and Bacterial Populations, *In vivo* (*Gallus gallus*)	Nutrients	5.9	2023
Original paper 5	Chia Phenolic Extract Appear to Improve Small Intestinal Functionality, Morphology, Bacterial Populations, and Inflammation Biomarkers *In vivo* (*Gallus gallus*)	Nutrients	5.9	2023

9 RESULTS

9.1 Paper 1: Effect of Chia (*Salvia hispanica* L.) Associated with High-Fat Diet on the Intestinal Health of *Wistar* Rats

nutrients

Article

Effect of Chia (*Salvia hispanica* L.) Associated with High-Fat Diet on the Intestinal Health of *Wistar* Rats

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Abstract: A direct correlation has been reported between excessive fat intake and the development and progression of various enteropathies. Plant foods may contain bioactive compounds and non-digestible dietary fiber, with potential to improve intestinal health. Chia is a good source of dietary fiber and bioactive compounds. Our study evaluated the role of chia flour associated with a high-fat diet (HFD) on colon histomorphometry, intestinal functionality and intestinal microbiome composition and function in *Wistar* rats. The study used 32 young male rats separated into four groups to receive a standard diet (SD) or HFD, with or without chia, for 35 days. At the end of the study, the cecum, cecal content and duodenum were collected. The consumption of chia increased the production of short-chain fatty acids and improved fecal moisture. Chia consumption improved the circular muscle layer in the SD group. The diversity and abundance of intestinal bacteria were not affected, but increased richness was observed in the microbiome of the SD+chia group. Moreover, chia consumption decreased the expression of proteins involved in intestinal functionality. Chia consumption improved intestinal morphology and functionality in young *Wistar* rats but was insufficient to promote significant changes in the intestinal microbiome in a short term of 35 days.

Keywords: chia seed; western diet; intestinal microbiota; intestinal morphology; SCFA; intestinal functionality

1. Introduction

The large consumption of a western diet, which is increased in fat and reduced in vegetables and fiber, is increasingly becoming a big risk factor for several chronic metabolic and inflammatory disorders involving different organs. The chronic high-fat diet (HFD) consumption can induce or aggravate many diseases that affect a wide range of organs besides obesity and metabolic syndrome [1,2]. In addition, a direct correlation has been reported between excessive fat intake and the development and progression of various enteropathies, with conditions that lead to the secretion of pro-inflammatory cytokines, which initiate and

maintain an inflammatory process, thus causing alteration in the intestinal microbiota, dysfunction of the epithelial barrier and reduced permeability and integrity of the intestinal mucosal barrier [2–4].

By manipulating external factors, the plasticity of the microbiota allows the reshaping of the architecture and biological outputs of intestinal microbes to improve human health. A relation between diet and the intestinal microbiota is observed, which leads to the conclusion that dietary factors are the most potent modulators of microbiota composition and function. Intestinal microorganisms, in turn, influence the absorption, metabolism and storage of nutrients, with effects on host physiology [5].

When it reaches the colon, the fiber diet is anaerobically fermented by the intestinal bacteria. Some nutritionally specialized bacteria in the phyla Firmicutes and Actinobacteria are considered to be important in initiating the degradation of the fiber diet, and the continued breakdown is attributed to certain abundant species within the phylum Bacteroidetes, and then they can produce short-chain fatty acids (SCFAs). SCFAs play a significant role in intestinal homeostasis and affect the body [5,6]. The most abundant SCFAs in the intestine are acetic acid, propionic acid and butyric acid [6,7]. Microbiota-accessible carbohydrates provide an energy source for intestinal bacteria, some of which are nutritionally specialized in degrading these carbohydrates and producing certain SCFA [6]. The consequent production of SCFAs benefits the host by serving as both recovered energy from otherwise inaccessible carbohydrates as well as potent regulatory molecules, with vast physiological effects, including energy homeostasis, lipid and carbohydrate metabolism, and suppression of inflammatory signals [5,6].

Plant foods may contain bioactive compounds and dietary fibers with potential effects on intestinal bacterial populations, gastrointestinal motility improvement, intestinal functionality and morphology, increased mucus production, number/diameter of goblet cells, surface area of villi and crypt depth. Such effects seem to result from the increased motility of the digestive tract, which leads to hyperplasia and/or hypertrophy of muscle cells [7–9].

Chia (*Salvia hispanica* L.) is a potentially bioactive food. Its consumption may reduce the risk and attenuate metabolic alterations, due to a series of health benefits, such as antidiabetic effects, antitumor potential, immunostimulant activity, antioxidant protection, cardiovascular and liver protection, remission of inflammation and reduced heart fat content [10–12]. Chia contains components with diverse actions, whose benefits are mainly due to their high nutritional value, high concentrations of lipids (32.16 g.100 g^{-1}), proteins (18.18 g.100 g^{-1}) and total dietary fiber (33.37 g.100 g^{-1}), besides insoluble fiber (30.47 g.100 g^{-1}), minerals and antioxidant compounds beneficial to health [13]. The intra-amniotic (*in ovo*) administration

of a soluble chia extract improved intestinal morphology, affected the intestinal microbiota and positively regulated the gene expression of proteins associated to mineral metabolism [8]. Furthermore, the relationship between chia seed peptides and the regulation of adipogenesis and inflammation has already been demonstrated [14]. Therefore, the objective of the present study was to evaluate the role of chia associated with a high-fat diet on colon histomorphometry, intestinal functionality and microbiome composition and function of young *Wistar* rats.

2. Materials and Methods

2.1. Sample Material

The seeds (*Salvia hispanica* L.) used in this study were grown in the state of Rio Grande do Sul (Brazil). The seeds were planted in January 2015, and the harvest was carried out in June 2015. The samples were packed and transported in cardboard boxes. Then, the samples were stored in hermetically sealed plastic bags until use, protected from light and frozen (−18 °C ± 1 °C). The seeds were ground up to a particle size of 850 μm to obtain flour, using a knife mill (Marconi Equipment, Brazil), in three replicates. Then, chia flour was packed in polyethylene aluminum bags and stored in a freezer (−18 °C ± 1 °C) until the chia was added to the diet.

2.2. Animals and Diets

Thirty-two newly weaned, 21-day-old male rats (Rattus norvegicus, *Wistar*, albinus variation) from the Central Animal Facility of the Center for Biological Sciences and Health, at the Federal University of Viçosa, Minas Gerais, Brazil, were separated into 4 groups (n = 8) randomized by body weight, using the WinPepi Program version 11.65 [15]. The rats were distributed into individual stainless-steel cages in a temperature-controlled environment (22 °C) and automatically controlled light and dark cycles of 12 h. The experimental diets were based either on the standard diet (AIN-93G) [16] or high-fat diet (Research Diets, New Brunswick, NJ, USA), with modifications. The animals received their respective experimental diets and deionized water ad libitum.

The standard diet was composed of 20% protein, 30% fat and 50% carbohydrate. The high-fat diet was prepared with the following proportions: 64% fat, 16% protein and 20% carbohydrate. The amount of chia was determined based on the human consumption of 40 g of chia/day (2 tablespoons). The chia seed was added to diets based on its composition, described by Da Silva (2017) [13], of lipids (32.16 g.100 g^{-1}), proteins (18.18 g.100 g^{-1}), total dietary fiber (33.37 g. 100 g^{-1}) and carbohydrates (4.59 g.100 g^{-1}). The other ingredients were added

in quantities sufficient to provide the planned amounts of proteins, lipids, carbohydrates, fiber and calories (Table 1).

Table 1. Compositions of the experimental diets.

	Experimental Diets			
Ingredients (g/kg of diet)	**SD**	**SD+chia**	**HFD**	**HFD+chia**
Albumin *	217.90	117.60	217.90	117.60
Chia flour	0.00	416.80	0.00	416.80
Dextrinized starch	132.00	132.00	132.00	115.10
Sucrose	100.00	100.00	100.00	100.00
Lard	0.00	0.00	200.00	200.00
Soybean oil (mL)	134.20	0.00	134.20	0.00
Microcrystalline cellulose	139.20	0.00	139.20	0.00
Mineral mix	35.00	35.00	35.00	35.00
Vitamin mix	10.00	10.00	10.00	10.00
L-cystine	3.00	3.00	3.00	3.00
Choline bitartrate	2.50	2.50	2.50	2.50
Corn starch	221.20	183.10	21.20	0.00
Total calories (kcal)	3700.42	3624.75	4700.42	4624.11
Caloric density (kcal g^{-1})	3.70	3.62	4.70	4.62

*Considering that albumin is 78% pure. SD: standard diet, HFD: high-fat diet. Means followed by different small letters in the same row differ significantly, according to Newman–Keuls post hoc test, at the 5% threshold of probability.

Each group received one of the following experimental diets: standard diet (SD), standard diet + chia flour (SD+chia), high-fat diet (HFD) or high-fat diet + chia flour (HFD+chia). Body weight gain and food consumption were monitored weekly during the experimental period. On the 35th day, after 12 h of fasting, the animals were anesthetized with

isoflurane (Isoforine, Cristália®), and then euthanized by cardiac puncture. Cecum, cecal content and duodenum were collected, weighed and stored at −80 °C prior to analysis. The colon segment was collected, flushed with phosphate buffered saline solution, fixed in formaline for 24 h and kept in 70% ethanol for histological analysis. The following fat depots were weighed: visceral, retroperitoneal, epididymal and abdominal adipose tissues. The adiposity was calculated as a percentage using the following formula: (visceral + retroperitoneal + epididymal + abdominal adipose tissues)/total body weight x 100 [17].

All the experimental procedures with animals were performed in accordance with Directive 86/609/EEC of November 24, 1986, in compliance with the ethical principles for animal experimentation. The study protocol was approved by the Ethics Committee of the Federal University of Viçosa (protocol 20/2017; date of approval: 13 July 2017).

2.3. Fecal Moisture

The moisture content in the feces, collected at the end of the experiment, was determined by the gravimetric method. The samples were oven-dried at 105 °C for 24 h [18].

2.4. Cecal Content pH

About 1 g of cecum feces was homogenized in 10 mL of distilled water, with the aid of vortex glass spheres. Subsequently, the glass electrode of the pHmeter (Bel, Italy) was inserted. The measurements were performed in duplicate [19].

2.5. IgA Quantification

In order to determine sIgA, the standard procedures were followed to prepare the cecal content homogenates, 1:5 (w/v). The cecal content samples were defrosted on ice. To prepare the suspensions, 200 mg feces was added to 800 µL of phosphate-buffered saline and homogenized with the aid of a vortex. It was used an Immunochron enzyme-linked immunosorbent assay (ELISA) to measure the mucosal immunity, based on the cecal content sIgA concentration [20].

2.6. Short Chain Fatty Acids (SCFA) Content

The analysis to determine the content of SCFA followed the methodology proposed by Siegfried and Ruckemmann (1984) [21], with modifications. Briefly, 500 mg of cecum feces were homogenized in MiliQ water, following a vortex shaking protocol. Next, the samples were centrifuged at 12,000× *g* for 10 min. The supernatants were transferred to new tubes, received the addition of calcium hydroxide and cupric sulfate and were vigorously shaken. The samples

were frozen and then thawed at room temperature for centrifugation. The supernatant was added with concentrated sulfuric acid and then frozen. Then, the samples were thawed, refrozen and finally thawed for centrifugation at 12,000× *g*, for 10 min. The supernatant was transferred to vials for analysis by high-performance liquid chromatography (HPLC).

The SCFAs were determined in a Dionex Ultimate 3000 Dual detector HPLC apparatus (Dionex Corporation, Sunnyvale, CA, USA) equipped with a refractive index detector Shodex RI-101, using a Bio-Rad HPX-87H column, 300 mm x 4.6 mm, maintained at 45 °C. The conditions that the analyses were performed were as follow: mobile phase sulfuric acid 5 mmol l^{-1}, flow rate 0.7 mL min^{-1} and injection volume 20 μL. Acetic, propionic and butyric acids were used to prepare the stock solutions of the standards. The SCFAs were prepared with a final concentration of 10 mmol/L.

2.7. *Colon Histomorphometry*

Semi-serialized histological fragments from proximal colon (3 μm thickness) were obtained on a semi-automated rotating microtome (Leica, Brazil) and stained by the hematoxylin/eosin technique. To measure the length and thickness of the crypts and the thickness of the circular and longitudinal muscle layers twenty random fields per animal were selected. The slides were examined under a AX70 photomicroscope (Olympus, Japan). The ImagePro-Plus® software system, version 4.5 (Media Cybernetics, Rockville, MD, USA) was used to processed the images.

2.8. *DNA Extraction and Sequencing*

The total genomic DNA was extracted from the cecum content samples, following a mechanical disruption and phenol/chloroform extraction protocol [22]. PCR amplicon libraries targeting the hypervariable V4 region of the 16S rRNA gene were produced using the primers 515F (5′GTGYCAGCMGCCGCGGTAA3′) and 806R (GGACTACNVGGGTWTCTAAT3′) and a barcoded primer set adapted for the Illumina MiSeq platform (Illumina, San Diego, California, CA, USA) [23,24]. Illumina MiSeq was used to load the samples onto an Illumina flow cell for paired-end sequencing reactions in the Environmental Sample Preparation and Sequencing Facility (ESPSF), at the Argonne National Laboratory (Lemont, IL, USA).

The sequences obtained for all samples in the present study were submitted to the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI), under the accession number PRJNA805271.

The Mothur software system v.1.44.3 was used to data processing and analysis [25]. The R1 and R2 paired-end reads were joined, and sequences smaller than 150 or greater than 300 bp were removed. Sequences with homopolymers with at least 8 nucleotides or containing ambiguous base pairs were also eliminated. Chimera sequences were detected and removed using UCHIME [26]. After cleaning the sequences, they were aligned with the 16S rRNA gene, using the SILVA database v.138 [27].

The Operational Taxonomic Units (OTUs) were grouped according to the sequence similarity cutoff. The coverage of all samples was assessed by Good's coverage estimator (bacteria > 97%). A normalized data table was used for calculating alpha and beta diversity and the relative abundance of OTUs. Alpha diversity was estimated by using Chao1, Shannon and Simpson indices. Beta diversity between dietary groups was assessed by Principal Coordinate Analysis (PoA), based on the Jaccard dissimilarity index [28]. Metagenome functional predictive analysis was carried out using PICRUSt2 software [29].

2.9. Determination of Gene Expression of Proteins Involved in Intestinal Health by Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR)

mRNA expression levels of genes in the intestinal tissue (duodenum) which are involved in intestinal health were analyzed by RT-qPCR. The SYBR Green PCR master mix from Applied Biosystems (Foster City, CA, USA) was employed, and the analyses were performed on the StepOne™ Real-Time PCR System (Thermo Fisher Scientific) by means of the measurement system involving SYBR-Green Fluorescence and Primer Express software (Applied Biosystems, Foster City, CA, USA). Sense and antisense primer sequences were ordered (Choma Biotechnologies) to amplify aminopeptidase (AP) (ID: 301368687), sucrose isomaltase (SI) (ID: 301368688), peptide transporter 1 (PepT1) (ID: 301368693) and sodium–glucose transport protein 1 (SGLT1) (ID: 301368686). The relative expression levels of mRNA were normalized to the endogenous control (beta-actin; Table 2). All the steps were performed under open conditions with RNase.

Table 2. Sequencing primers used in the RT-qPCR analysis.

Genes	**Oligonucleotide (5′-3′)**	
	Forward	**Reverse**

Beta-actin	TTCGTGCCGGTCCACACCC	GCTTTGCACATGCCGGAGCC
AP	CTCTCTCCTCAAACACATGAA	AGTTCAGGGCCTTCTCATATTC
SI	CCTCCAGAACACAATCCCTATAC	GGAGAGGTGAGATGGATTAGA
PepT1	CCTGGTCGTCTCATCATATT	TTCTTCTCATCCTCATCGAACTG
SGLT1	CATCCAGTCCATCACATTAC	CAATCAGGAAGCCGAGAATCA

AP: aminopeptidase; SGLT1: sodium–glucose transport protein 1; SI: sucrose isomaltase; PepT1: peptide transporter 1.

2.10. Statistical Analysis

Food consumption, body weight, colonic histomorphometry characteristics and concentrations of SCFA data were first submitted to a Kolmogorov–Smirnov normality test. Next, a one-way analysis of variance (ANOVA) was applied, followed by the Newman–Keuls post hoc test to compare all test groups. The experimental treatments were arranged in a completely randomized design, with eight repetitions. The data are presented as means ± standard deviation, and statistical significance was established at $p < 0.05$. Correlations between biological markers and intestinal parameters were assessed by Pearson's correlation test. The biochemical parameters and stress oxidative markers were previously carried out and published [30].

Regarding the microbiome results, the Chao1, Shannon and Simpson indexes were used to estimates differences between alpha diversity, ANOVA was used to analyse the differences between the groups. The differences between beta diversity were analyzed by the pairwise PERMANOVA test. Statistically significant p-values associated with microbial clades and functions were corrected for multiple comparisons using the Benjamini–Hochberg false discovery rate (FDR) correction. The statistical analysis was performed using SPSS version 20.0. The level of significance was established at $p < 0.05$.

3. Results

3.1. Data of Animals

The food consumption was higher in the rats fed the standard diet (SD and SD+chia) than in the rats fed HFD (HFD and HFD+chia). The animals that were fed with the HFD containing chia (HFD+chia) presented higher food consumption than those of the HFD group.

The animals fed the SD and HFD without chia (SD and HFD) presented lower final weight than those in the groups fed chia. Adiposity, cecum weight and cecal content pH did not differ between the experimental groups. The consumption of chia (SD+chia x SD and HFD+chia x HFD) increased the moisture concentration in the feces of the animals and did not alter the IgA concentration, while the SD group presented the lowest IgA concentration (Table 3). Furthermore, chia consumption increased the concentration of acetic, propionic and butyric acids in the cecal content (Figure 1).

Table 3. Biometric data, consumption data, fecal moisture, cecal content pH and IgA concentration.

	SD	**SD+chia**	**HFD**	**HFD+chia**
Food consumption (g/day)	14.74 ± 0.86 ^{a}	15.46 ± 1.20 ^{a}	10.86 ± 0.49 ^{c}	12.29 ± 0.93 ^{b*}
Final weight (g)	221.69 ± 15.25 ^{b}	241.10 ± 15.65 ^{a*}	208.76 ± 11.95 ^{b}	240.11 ± 16.89 ^{a*}
Adiposity (% of body weight)	2.32 ± 0.60 ^{a}	2.66 ± 0.39 ^{a}	2.69 ± 0.49 ^{a}	2.96 ± 0.46 ^{a}
Cecum weight (g)	4.20 ± 0.71 ^{a}	4.76 ± 0.60 ^{a}	4.25 ± 0.40 ^{a}	4.06 ± 0.83 ^{a}
Cecal content pH	7.23 ± 1.28 ^{a}	7.33 ± 0.27 ^{a}	6.70 ± 0.21 ^{a}	7.13 ± 0.45 ^{a}
Fecal moisture (%)	16.36 ± 3.86 ^{c}	37.65 ± 5.37 ^{a*}	14.36 ± 6.41 ^{c}	28.01 ± 5.97 ^{b*}
IgA (ng)	271.09 ± 95.70 ^{b}	555.50 ± 193.67 ^{a}	751.83 ± 278.88 ^{a}	681.17 ± 155.91 ^{a}

Values referring to means ± SD, n = 8/group. SD: standard diet; SD+chia: standard diet + chia; HFD: high-fat diet; HFD+chia: high-fat diet + chia. The data were analyzed by one-way ANOVA. Means followed by different small letters in the same row differ significantly, according to Newman–Keuls post hoc test, at the 5% threshold of probability. * Indicates differences between the groups by the t-test, at 5% probability, in the comparison of the groups that received the same diet, either with or without chia (SD x SD+chia and HFD x HFD+chia).

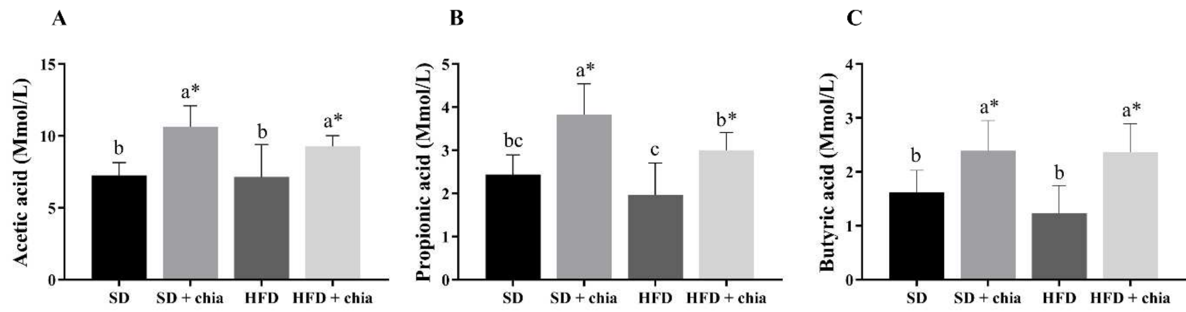

Figure 1. Short-chain fatty acid (SCFA) concentration in cecal contents of young *Wistar* rats, after 35 days of treatment (n = 8). (**A**) Concentration of acetic acid; (**B**) concentration of propionic acid; (**C**) concentration of butyric acid. SD: standard diet; SD+chia: standard diet + chia; HFD: high-fat diet; HFD+chia: high-fat diet + chia. The data were analyzed by one-way ANOVA. Means followed by different small letters in the same row differ significantly, according to Newman–Keuls post hoc test, at 5% threshold of probability. * Indicates differences between the groups by the t-test, at 5% probability, in the comparison of the groups that received the same diet, either with or without chia (SD x SD+chia and HFD x HFD+chia).

3.2. Colonic Histomorphometry Characteristics

The HFD consumption reduced the longitudinal muscle layer, circular muscle layer, crypt length and crypt thickness. On the other hand, when associated with high-fat diet, chia consumption reduced the longitudinal muscle layer. Chia consumption with a standard diet increased the circular muscle layer compared to all the other groups. However, in the high-fat groups, chia consumption reduced this measurement. The crypt length was increased by chia consumption in the group that received HFD, but in the group fed the standard diet, the crypt length and crypt thickness were reduced by chia consumption (Table 4).

Table 4. Colonic histomorphometry characteristics of young *Wistar* rats after 35 days of treatment.

	SD	SD+chia	HFD	HFD+chia
LML (μm)	49.16 ± 12.48 ^{a}	52.65 ± 26.90 ^{a}	26.02 ± 11.36 ^{b*}	22.68 ± 10.94 ^{b}
CML (μm)	111.88 ± 35.84 ^{b}	127.44 ± 70.86 ^{a}	62.32 ± 24.37 ^{c*}	50.01 ± 24.13 ^{d}

Crypt length (µm)	180.53 ± 41.33 ^{a*}	163.67 ± 44.52 ^{b}	103.39 ± 13.97 ^{d}	121.51 ± 30.96 ^{c*}
Crypt thickness (µm)	45.57 ± 7.64 ^{a*}	36.00 ± 13.53 ^{b}	26.72 ± 5.67 ^{c}	25.41 ± 6.08 ^{c}

Values referring to means ± SD, n = 8/group. SD: standard diet; SD+chia: standard diet + chia; HFD: high-fat diet; HFD+chia: high-fat diet + chia; LML: longitudinal muscle layer; CML: circular muscle layer. The data were analyzed by one-way ANOVA. Means followed by different small letters in the same row differ significantly, according to the Newman–Keuls post hoc test, at the 5% threshold of probability. * Indicates differences between the groups by the t-test, at 5% probability, in the comparison of the groups that received the same diet, either with or without chia (SD x SD+chia and HFD x HFD+chia).

3.3. Intestinal Microbiota Analysis

Regarding the intestinal microbiota analysis, the sequencing of the 16S rRNA gene from the fecal samples generated 731.297 raw sequences. Following the filtering and cleaning of the sequences, 569.406 sequences of suitable quality were obtained. The Good's coverage obtained in the samples was >99%, which indicates adequate sequencing coverage. The summary of the sample sequencing data is shown in the Supplementary Table S1.

Table S1. Sequencing data at the end of 35 days of treatment, according to each experimental group.

Groups	**Good's coverage**	**Raw sequences**	**After filtering and cleaning**		**After normalization**	
		Reads	Reads	OTUs	Reads	OTUs
SD	0.998 ± 0.000	29399 ± 3650	22232 ± 2825	316 ± 24	15324 ± 7	316 ± 24
SD+chia	0.997 ± 0.001	35042 ± 5041	27337 ± 3937	404 ± 51	15335 ± 39	394 ± 67
HFD	0.997 ± 0.000	25994 ± 5565	20763 ± 3619	300 ± 78	15318 ± 8	300 ± 78
HFD+chia	0.997 ± 0.000	31449 ± 7733	24569 ± 5404	396 ± 78	15313 ± 32	383 ± 69

Values referring to means ± SD, n = 8/group. SD: standard diet; SD+chia: standard diet + chia; HFD: high fat diet; HFD+chia: high fat diet + chia.

The richness and diversity indexes were used to evaluate the alpha diversity of the microbial samples. No difference was observed in the Chao1, Shannon and Simpson indexes among the experimental groups (Figure 2A–C). However, when assessed pairwise (SD x SD+chia and HFD x HFD+chia), the Chao1 index show an increase in richness in the intestinal microbiota of the animals fed SD+chia compared to the SD group (Figure 2A).

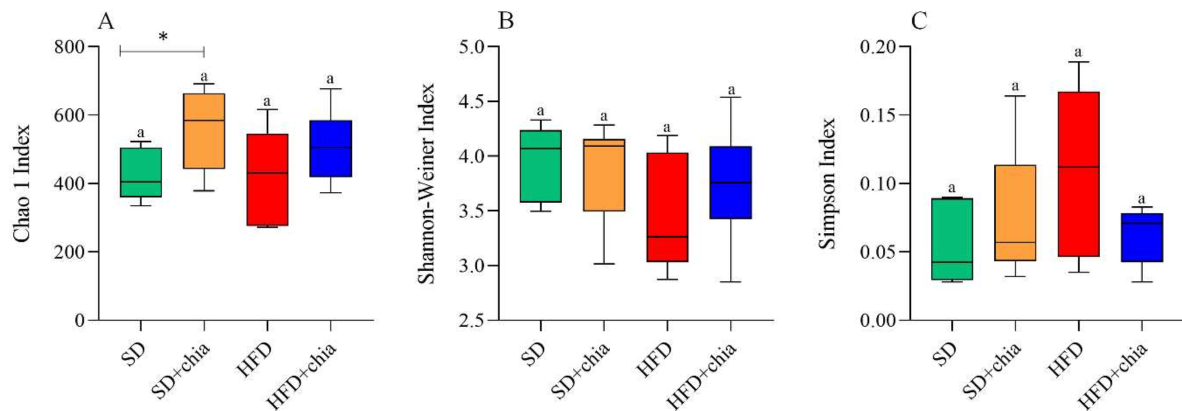

Figure 2. Alpha diversity metrics of bacterial communities in the cecum faces. n = 6 per group. (A) Chao1 index; (B) Shannon-Weiner Index; (C) Simpson Index; SD: standard diet; SD+chia: standard diet+chia; HFD: high-fat diet; HFD+chia: high-fat diet+chia. Treatment groups indicated by the same letter are not significantly different ($p < 0.05$). * Indicates differences between the groups by the t-test, at 5% probability, in the comparison of the groups that received the same diet, either with or without chia (SD x SD+chia and HFD x HFD+chia).

According to the beta diversity analyses, calculated by using Jaccard distances, there was some variation in bacterial communities in response to the consumption of the four types of diet at the level of OTU, phyla and genera, as indicated by the PCoA analysis and PERMANOVA (Figure 3A–C).

The spatial ordination of the OTUs (Figure 3A) indicated differences in the clustering of the samples of the different experimental groups. The data based on their collections of sequences presented differences in the distance metrics among the SD group compared to SD+chia, and between the HFD group and HFD+chia. However, after FDR correction, the clustering of OTU sequences presented no difference. Spatial ordination at the phylum level (Figure 3B) indicated no changes between all treatment groups or between the HFD and HFD+chia groups. Although PERMANOVA identified a significant difference between the

clustering of phyla from SD and SD+chia groups, no difference was verified after FDR correction.

The statistical data revealed that before FDR correction, the Lachnospiraceae family was enriched in the group fed HFD+chia compared to the HFD group. In addition, the Muribaculaceae family and the genus *Roseburia* were enriched in the group fed SD+chia compared to the SD group, which indicates a beneficial effect of chia consumption on the SCFA-producing bacteria (Supplementary Table S2).

Table S2. Significantly different families and genus identified prior to FDR correction, at the end of 35 days of treatments.

	SD	**SD+chia**	**HFD**	**HFD+chia**
Lachnospiraceae	27.34 ± 3.49[a]	26.61 ± 1.50[a]	22.78 ± 1.89[b]	26.97 ± 1.92[a]
Muribaculaceae	9.68 ± 1.39[b]	12.88 ± 2.96[a]	12.63 ± 1.73[a]	11.98 ± 1.76[ab]
Roseburia	0.42 ± 0.14[b]	0.68 ± 0.21[a]	0.48 ± 0.14[ab]	0.71 ± 0.24[a]

Values refer to mean relative abundance ± SD, n = 6/group. SD: standard diet; SD+chia: standard diet + chia; HFD: high fat diet; HFD+chia: high fat diet + chia. [a,b] Treatment groups not indicated by the same letter are significantly different ($p<0.05$).

The spatial ordination at the genus level (Figure 3C) presented differences in the distance metrics among the experimental groups. However, as previously observed at the OTU and phylum levels, after FDR correction, these differences were lost. It is important to highlight that the *Corynebacterium* genera concentration differs between the groups fed with HFD and HFD+chia.

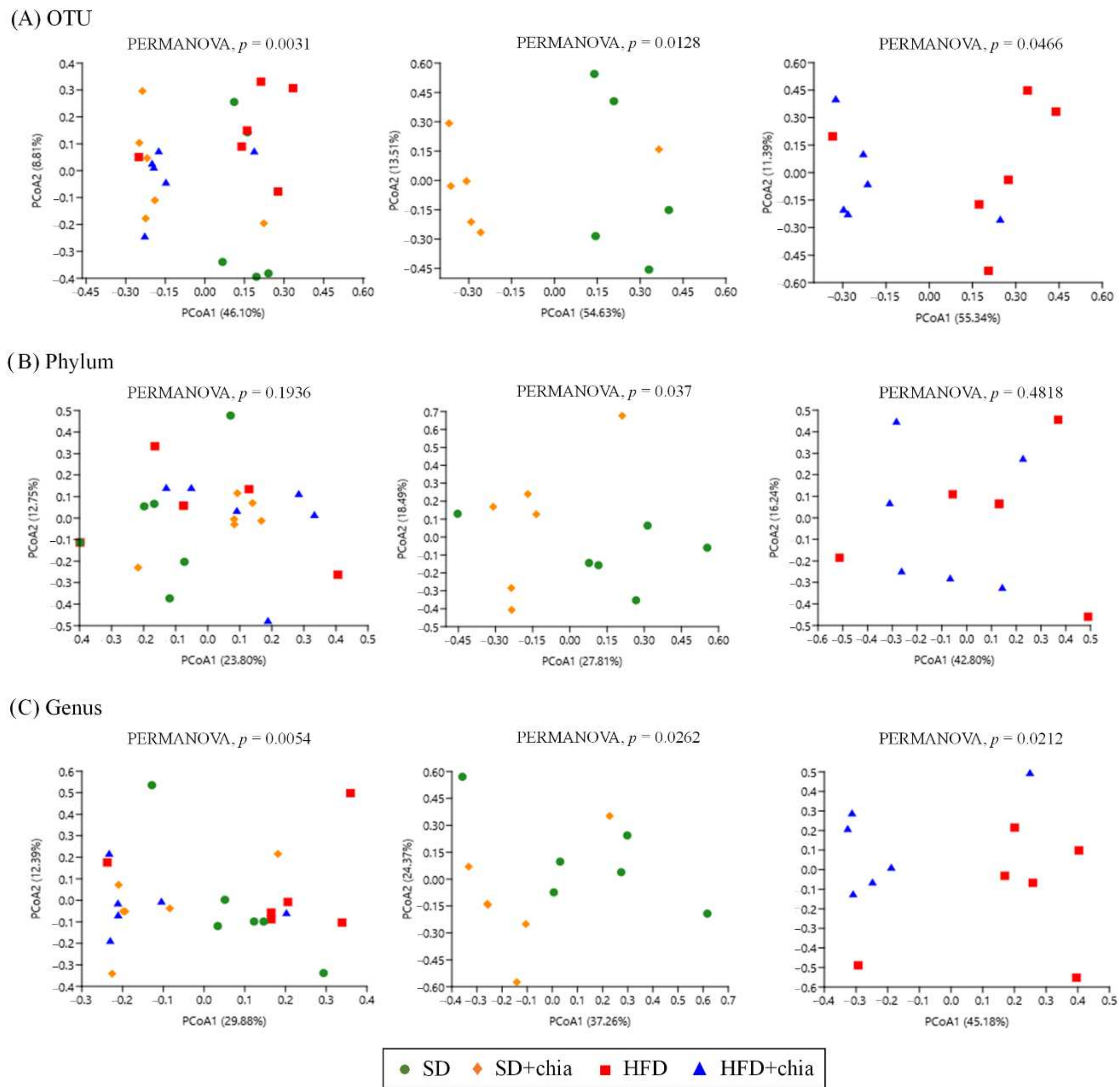

Figure 3. Changes in β-diversity of the cecal microbiome of young *Wistar* rats after 35 days of treatment. (**A**) Principal Coordinate Analysis (PCoA) based on the Jaccard distance at OTU level. (**B**) Principal Coordinate Analysis (PCoA) based on Jaccard distance at phylum level. (**C**) Principal Coordinate Analysis (PCoA) based on Jaccard distance at genus level.

The taxonomic analysis of the bacterial community in response to chia flour consumption revealed the existence of 18 phyla, 30 classes, 65 orders, 93 families and 193 genera. The stratification of phyla, genus and the Firmicutes to Bacteroidetes ratio is plotted in Figure 4. Although no difference was found in the relative abundance of the identified phyla and genus between the experimental groups, after FDR correction, we observed the dominancy of phyla Firmicutes (63–68% of relative abundance) and Bacteroidetes (19–22% of relative

abundance) composing the intestinal microbiota of young rats (Figure 4A), and the dominancy of genera from the Muribaculaceae family, and *Bacteroides* (Figure 4C), identified after 35 days of treatment. Furthermore, no difference was observed in the Firmicutes to Bacteroidetes ratio between the experimental groups (Figure 4B).

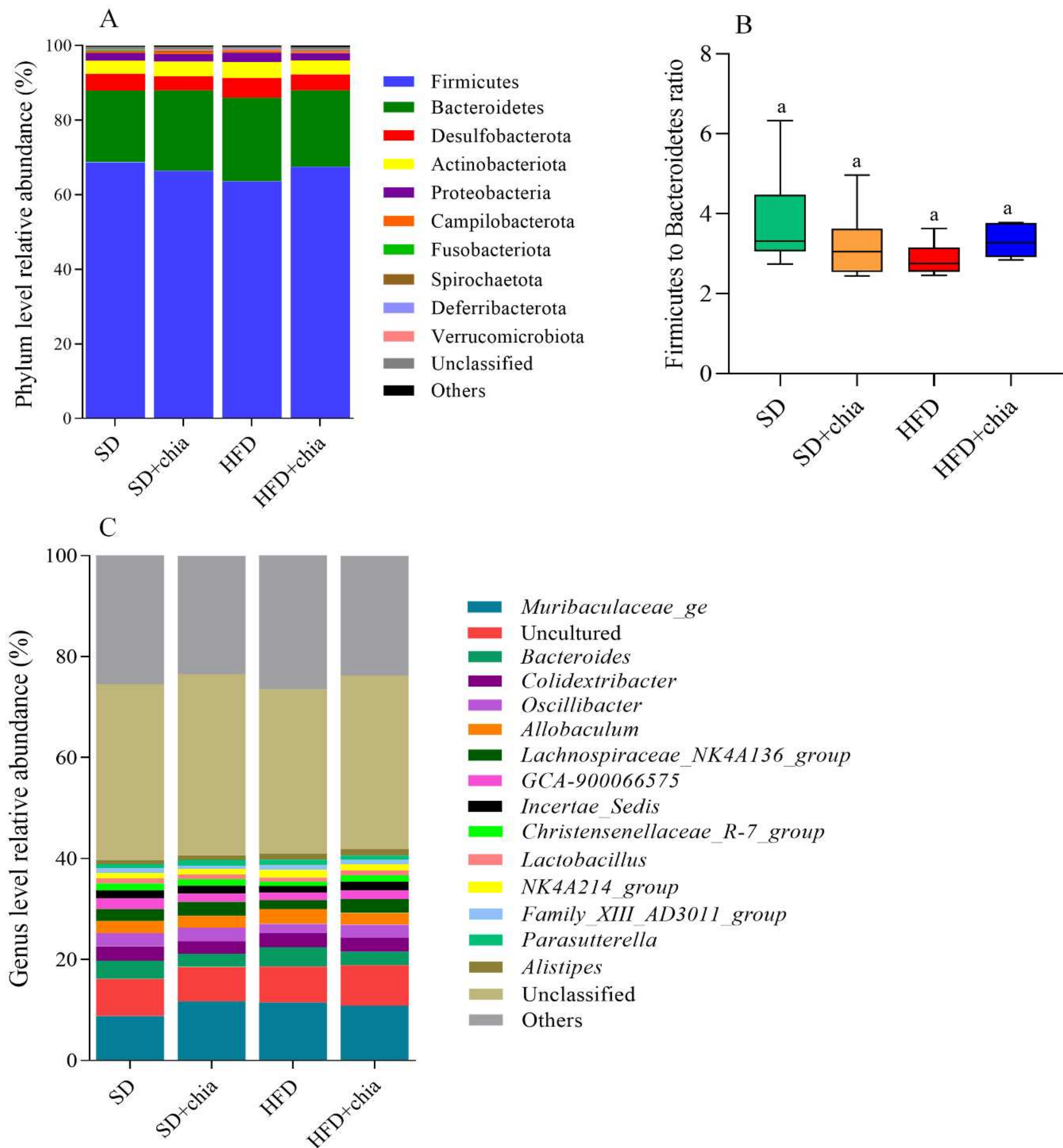

Figure 4. Relative abundances of bacterial microbiota composition at phylum and genera level of young *Wistar* rats, after 35 days of treatment. (**A**) Relative abundance of each identified phylum. (**B**) Firmicutes to Bacteroidetes ratio. (**C**) Genera relative abundance. n = 6/group. SD: standard diet; SD+chia: standard diet + chia; HFD: high-fat diet; HFD+chia: high-fat diet +

chia. Only phyla with abundance >0.2% and genera with abundance >1% in at least one group are displayed. The data were analyzed with FDR correction. Treatment groups indicated by the same letter are not significantly different ($p < 0.05$). * Indicates differences between the groups by the t-test, at 5% probability, in the comparison of the groups that received the same diet, either with or without chia (SD x SD+chia and HFD x HFD+chia).

We investigated whether chia treatments affected the genetic capacity of the microbiota and explored possible functional changes. PICRSt2 was used for the functional predictive analysis of the metagenome and revealed the most abundant pathways (Figure 5), but no difference was detected between the treatments.

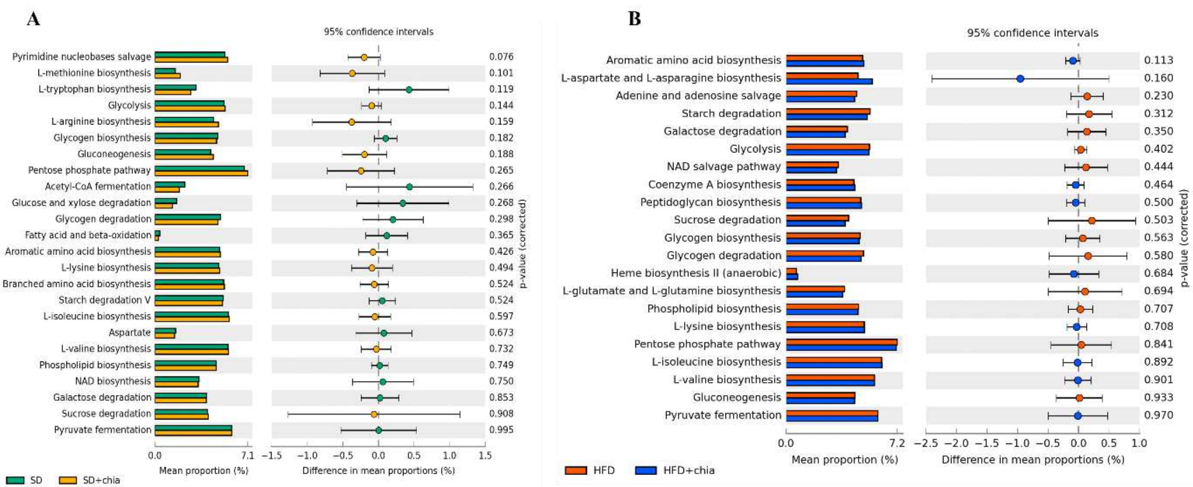

Figure 5. Difference in the relative abundance of the most abundant KEGG microbial metabolic pathways in the microbiota of young *Wistar* rats, after 35 days of treatment. (**A**) Enriched pathways between the SD and SD+chia treatment groups. (**B**) Enriched pathways between the HFD and HFD+chia treatment groups. Extended error bar plot was performed by bioinformatic software (STAMP). Welch's two-sided test was used and Welch's inverted was 0.95. n = 6/group. SD: standard diet; SD+chia: standard diet + chia; HFD: high-fat diet; HFD+chia: high-fat diet + chia.

3.4. BBM Functional Proteins

Regarding intestinal functionality, we observed that the group that consumed HFD presented higher gene expression for all intestinal genes evaluated. Furthermore, chia consumption (SD+chia and HFD+chia) reduced PepT1, AP, SGLT1 and SI mRNA gene expression in relation to the control diets (SD and HFD) (Figure 6).

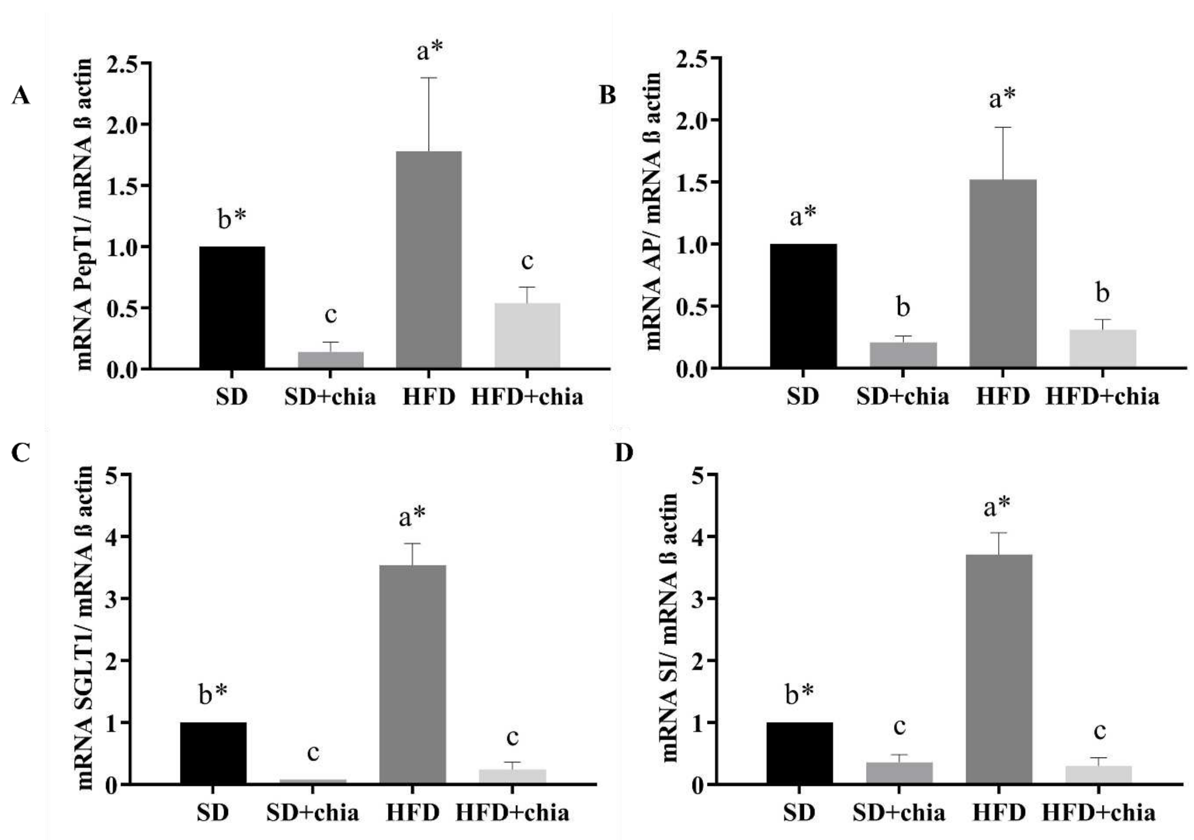

Figure 6. Gene expression of brush border membrane functional proteins in the intestinal tissue. RT-qPCR analysis. (**A**) PepT1 expression, (**B**) AP expression, (**C**) SGLT1 expression and (**D**) SI expression. ST: standard diet; ST+chia: standard diet + chia; HFD: high-fat diet; HF+chia: high-fat diet + chia; PepT1: peptide transporter 1; AP: aminopeptidase; SGLT1: sodium–glucose transport protein 1; SI: sucrose isomaltase. The data were analyzed by one-way ANOVA. Means followed by different small letters in the same row differ significantly, according to the Newman–Keuls post hoc test, at the 5% threshold of probability. * Indicates differences between the groups by the t-test, at 5% probability, in the comparison of the groups that received the same diet, either with or without chia (SD x SD+chia and HFD x HFD+chia).

3.5. Pearson Correlation Analysis

We performed the Pearson correlation analysis to assess the relationship between changes in intestinal microbial abundance, gut health parameters, oxidative stress markers and biochemical variables. When correlations were assessed, Bacteroides were negatively correlated with the Muribaculaceae family (r = −0.487), Roseburia (r = −0.480) and butyric acid (r = −0.496). The Lachnospiraceae family was negatively correlated with total cholesterol (TC) (r = −0.507). The Muribaculaceae family was positively correlated with IgA (r = 0.482). Roseburia was positively correlated with acetic acid (r = 0.602), propionic acid (r = 0.471), catalase (CAT) (r = 0.485) and malondialdehyde (MDA) (r = 0.495). Acetic acid was positively correlated with propionic acid (r = 0.836), butyric acid (r = 0.649), fecal moisture (r = 0.662) and total antioxidant capacity of plasma (TAC) (r = 0.475), and negatively correlated with TC (r = −0.426). Propionic acid was positively correlated with butyric acid (r = 0.664) and fecal moisture (r = 0.711), CAT (r = 0.590), and negatively correlated with TC (r = −0.515). Butyric acid was positively correlated with fecal moisture (r = 0.581) and TAC (r = 0.486), and negatively correlated with TC (r = −0.423). The fecal moisture was positively correlated with the cecal content pH (r = 0.785) and CAT (r = 0.576), and negatively correlated with TC (r = −0.598). Superoxide dismutase (SOD) was positively correlated with MDA (r = 0.597). Finally, CAT was positively correlated with MDA (r = 0.515) and glucose (r = 0.391), and negatively correlated with TC (r = −0.427) (Figure 7).

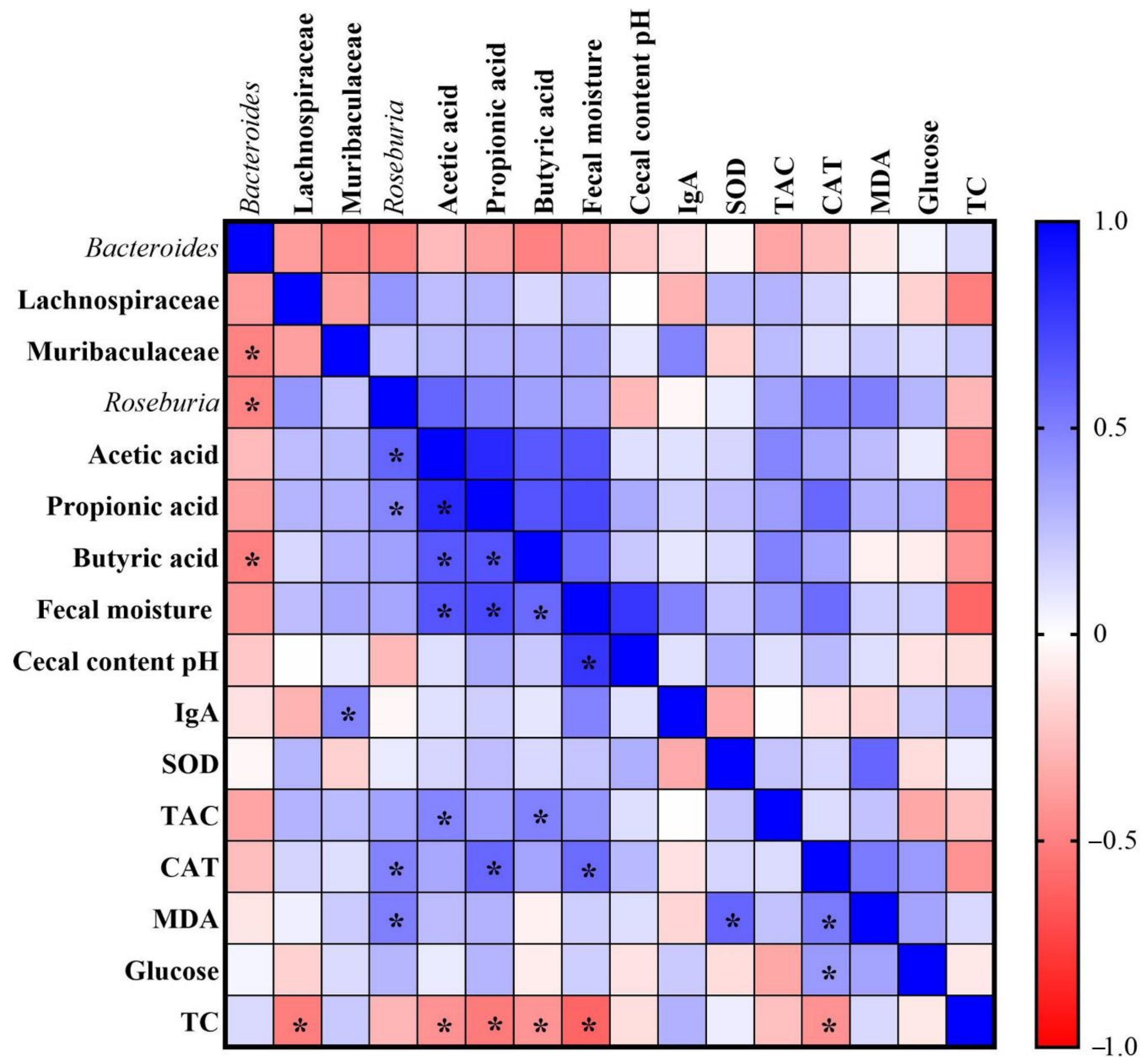

Figure 7. Heatmap of Pearson correlation analysis. IgA: immunoglobulin; SOD: superoxide dismutase; TAC: total antioxidant capacity of plasma; CAT: catalase; MDA: malondialdehyde; TC: total cholesterol. * Indicates statistically significant difference.

4. Discussion

Chia is a good source of bioactive compounds and dietary fiber [13,31]. However, the potential effects of chia associated with an inflammatory condition on the intestinal microbiota composition, intestinal morphology and intestinal functionality have not been investigated. Thus, the present study focused on evaluating the effect of chia consumption associated with HFD, for 35 days, on the gut health of young *Wistar* rats. Our study revealed that the intake of chia with the SD or HFD increased the production of acetic acid, propionic acid and butyric

acid and increased fecal moisture. The consumption of HFD affects the intestinal morphology, reducing the longitudinal and circular muscle layer and the length and thickness of the crypts. Chia consumption increased the crypt length in the group that received HFD and improved circular muscle layer in the group that received SD. The diversity and abundance of intestinal bacteria were not affected, but increased richness was observed in the intestinal microbiome of animals fed SD+chia compared to the SD group. Additionally, chia consumption reduced the expression of proteins involved in intestinal functionality.

The lower food consumption observed in animals fed HFD can be attributed to higher energy density and greater satiety during the experimental period, as previously reported [12,30]. When chia was consumed with HFD, the food consumption was increased, as was the final weight. In the group that was fed SD, although the food consumption was not altered by chia consumption, the SD+chia group presented higher final weight, but no difference in adiposity was observed between groups. This leads us to assume that there was an increase in the muscle mass of these animals. The study of Grancieri *et al*. (2022) [32] observed that the incorporation of the digested protein of chia into a normal diet increased body weight and contributed to muscle mass gain in the animals, which impacted weight gain without increasing body fat. The skeletal muscle plays a major role in fatty acid uptake and oxidation, while HFD can increase the susceptibility to loss of muscle mass and the degradation of proteins [33]. Chia consumption, when associated with SD and HFD, increased fecal moisture, probably due to the higher content of dietary fibers present in food. The gel-forming dietary fibers present sufficient water-holding capacity and increase fecal bulking action [34]. The increased weight of the feces makes it easier for them to pass through the colon and be expelled from the body, thus alleviating problems such as constipation. Here, the increased moisture in groups that were fed chia can be attributed to chia's ability to absorb water, which adds bulk to the feces. Chia secrets a mucilage when it becomes wet, a gelatinous and viscous substance after water absorption, which can increase fecal moisture [34].

In general, a high amount of soluble and insoluble dietary fiber increases intestinal motility and fecal volume, which tends to increase the thickness of muscle layers. The circular muscle layer was higher in the group fed SD+chia than in the SD group. This increase was also observed in female ovariectomized *Wistar* rats [35]. This result is probably due to the increased motility of the digestive tract, enabled by the intact form of the dietary fiber found in chia, besides the formation of gel, promoted by the soluble dietary fiber fraction, which leads to the hypertrophy of muscle cells [36].

The consumption of dietary fiber provides a substrate for microbial activity and affects the intestinal microbiota by altering bacterial fermentation and fermentation products, such as SCFA [5,6]. In our study, increased SCFA production was observed in groups that were fed chia. These changes in total SCFAs indicate that the dietary fiber from chia seed could be utilized by the microbiota [37]. SCFAs, such as butyric acid, acetic acid and propionic acid, are the most abundant fecal metabolites. It is important to mention that acetic acid was the most abundant SCFA, which is in agreement with previous reports by Tamargo *et al*. (2018) [37]. Different SCFA production patterns are possibly dependent on the type of fiber. These fermentation products are extremely important for host health, since butyric acid, apart from serving as the primary energy source for colonocytes, also improves the integrity of intestinal epithelial cells by promoting tight junctions and cell proliferation. Both acetic acid and propionic acid also aid in anti-inflammatory processes and cytokine production [38]. It is suggested that acetic acid plays an important regulatory role in body weight control and insulin sensitivity, acting as a direct mediator in lipid metabolism and glucose homeostasis [39]. The production of SCFAs can reduce the intestinal pH, which may increase mineral solubility and absorption [8,40]. However, in the present study, the cecal content pH was not altered. In our study, the Pearson's correlation indicated that SCFAs were positively correlated with fecal moisture and antioxidant capacity, and negatively correlated with cholesterol.

In our study, the consumption of chia did not affect the immunoglobulin A (IgA) concentration. IgA is abundant in the intestine and plays an essential role in the defense of the intestinal mucosa against harmful pathogens. It is suggested that diet and intestinal microbiota are involved in the regulation of IgA production [41,42]. Our result is in agreement with another study that also evaluated the impact of chia consumption on intestinal health and found that there was no change in the concentration of IgA [35]. Nakajima *et al*. (2020) [43] revealed that the microbiota in the large intestine is involved in IgA induction by dietary fiber, and the amount of IgA was considerably higher in the intestinal contents of mice that were fed a diet with soluble dietary fiber than in those that were fed a diet with insoluble dietary fiber. The concentration of dietary fiber in chia is mainly formed by insoluble fiber [13], which can explain why the IgA concentration was not altered in our animals.

The diversity and abundance of intestinal bacteria were not affected when the Chao1, Shannon and Simpson indexes were calculated among the four experimental groups. However, the Chao1 index indicated increased richness in the intestinal microbiome of the animals fed a standard diet+chia (SD+chia) compared to the SD group, in agreement with our findings from another study [35]. Richness is related to the number of different species, and high richness of

the intestinal microbiome is associated with healthy host metabolism, while their absence is aligned with unhealthy outcomes [38,44–46]. In the same group (SD+chia x SD), the Muribaculaceae family and the genus *Roseburia* were enriched before FDR correction. Studies suggest that the Muribaculaceae family have members with a functional potential in the intestine, which is the ability to degrade dietary carbohydrates and ferment polysaccharides into SCFAs [47,48]. *Roseburia* was positively correlated with acetic acid and propionic acid and has also been reported to produce SCFAs, mainly propionic acid [38], which is in accordance with our results. Therefore, our results indicated a beneficial effect of chia consumption on the SCFA-producing bacteria. It is important to highlight that *Corynebacterium* genus concentration varies between the groups fed with HFD and HFD+chia. Chia consumption was able to reduce the concentration of the genus. This is a positive event, since *Corynebacterium* can produce phospholipase D, an exotoxin that degrades lipids in cell membranes, which may increase cellular permeability and thereby facilitate the spread of the pathogen in the tissues [49]. In addition, according to the functional analysis of the microbiota, we observed the KEGG metabolic pathways that were more abundant, although no differences were detected between the treatments. This fact can be explained by the short time of chia consumption by the animals (35 days).

When we evaluated the intestinal functionality, we found that the HDF increased the expression of all genes evaluated, and chia consumption (HFD+chia) decreased the gene expression, which was similar to the group that consumed SD+chia. Chia consumption reduced the expression of SI and SGLT1, which are genes related to carbohydrate digestion and absorption. Studies have reported that fasting plasma glucose concentration is reduced after chia consumption [36,50,51]. Thus, chia intake may reduce carbohydrate absorption/digestion through the downregulation of SI and SGLT1 gene expression and the consequent reduction in SI, which suppresses the rapid production of glucose on the surface of the brush border membrane, in combination with decreased glucose absorption by reducing the relative expression of SGLT1 [52]. The same result was observed for AP and PepT1 protein gene expression. The function of PepT1 is to transport peptides to the enterocyte. AP is an exopeptidase responsible for cleaving amino acids from the N-terminus of peptides [53]. In our study, we observed that chia consumption reduced PepT1 and AP gene expression, probably due to the fact that the organism is in homeostasis, which does not require the increase in the expression of these genes to perform its function with regard to protein metabolism.

5. Conclusions

Chia consumption in standard and high-fat diet increased the production of short-chain fatty acids (acetic acid, propionic acid and butyric acid) and improved fecal moisture. Furthermore, chia consumption improved intestinal morphology, increasing the circular muscle layer in the SD group and the crypt length in the group that received HFD. Besides this, chia decreased the gene expression of SGLT1, SI, AP and PepT1 in all groups. The 35-day intervention in young male *Wistar* rats did not affect the diversity and abundance of intestinal bacteria but promoted an increase in richness in the SD+chia group. Therefore, further studies using a longer intervention period are needed to clarify the effects of chia on the intestinal microbiome.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Sequencing data at the end of 35 days of treatment, according to each experimental group; Table S2: Significantly different families and genus identified prior to FDR correction, at the end of 35 days of treatments.

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9.2 Paper 2: Effect of chia four associated with high fat diet on intestinal health in female ovariectomized *Wistar* rats

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ORIGINAL CONTRIBUTION

Effect of chia flour associated with high fat diet on intestinal health in female ovariectomized *Wistar* rats

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Abstract

Purpose The present study aimed to evaluate the effect of chia flour associated with a high fat diet on intestinal health in female ovariectomized *Wistar* rats.

Methods The study was conducted with 32 adult female ovariectomized *Wistar* rats, which were separated into four groups: standard diet (ST), standard diet with chia (STC), high fat diet (HF) and high fat diet with chia (HFC) for 18 weeks. Cecum content pH, short chain fatty acid content, brush border membrane functionality and morphology and the gut microbiota were evaluated.

Results This study demonstrated that the consumption of chia flour increased the production of acetic and butyric acids, the longitudinal and circular muscle layers and crypt thickness. It also improved the expression of aminopeptidase (AP) and sucrose-isomaltase (SI) and decreased the cecum content pH. Further, the consumption of chia improved richness and decreased diversity of the microbiota. Operational Taxonomic Units (OTUs) clustering indicated difference between the ST and STC groups. In the linear discriminant analysis effect size (LEfSe) analysis, the *Bacteroides* genus and members of the Muribaculaceae and Lachnospiraceae families were enriched in the STC treatment group. The STC group demonstrated the enrichment of Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways related to peptidoglycan and coenzyme A biosynthesis.

Conclusion Our results suggest that chia flour, which is rich in dietary fiber and phenolic compounds, presented potential properties to improve intestinal health.

Keywords Chia seed · Short chain fatty acids · Gut microbiome · Diversity analysis · Intestinal functionality · Gut health

1. Introduction

The intestine is the largest vital epithelial organ. To perform its functions, the intestinal epithelium needs to form a barrier, which depends on a well-balanced cellular homeostasis, orchestrated by an interaction and balance between differentiation, renewal, proliferation, and the intestinal ecosystem. Disruption of the balance in intestinal homeostasis is associated with a wide range of pathological changes, including metabolic disorders and inflammatory and autoimmune diseases [1].

Excessive consumption of a western diet, high in fat and low in vegetables and dietary fiber, is one of the main risk factors for various metabolic and chronic inflammatory disorders involving different organs. In addition to obesity and metabolic syndrome, many diseases that

affect multiple organs are induced or worsened by the chronic consumption of a high-fat diet (HFD) [2–4]. Saturated fat, in addition to triggering various harmful metabolic responses to the body, is harmful to the intestine, since it increases the permeability of the organ, which leads to deleterious effects on the intestinal microbial ecosystem. These effects cause intestinal dysbiosis and impair the health status of individuals [5].

Growing interest in the dynamic role of microbiome disorders in health has shown that the gut microbiome is still linked to menopause in women [6,7]. Estrogen and its receptors, in addition to reproductive functions, play a multifactorial role in inflammation, autoimmunity and in the physiology of the gastrointestinal tract, contributing to the prevention and/or progression of various conditions in this tissue such as inflammatory bowel diseases. At low estrogen levels (as seen after menopause) pro-inflammatory pathways are stimulated [8]. These hormonal changes are also related to greater body adiposity, which can be aggravated by the consumption of a high-fat diet [9] and be an aggravating factor in inflammation. The post-menopausal period can affect the abundance of some genera, microbial diversity, Firmicute/Bacteroidete ratio, permeability and intestinal motility in humans. Thus, a relationship between the gut microbiome and systemic health is suggested, which may be negatively affected by menopausal changes and inflammation [6,7].

The *Salvia* genus, native to southern Mexico and northern Guatemala, comprises approximately 900 seed species of the Lamiaceae family, 61 of which are cultivated in Brazil. Among the species of the *Salvia* genus, chia (*Salvia hispanica* L.) is an herbaceous plant with high nutritional and functional value [10]. It is a source of protein, dietary fiber, polyunsaturated fatty acids, phenolic compounds, vitamins and minerals [11]. In relation to the content of dietary fiber, it is important to highlight that about 30% of the dietary fiber is composed of insoluble fiber and 3% of soluble fiber [11]. Moreover, chia has a substantial amount of phytochemicals, such as rosmarinic acid, rosmarinyl glucoside and ferulic acid [12], vitamin E, flavones and flavanones [11]. Chia consumption has proven antihyperlipidemic [13], anti-inflammatory [14], hypoglycemic [15], and cardioprotective *in vivo* activity [16]. Some studies with humans demonstrated that chia consumption can induce short-term satiety in healthy individuals [17] and have a beneficial effect on some markers of the lipid and fatty acid profile [18]. Besides, the literature reveals that the concentration of dietary fiber present in this food is able to improve intestinal morphology and health when associated with a standard diet, which indicates that chia is capable of inducing beneficial changes in the microbial composition [15].

Despite the available knowledge about the effect of chia consumption on health parameters and intestinal morphology, the effects of this food on intestinal health after

menopause changes are still undetermined. We hypothesized that chia consumption may improve intestinal health parameters in animals that have undergone the characteristic changes of menopause and fed a high-fat diet. Therefore, the present study aimed to evaluate the effect of chia flour associated with a high fat diet on intestinal health in female ovariectomized *Wistar* rats. Our primary outcome was to assess the effects of chia consumption on parameters of measurements of intestinal health (brush border membrane functionality, microbiota composition, intestinal functionality and histomorphometric measurement) during the post menopause, the secondary outcome was analysing the biometric and intake measurements and correlation analysis.

2. Material and Methods

2.1. Sample material

Chia seeds (*Salvia hispanica* L.) grown in the state of Rio Grande do Sul (Brazil) were used in the study. The seeds were ground up in three replicates, using a knife mill (Marconi Equipment, Brazil) to a particle size of 850 µm to obtain flour. Subsequently, chia flour was packed in polyethylene aluminum bags and stored in a freezer (-18°C ± 1°C) until the diet preparation.

2.2. Animals and diets

Thirty-two 21-day-old female rats (*Rattus norvegicus*, *Wistar*, albinus variation), newly weaned, from the Central Animal Facility of the Center for Biological Sciences and Health at the Federal University of Viçosa, Minas Gerais, Brazil, were systematically subdivided into 4 groups (n=8). Randomization of animals was performed based on the body weight of the animals, using the WinPepi Program version 11.65, to obtain the same weight average at the beginning of the study and to maintain homogeneity between groups. So that the average weight did not exceed 5 g per group. To calculate the estimated number of animals per group it was used descriptive studies, as detailed by Fontelles *et al*. [19]. The animals were distributed into individual stainless-steel cages, one rat per cage in a controlled temperature environment (22°C) and automatically controlled light and dark cycles of 12 hours. The animals received deionized water *ad libitum* and their respective experimental diets was offered in feeders and was available *ad libitum* to the animals.

The experimental diets were based either on the standard diet [20] or high-fat diet (Research Diets, New Brunswick, NJ), with modifications. Chia was added to the diets (STC and HFC) to replace a 100% of oil and dietary fiber (Table 1). The standard diet was composed of 19% protein, 17% fat and 64% carbohydrate. The high fat diet was prepared in the following proportions: 51% fat, 15% protein and 34% carbohydrate. The amount of chia offered in the

diets was based on its composition: lipids ($32.16g.100g^{-1}$), proteins ($18.18g .100g^{-1}$), total dietary fiber ($33.37g. 100g^{-1}$), soluble fiber ($2.89g. 100g^{-1}$), insoluble fiber ($30.47 g. 100g^{-1}$) and carbohydrates ($4.59g.100g^{-1}$) [11]. The other ingredients were added in sufficient quantities to provide the planned amounts of lipids, proteins, carbohydrates, fiber and calories (Table 1).

Table 1. Composition of the experimental diets (g/kg of diet).

	Induction Diets		**Experimental Diets**			
Ingredients	**ST**	**HF**	**ST**	**STC**	**HF**	**HFC**
Chia (g)	-	-	-	232.60	-	232.60
Albumin (g)*	179.50	179.50	179.50	133.70	179.50	133.70
Dextrinized starch (g)	155.00	155.00	155.00	155.00	155.00	155.00
Sucrose (g)	100.00	100.00	100.00	100.00	100.00	100.00
Soybean oil (mL)	40.00	40.00	70.20	-	70.20	-
Lard (g)	-	240.00	-	-	195.00	195.00
Microcrystalline cellulose (g)	50.00	50.00	86.00	-	86.00	-
Mineral mix (g)	35.00	35.00	35.00	35.00	35.00	35.00
Vitamin mix (g)	10.00	10.00	10.00	10.00	10.00	10.00
L-cystine (g)	1.80	1.80	1.80	1.80	1.80	1.80
Choline bitartrate (g)	2.50	2.50	2.50	2.50	2.50	2.50
Corn starch (g)	420.00	178.45	347.50	319.40	151.00	122.90
Cholesterol	-	1.50	-	-	1.50	1.50
Nutritional composition						
Total calories (kcal)	3778.00	4971.80	3759.80	3717.48	4728.80	4686.48
Caloric density (kcal/g)	3.78	4.97	3.76	3.71	4.73	4.69

*Purity of 78%. ST: standard diet; STC: standard diet + chia; HF: high fat diet; HFC: high fat diet + chia. Means with different letters in the same row indicate a significant difference ($p \leq 0.05$), according to Newman-Keuls test.

During the first 7 weeks, the animals received standard AIN-93M diet [20] (n=16), or high fat diet (n=16), according to their group. After that period, the rats (n=32) were subjected to ovariectomy (OVX). The rats maintained their respective diets for 3 weeks to recover from surgery. At 10 weeks, the OVX animals were redistributed into 4 groups (n=8), and each group received one of the following diets for 8 weeks: standard diet (ST); standard diet + chia (STC); high-fat diet (HF); high-fat diet + chia (HFC). Each single animal was considered an experimental unit.

Body weight and food intake were monitored weekly during the experimental period to determine the feed efficiency ratio (FER) [15]. On the 126th day, after 12 hours of fasting, the animals were anesthetized with isoflurane (Isoforine, Cristália®). The cecum weight was measured, its content was collected in a sterile microtube, immediately frozen in liquid nitrogen and stored at -80 °C for analysis. The duodenum was collected, part of which was flushed with phosphate buffered saline solution, fixed in formaline for 24 h and kept in ethanol 70% for histological analysis, while other part was stored at −80°C before analysis. The order of the analyzes was done randomly. The person who performed the analysis of the results was not blind, but the results were analyzed by more than one person. All the experimental procedures with animals were performed in accordance with the Directive 86/609/EEC of November 24, 1986, in compliance with the ethical principles for animal experimentation. The study protocol was approved by the Ethics Committee of the Federal University of Viçosa (Protocol 20/2017; date of approval: July 13th, 2017).

2.3. Cecum content pH

About 1g of cecum content was homogenized in 10 mL of distilled water, with the aid of vortex glass spheres, for cecum content pH analysis. Subsequently, the glass electrode of the pHmeter (Bel, Italy) was inserted. The measurements were performed in duplicate [21].

2.4. IgA quantification

For the determination of sIgA, 1:5 (w/v) fecal homogenates were prepared according to standard procedures. The fecal samples were defrosted on ice. Suspensions were prepared by adding 200mg feces to 800μL of phosphate-buffered saline and homogenizing with the aid of a vortex. The mucosal immunity was evaluated based on the fecal sIgA concentration, which was measured using an Immunochron enzymelinked immunosorbent assay (ELISA) [22]. Total protein in the sample homogenate was quantified by the Bradford method [23]. The results were expressed in ng of IgA per milligram of protein.

2.5. Short chain fatty acids (SCFA) content

Cecum content samples (~500 mg) were homogenized in 1 mL of Milli-Q water for analysis, with the aid of vortex, and centrifuged at 12.000 g, for 10 min. The supernatant was removed, and the other steps were performed as described by Siegfried, Ruckemann and Stumpf (1984) [24]. Subsequently, the samples were analyzed by high performance liquid chromatography (HPLC), using a Dionex Ultimate 3000 Dual detector HPLC apparatus (Dionex Corporation, Sunnyvale, CA, USA) equipped with a refractive index detector Shodex RI-101, using a Bio-Rad HPX-87H column, 300 mm x 4.6 mm maintained, at 45°C. The analyses were performed isocratically under the following conditions: mobile phase sulfuric acid 5 mmol l-1, flow rate 0.7 mL min-1 and injection volume 20 µl. Stock solutions of the standards were prepared using the acetic, propionic and butyric acid. All SCFA were prepared with a final concentration of 10 mmol/L.

2.6. mRNA extraction from intestinal tissue and cDNA synthesis

The duodenum was macerated in liquid nitrogen under RNAse free conditions, and the samples were aliquoted for total RNA extraction. Total RNA was extracted with the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The extracted mRNA was used to synthesize the cDNA with the M-MLV reverse transcription kit (Invitrogen Corp., Grand Island, NY) [18].

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

mRNA expression levels of genes in the intestinal tissue that are involved in intestinal health were analyzed by RT-qPCR. The SYBR Green PCR master mix from Applied Biosystems (Foster City, CA) was employed, and the analyses were performed on the StepOne™ Real-Time PCR System (Thermo Fisher Scientific) by means of the measurement system involving SYBR-Green Fluorescence and Primer Express software (Applied Biosystems, Foster City, CA). Sense and antisense primer sequences were ordered (Choma Biotechnologies) to amplify AP (ID: 301368687), SI (ID: 301368688), PepT1 (ID: 301368693) and SGLT1 (ID: 301368686). The relative expression levels of mRNA were normalized to the endogenous control (beta-actin; Table 2).

Table 2. Sequencing primers used in the RT-qPCR analysis.

Genes	**Oligonucleotide (5’-3’)**	
	Forward	**Reverse**

Beta-actin	TTCGTTGCCGGTCCACACCC	GCTTTGCACATGCCGGAGCC
AP	CTCTCTCCTCAAACCACATGAA	AGTTCAGGGCCTTCTCATATTC
SI	CCTCCAGAACACAATCCCTATAC	GGAGAGGTGAGATGGGATTAGA
PepT1	CCTGGTCGTCTGCATCATATT	TTCTTCTCATCCTCATCGAACTG
SGLT1	CATCCAGTCCATCACCAGTTAC	CAATCAGGAAGCCGAGAATCA

AP: amino peptidase; SGLT1: sodium-glucose transport protein 1; SI: sucrose isomaltase; PepT1: peptide transporter 1.

2.7. Histomorphometric analysis

Semi-serialized histological duodenum fragments, 3 µm-thick, were obtained on a semi-automated rotating microtome (Leica, Brazil) and stained by hematoxylin and eosin technique. Slides were examined under a AX70 photomicroscope (Olympus, Japan). Twenty random fields per animal were selected to measure crypt depth, crypt thickness and thickness of the circular and longitudinal muscle layers. Only crypts with definite and visible connective epithelium were used, and the images were processed using the ImagePro-Plus® software version 4.5 (Media Cybernetics, Rockville USA) [15].

2.8. DNA extraction and sequencing

Total genomic DNA was extracted from the cecum content samples, following a mechanical disruption and phenol/chloroform extraction protocol [26]. PCR amplicon libraries targeting the hypervariable V4-region of the 16S rRNA gene were produced using the primers 515F (5'GTGYCAGCMGCCGCGGTAA3') and 806R (GGACTACNVGGGTWTCTAAT3') and a barcoded primer set adapted for the Illumina MiSeq plataform (Illumina, San Diego, California, USA) [27,28]. The samples were loaded onto an Illumina flow cell for paired-end sequencing reactions, using the Illumina MiSeq platform in the Environmental Sample Preparation and Sequencing Facility (ESPSF), at the Argonne National Laboratory (Lemont, Illinois, USA).

The sequences obtained for all samples in the present study were submitted to Sequence Read Archive (SRA), at the National Center for Biotechnology Information (NCBI), under the accession number PRJNA805268.

Data processing and analysis were performed using Mothur software v.1.40.0 [29]. In summary, the R1 and R2 paired-end reads were joined, and sequences smaller than 150 or greater than 300 bp were removed. Chimera sequences were detected and removed using

UCHIME [30]. After cleaning, the sequences were aligned with the 16S rRNA gene, using the *Si*LVA database v.138 [31].

The Operational Taxonomic Units (OTUs) were grouped with a 97% sequence similarity cutoff. To correct sampling bias due to unequal amplicon library sizes, the samples were normalized for the lowest number of sequences produced from any sample. Alpha diversity was estimated by using Chao1, Shannon and Simpson indices, and beta diversity was assessed by Principal Coordinate Analysis (PCoA) based on the Jaccard dissimilarity index [32]. The taxonomy was evaluated in each experimental group and the Firmicutes/ Bacteroidetes ratio was calculated. The final analysis investigation of relative abundances at all taxonomic levels was carried out using the linear discriminant analysis effect size (LEfSe) method to investigate bacterial biomarkers that could represent differences in the gut microbiota of the experimental treatments. The PICRUSt2 software system was used to carry out metagenome functional predictive analysis to investigate any alterations in the metagenomic potential of the gut microbiota. The Greengenes database was used to identify the 16S rRNA gene copy number to normalize feature abundance, and KEGG ortholog prediction was calculated.

2.9. Statistical analysis

The biological data were initially submitted to a Kolmogorov-Smirnov normality test. Then, a *one-way* analysis of variance (ANOVA) was applied, followed by the *post-hoc* of Newman-Keuls to compare all experimental groups to verify the effect of standard and high fat diets on intestinal health. Further, the *t*-test was used to compare the pairs ST x STC, and HF x HFC (the same diet with or without chia) to verify the effect of chia on intestinal health. The correlations between biological and gut microbial markers were assessed by the Pearson's correlation test. The inflammatory and stress oxidative markers have already been carried out and published [14]. The analysis was performed in Graphpad Prism version 9.0.

For the microbiome results, Chao1, Shannon and Simpson indices were used to calculate bacterial richness within the samples, while differences between the groups were analyzed by ANOVA. Differences between Beta-diversity indices were analyzed by the Pairwise PERMANOVA test. Statistically significant *p*-values associated with microbial clades and functions were corrected for multiple comparisons using the Benjamini Hochberg false discovery rate (FDR) correction. Statistical analysis was performed using SPSS version 20.0. The level of significance of all analyses was established at $p < 0.05$.

3. Results

The weight gain (Figure 1A) and feed efficiency ratio (Figure 1C) in groups fed with high fat diet (HF and HFC) were higher ($p < 0.05$) than in groups fed with standard diets (ST

and STC). However, the food intake was lower ($p < 0.05$) in animals that consumed higher amounts of fat, regardless of the presence of chia (Figure 1B). Besides, chia consumption did not change ($p > 0.05$) the weight gain, food intake or feed efficiency ratio of the animals.

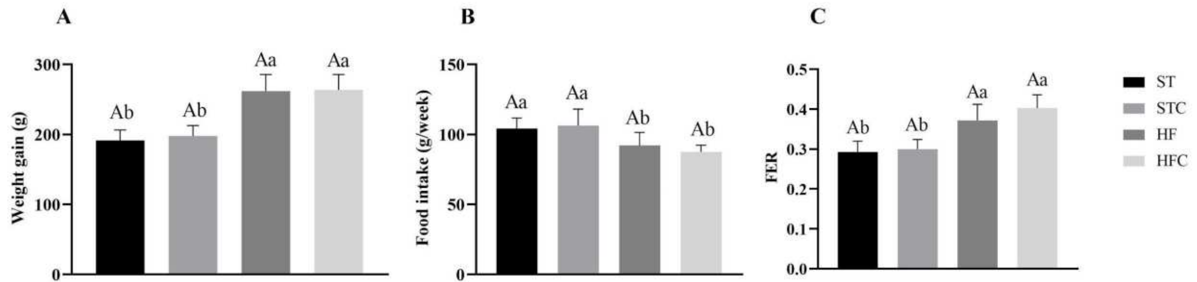

Figure 1. (A) Weight gain, (B) Food intake and (C) FER. ST: standard diet; STC: standard diet + chia; HF: high fat diet; HFC: high fat diet + chia. FER: feed efficiency ratio. Data expressed as mean ± standard deviation (n=8/group). Means followed by the same lowercase letter do not differ significantly according to the Newman-Keuls test at the 5% threshold of probability. Means followed by the same capital letter do not differ from each other, by the *t*-test at 5% probability, compared to the groups that received the same diet, either with or without chia.

The cecum content pH was lower ($p < 0.05$) in groups fed with chia. The cecum weight and the IgA concentration did not differ ($p > 0.05$) among the experimental groups (Table 3). Among the organic acids analyzed, acetic acid increased ($p < 0.05$) in the cecal content of the STC group, compared to the ST group, and no difference ($p > 0.05$) was observed in the HF group in relation to the ST group. However, HFC presented lower concentration of acetic acid in relation to the STC group. Butyric acid increased in groups fed with chia (STC and HFC) in relation to the control group (ST). In addition, the comparison between ST and STC revealed that chia consumption was able to increase ($p < 0.05$) butyric acid, which was also observed in high fat diet groups. The propionic acid cecal concentration increased ($p < 0.05$) among the groups that were fed a high fat diet (HF and HFC) in relation to standard diet (ST and STC) (Table 3).

The crypt depth was lower ($p < 0.05$) in the STC group. Chia seed consumption increase the crypt thickness. The circular muscle layer was increased by the consumption of chia in animals that were fed a standard diet and did not differ among the high fat groups ($p > 0.05$). Chia increased the longitudinal muscle layer in the group fed with a standard diet. However, HFC group present a reduction ($p < 0.05$) on longitudinal muscle layer in comparison to the HF group, but the value did not differ in relation to STC group. Among the groups that fed a

standard diet, those who fed STC presented higher longitudinal muscle layer compared to ST (Table 3).

Table 3. Body weight, cecum content pH, cecum weight, IgA concentration, short chain fatty acids and histomorphometry characteristics of animals.

Variables	Groups			
	ST	**STC**	**HF**	**HFC**
Inicial body weight (g)	101.20 ± 5.30 ^{Aa}	91.20 ± 10.60 ^{Aa}	102.10 ± 10.05 ^{Aa}	94.94 ± 15.61 ^{Aa}
Final body weight (g)	281.60 ± 20.09 ^{Ab}	286.44 ± 28.19 ^{Ab}	348.02 ± 26.51 ^{Aa}	349.39 ± 20.53 ^{Aa}
Cecum content pH	9.04 ± 0.26^{Aa}	8.52 ± 0.51^{Bb}	9.03 ± 0.20^{Aa}	8.36 ± 0.39^{Bb}
Cecum weight (g)	4.33 ± 0.68^{Aa}	4.32 ± 0.51^{Aa}	3.73 ± 0.63^{Aa}	3.69 ± 0.93^{Aa}
IgA (ng IgA/mg protein)	20.42 ± 8.63^{Aa}	29.48 ± 8.92^{Aa}	24.50 ± 8.16^{Aa}	33.34 ± 15.71^{Aa}
Short Chain Fatty Acids (mM)				
Acetic Acid	6.72 ± 2.81^{Bb}	12.05 ± 2.91^{Aa}	9.75 ± 0.61^{Aab}	7.06 ± 1.41^{Ab}
Butyric Acid	0.42 ± 0.13^{Bb}	1.25 ± 0.19^{Aa}	0.65 ± 0.28^{Bb}	1.14 ± 0.32^{Aa}
Propionic Acid	4.19 ± 2.55^{Ab}	6.66 ± 3.73^{Ab}	13.66 ± 3.55^{Aa}	13.14 ± 3.88^{Aa}
Crypt depth (µm)	145.45 ± 46.74^{Aa}	132.33 ± 33.69^{Bb}	144.87 ± 40.10^{Aa}	135.48 ± 35.52^{Aab}
Crypt thickness (µm)	21.36 ± 4.76^{Bb}	24.17 ± 4.12^{Aa}	21.92 ± 4.60^{Bb}	23.78 ± 4.44^{Aa}
CML (µm)	38.89 ± 12.88^{Bc}	52.87 ± 15.79^{Aa}	41.20 ± 10.35^{Abc}	43.50 ± 11.86^{Ab}
LML (µm)	118.40 ± 24.12^{Bc}	147.85 ± 36.47^{Ab}	167.21 ± 45.87^{Aa}	151.11 ± 47.04^{Bb}

Values represent means ± SD. ST: standard diet; STC: standard diet + chia; HF: high fat diet; HFC: high fat diet + chia. LML: longitudinal muscle layer; CML: circular muscle layer. Data expressed as mean ± standard deviation (n=8/group). Means followed by the same lowercase letter in the row do not differ significantly according to the Newman-Keuls test, at the 5% threshold of probability. Means followed by the same capital letter do not differ from each other, by the *t*-test at 5% probability, compared to the groups that received the same diet, either with or without chia.

SGLT1, AP and SI mRNA gene expression were higher in the group fed with STC. The consumption of a high fat diet (HF and HFC) reduced the SGLT1 gene expression, compared to the consumption of a standard diet (ST and STC). Chia consumption increased the mRNA gene expression of AP and SI and decreased the expression of PepT1, when consumed in a standard diet. When consumed in a high fat diet, the mRNA gene expression of SGLT1 and SI was decreased (Figure 2).

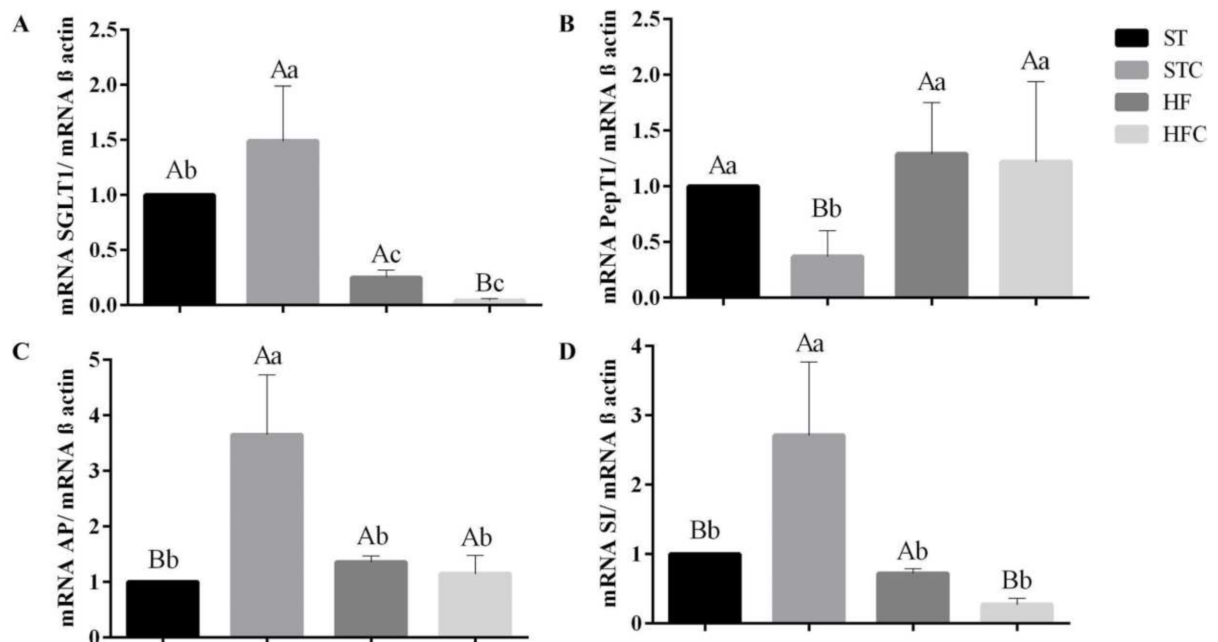

Figure 2. Effect of chia consumption (standard diet and high fat diet) on the gene expression of proteins in the intestinal tissue. RT-qPCR analysis. (A) SGLT1 expression, (B) PepT1 expression, (C) AP expression and (D) SI expression. ST: standard diet; STC: standard diet + chia; HF: high fat diet; HFC: high fat diet + chia; SGLT1: sodium-glucose transport protein 1; PepT1: peptide transporter 1; AP: amino peptidase; SI: sucrose isomaltase. Data expressed as mean ± standard deviation (n=8/group). Means followed by the same lowercase letter did not differ significantly according to the Newman-Keuls test, at 5% probability. Means followed by the same capital letter do not differ from each other, according to the *t*-test, at 5% probability, in the comparison of the groups that received the same diet, either with or without chia.

The 16S rRNA gene sequencing from the cecal content generated 569.661 raw sequences. After filtering and cleaning, 430.127 good quality sequences were obtained. The Good's coverage estimator was > 99% across samples, which indicates that the current

sequencing depth could represent most of the bacterial community in the experimental groups (Supplementary Table).

Supplementary Table 1. Sequencing data at the end of treatment, according to each experimental group

Groups	Good's coverage	Raw sequences	After filtering and cleaning		After normalization	
		Reads	Reads	OTUs	Reads	OTUs
ST	0.998 ± 0.001	24522 ± 4097	18283 ± 3143	251 ± 61	10811 ± 23	244 ± 59
STC	0.997 ± 0.001	24259 ± 2436	17755 ± 1901	319 ± 62	10818 ± 14	319 ± 62
HF	0.997 ± 0.001	24271 ± 3496	18400 ± 2251	285 ± 44	10809 ± 34	276 ± 46
HFC	0.996 ± 0.003	21891 ± 10141	17250 ± 7554	293 ± 39	10805 ± 22	283 ± 36

Values represent means ± SD. ST: standard diet; STC: standard diet + chia; HF: high fat diet; HFC: high fat diet + chia.

Alpha diversity was assessed by using the Chao, Shannon, and Simpson indexes. There was no difference in the richness and diversity indexes among the experimental treatments ($p > 0.05$) (Figure 3A, B, and C). However, after comparing the pairs, considering the type of diet (ST x STC, and HF x HFC), Chao1 indicated an increased richness in the STC group, compared to the ST group ($p<0.05$) (Figure 3A), and the Simpson index revealed decreased dominance ($p<0.05$) in the STC group, compared to the ST control group (Figure 3C).

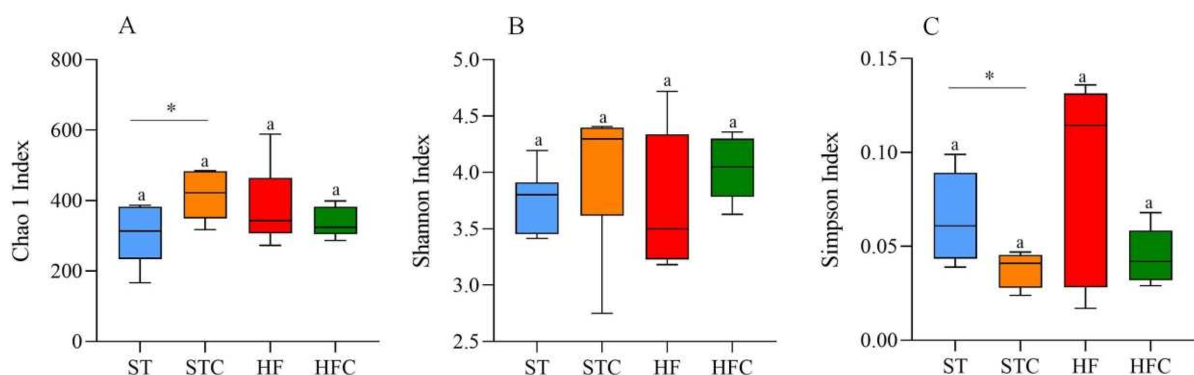

Figure 3. Alpha-diversity metrics of bacterial communities in the cecum faces. Data expressed as median; bars = minimum and maximum value. n = 6/group (ST, STC and HF groups), n = 5/group (HFC group). ST: standard diet; STC: standard diet + chia; HF: high fat diet; HFC: high fat diet + chia. Treatment groups indicated by the same letter are not significantly different ($p < 0.05$). * Indicates differences between the groups ST and STC (paired *t*-test, $p < 0.05$).

Principal Coordinate Analysis (PCoA) was used to assess beta diversity. Based on the Jaccard dissimilarity index and PERMANOVA, we observed some significant results ($p < 0.05$) in the clustering of samples, for OTUs and genus (Figure 4 A and C). The OTUs clustering of the four treatments indicated difference between the ST and HF groups ($p \leq 0.05$), but no difference was verified between the other experimental groups ($p > 0.05$). Accordingly, the pairwise comparison revealed difference between the ST and STC treatments ($p = 0.02$), which indicates a potential effect of the chia flour on the gut microbiome taxa (Figure 4A). Spatial ordination at phylum level (Figure 4B) indicated no significant changes between all treatment groups and in pairwise clustering ($p > 0.05$). However, at genus level (Figure 4C), we observed difference in the distance metrics among the experimental groups ($p < 0.001$), with significant distinction between the ST and HFC groups ($p = 0.02$). After a pairwise clustering according to the type of diet consumed, there was significant difference by PERMANOVA ($p < 0.05$). However, after the correction of the data by FDR, this difference was lost ($p > 0.05$).

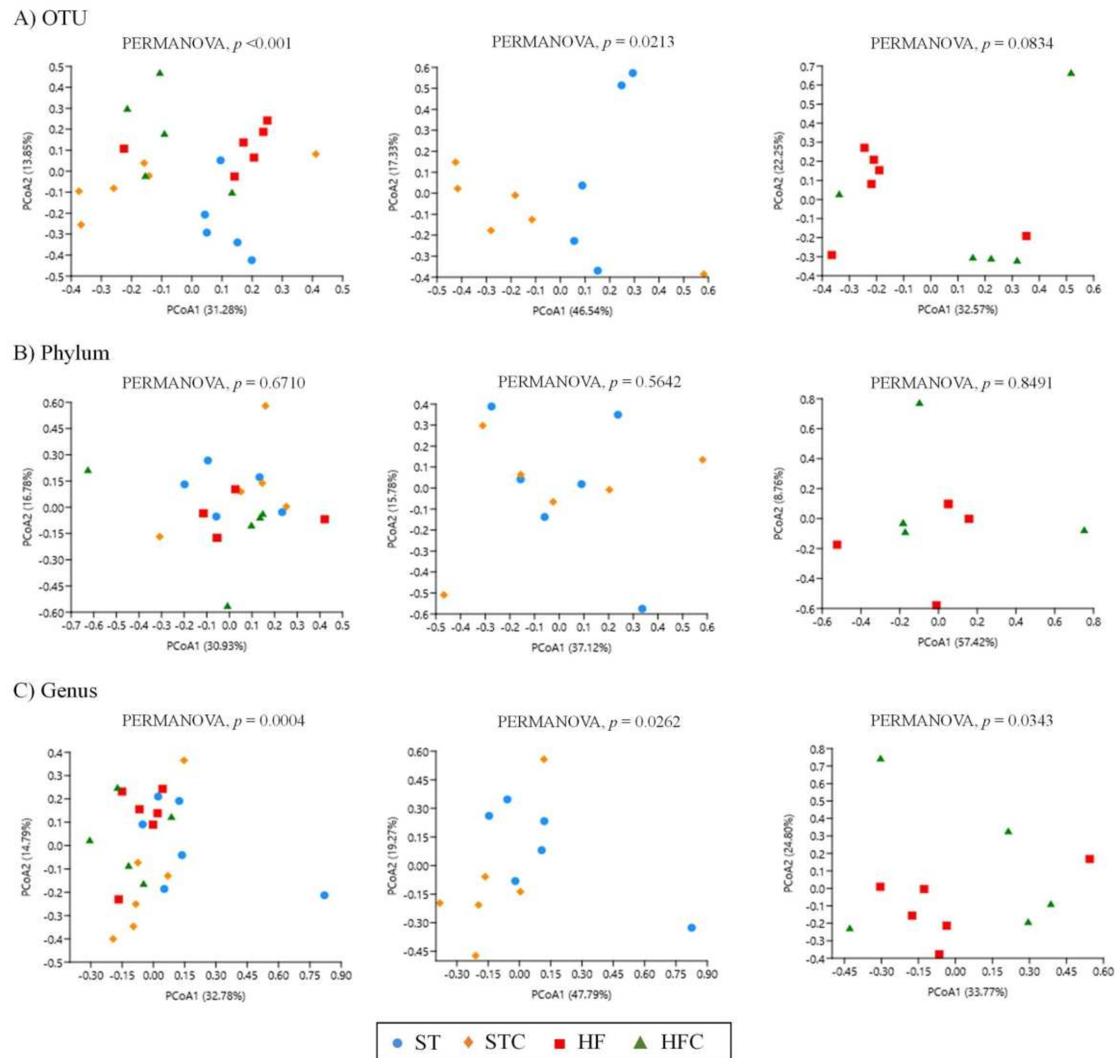

Figure 4. Changes in the β-diversity of the cecal microbiome of adult *Wistar* rats according to each treatment group. A) Principal Coordinate Analysis (PCoA) based on the Jaccard distance at OUT level. B) PCoA based on Jaccard distance at the Phylum level. C) PCoA based on Jaccard distance at Genus level. n = 6/group (ST, STC and HF groups), n = 5/group (HFC group). ST: standard diet; STC: standard diet + chia; HF: high fat diet; HFC: high fat diet + chia.

The taxonomic classification of the samples presented 17 phyla, 25 classes, 61 orders, 100 families and 199 genera. The stratification of the phyla that comprised more than 0.2% of relative abundance, after FDR correction, is exhibited in Figure 5A. There was no statistical difference at the level of phylum and genus when all treatment groups were compared nor after a pairwise comparison (p>0.05). However, the Firmicutes to Bacteroidetes ratio was lower in the ST group, compared to the STC group (p<0.05) (Figure 5B). The sequencing revealed that the four groups were mainly dominated by the Firmicutes (76-80% of relative abundance) and

Bacteroidetes (11-14% of relative abundance) phyla. At genus level (Figure 5C), we observed a dominancy of genera from Muribaculaceae (5-7% of relative abundance) and Lachnospiraceae (3-5% of relative abundance) families, and *Oscillibacter* and *Bacteroides* (1-2% of relative abundance) (Figure 5C), identified after the treatment period.

The analysis performed without FDR correction revealed differences related to the abundance of Bacteroidetes (p=0.03), which were found in large numbers in the cecal content of the animals (>10%). In addition, *Lachnospiraceae_NK4A136_group* exhibited difference between the groups (p=0.01), when the FDR correction was disregarded. However, all differences disappeared after FDR correction (p>0.05).

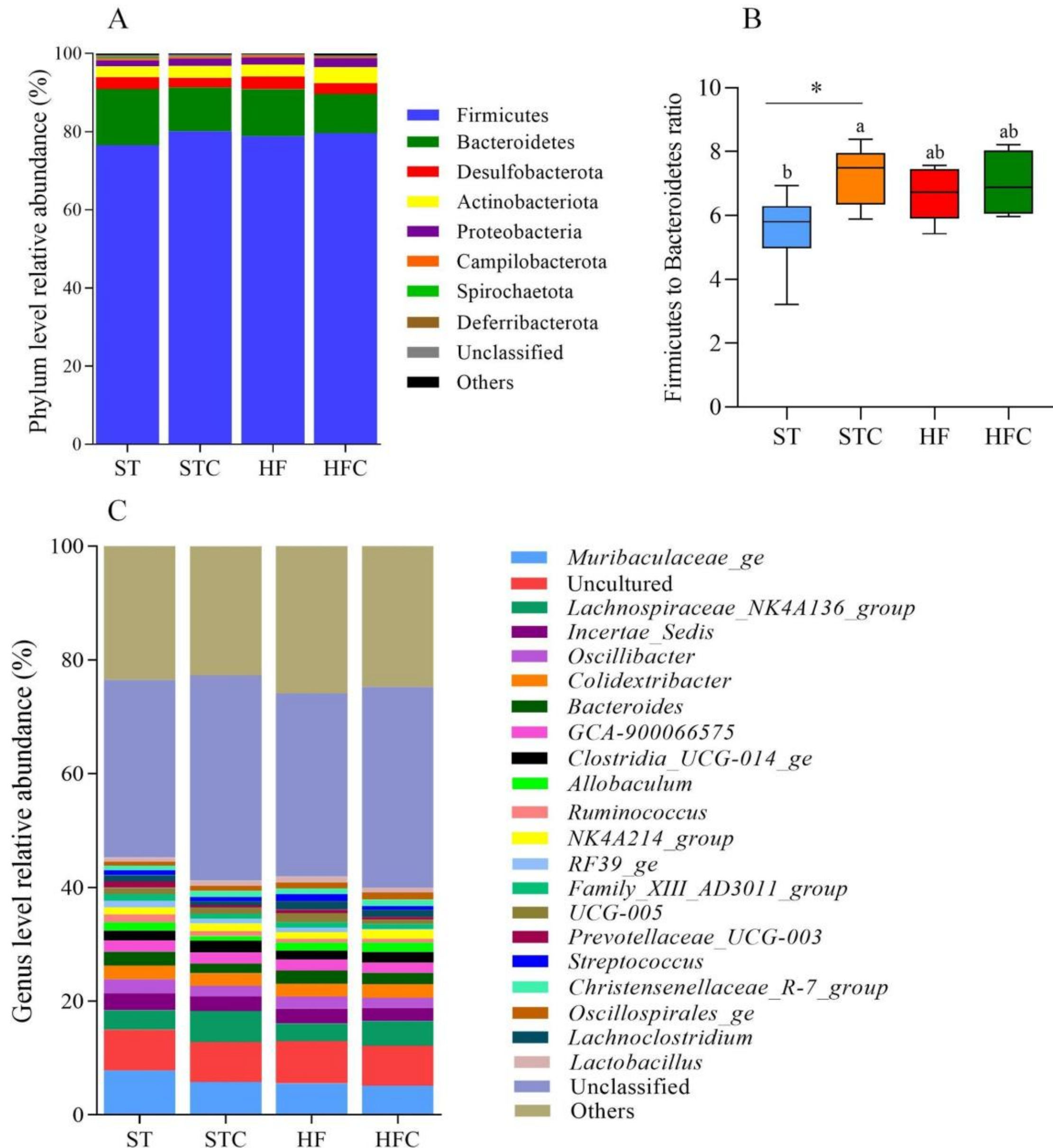

Figure 5. Relative abundances of bacterial microbiota composition at phylum and genera level of adult female *Wistar* rats. A: Relative abundance of each identified phylum; B: Firmicutes/Bacteroidetes ratio; C: Genera samples displayed according to each experimental group (ST, STC, HF and HFC). Data expressed as median; bars = minimum and maximum value. n = 6/group (ST, STC and HF groups), n = 5/group (HFC group). ST: standard diet; STC: standard diet with chia; HF: high fat diet; HFC: high fat diet with chia. Only phyla with abundance > 0.2% and genera with abundance > 1% in at least one group were displayed. Data were analyzed with an FDR correction. * Indicates differences between the groups ST and STC (paired *t*-test, $p < 0.05$).

The linear discriminant analysis effect size (LEfSe) showed that the *Bacteroides* genus and members of the Muribaculaceae and Lachnospiraceae families were significantly enriched ($p<0.05$) in the STC treatment group, compared to the ST control group (Figure 6A). In the HFC group, *Faecalibaculum* and *Ruminococcus* genera and members of the Lachnospiraceae family were significantly enriched ($p<0.05$), compared to the HF control group (Figure 6B).

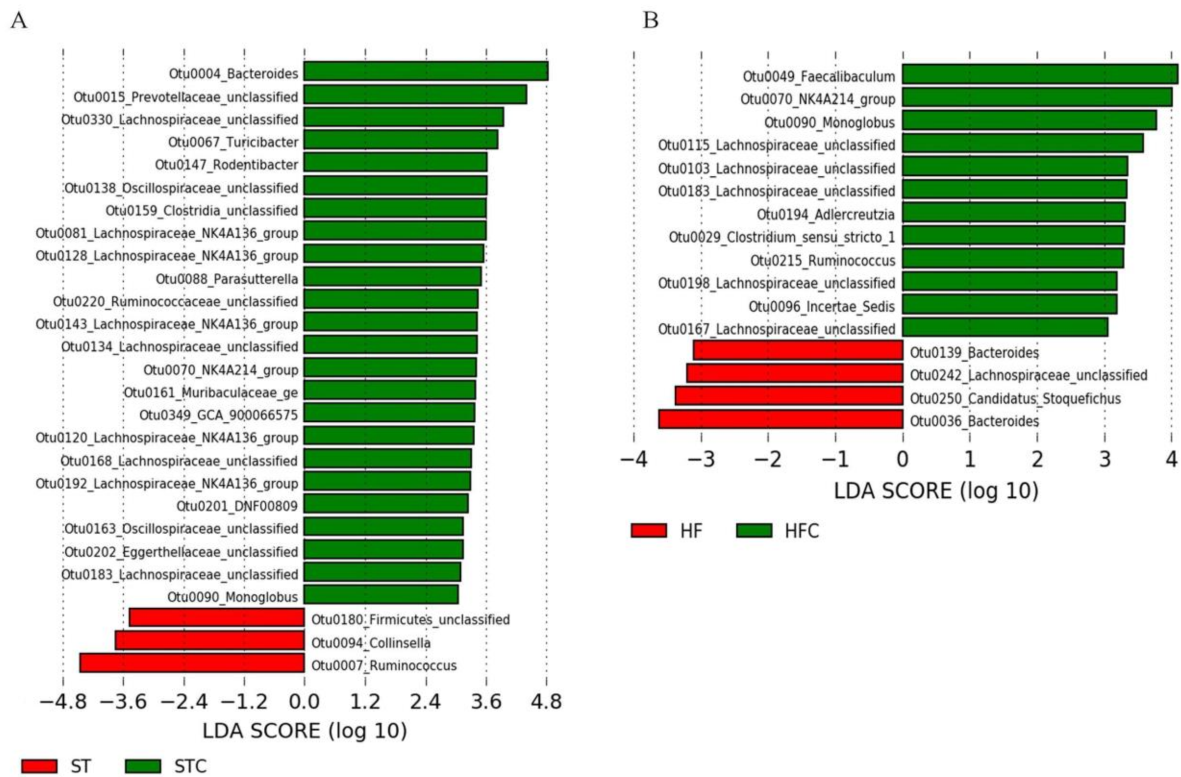

Figure 6. LEfSe method used to compute Linear discriminant analysis (LDA) scores of the relative abundance difference between the experimental groups. A: LDA scores of the relative abundance difference between the STC group and the ST control group; B: Computed LDA

scores of the relative abundance difference between the HFC group and the HF control group. ST: standard diet; STC: standard diet with chia; HF: high fat diet; HFC: high fat diet with chia. n = 6/group (ST, STC and HF groups), n = 5/group (HFC group).

The STC group demonstrated significant enrichment ($p<0.05$) of KEGG metabolic pathways related to peptidoglycan and coenzyme A biosynthesis, compared to the ST control group. However, there was no difference ($p>0.05$) in the other KEGG metabolic pathways or in the pathways between the HF and HFC groups (Figure 7A and B).

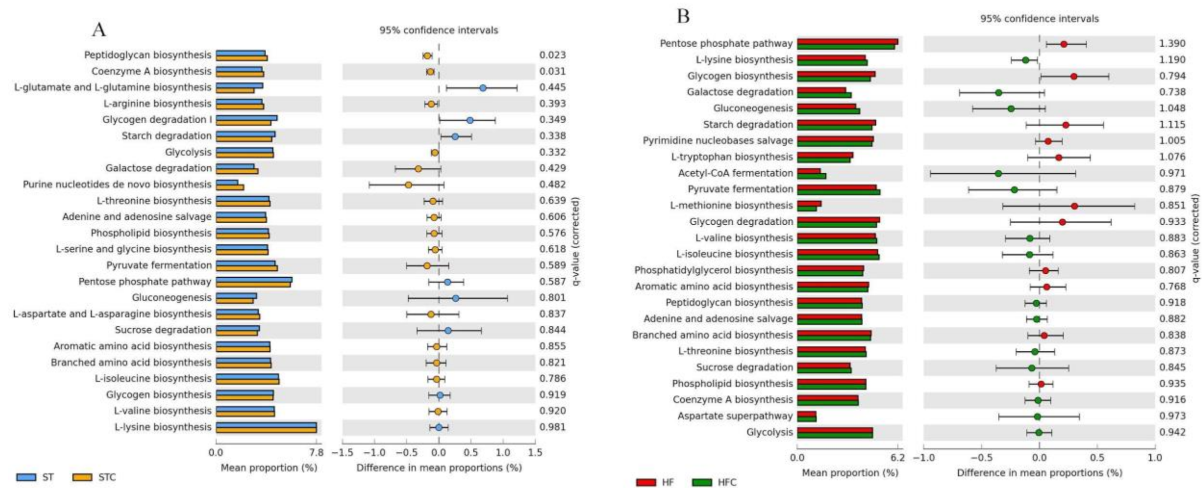

Figure 7. Difference in the relative abundance of the most abundant KEGG microbial metabolic pathways in the microbiota of adult *Wistar* rats. A: Enriched pathways between the ST and STC treatment groups; B: Enriched pathways between the HF and HFC treatment groups. Extended error bar plot was performed by bioinformatic software (STAMP) with FDR correction. Welch's two-sided test was used and Welch's inverted was 0.95. ST: standard diet; STC: standard diet with chia; HF: high fat diet; HFC: high fat diet with chia. n = 6/group (ST, STC and HF groups), n = 5/group (HFC group). Data express in 95% confidence interval.

The Pearson correlation analysis was used to assess the relationship between changes in gut microbial abundance, intestinal health parameters and inflammatory and oxidative stress markers. Changes in the Lachnospiraceae family were negatively correlated with the Muribaculaceae family ($r = -0.667$) and *Bacteroides* ($r = -0.429$), and positively correlated with butyric acid ($r = 0.536$) and catalase (CAT) ($r = 0.469$). The Muribaculaceae family was positively correlated with *Ruminococcus* ($r = 0.638$) and total antioxidant capacity of plasma (TAC) ($r = 0.505$), and negatively correlated with propionic acid ($r = -0.571$) and superoxide

dismutase (SOD) (r = -0.487). *Oscillibacter* was negatively correlated with propionic acid (r = -0.575) and positively correlated with TAC (r = 0.601). *Faecalibaculum* was negatively correlated with cecum content pH (r = -0.468); Bacteroides was negatively correlated with butyric acid (r = -0.576). *Ruminococcus* was negatively correlated with tumor necrosis factor alpha (TNF-α) (r = -0.543) and SOD (r = -0.530) and positively correlated with TAC (r = 0.520). Acetic acid was positively correlated with butyric acid (r = 0.559); propionic acid was negatively correlated with TAC (r = -0.743) and positively correlated with SOD (r = 0.568) and thiobarbituric acid reactive substances (TBARS) (r = 0.619); butyric acid was negatively correlated with cecum content pH (r = -0.649) and positively correlated with IgA (r = 0.577). Cecum content pH was negatively correlated with CAT (r = -0.546). TNF-α was positively correlated with Il-1β (r = 0.6705), SOD (r = 0.493) and TBARS (r = 0.546). Il-1β was negatively correlated with TAC (r = -0.457) and positively correlated with TBARS (r = 0.657). TAC was negatively correlated with TBARS (r = -0.395); and SOD was positively correlated with TBARS (r = 0.794) (Figure 8).

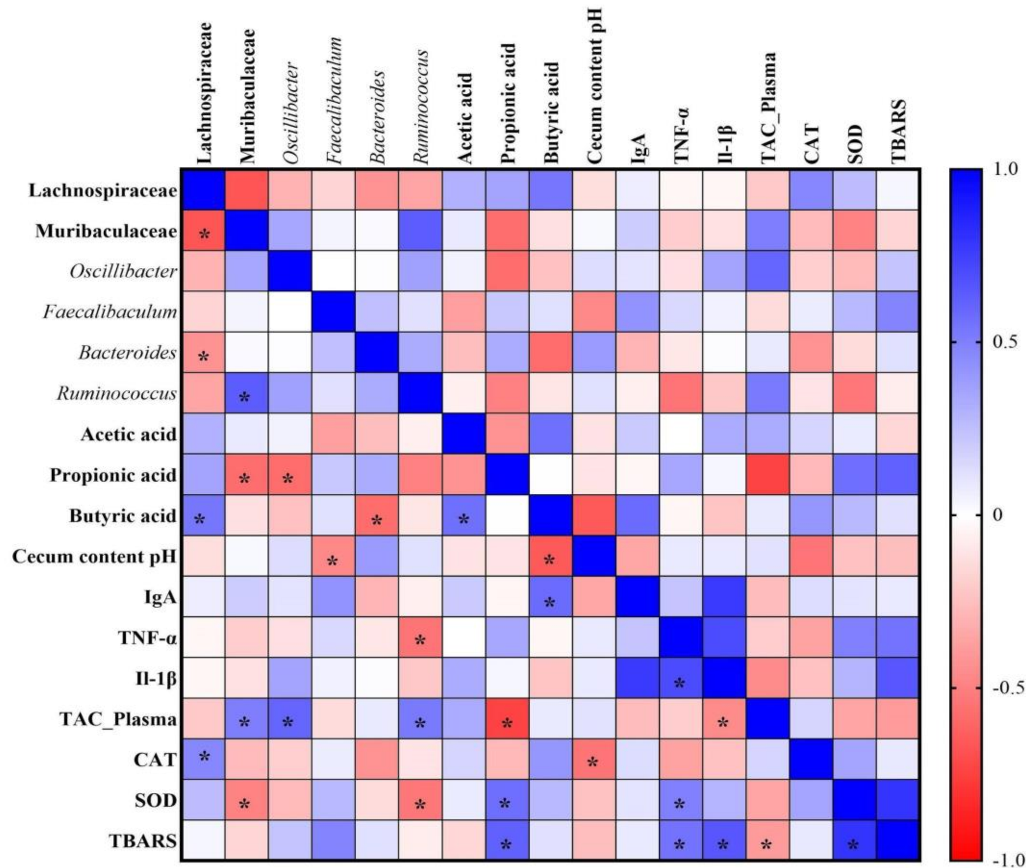

Figure 8. Heatmap of Pearson correlation analysis. IgA: immunoglobulin; TNF-α: tumor necrosis factor alpha; Il-1β: Interleukin 1β; TAC_Plasma: total antioxidant capacity of plasma;

CAT: catalase; SOD: superoxide dismutase; TBARS: thiobarbituric acid reactive substances. *Indicates statistically significant difference ($p < 0.05$). n = 6/group (ST, STC and HF groups), n = 5/group (HFC group).

4. Discussion

Few studies in the literature have evaluated the impact of chia consumption on intestinal parameters. However, due to chia chemical composition, mainly the amount of dietary fiber, polyunsaturated fatty acids, phenolic compounds and other bioactive compounds, it is suggested that this food may have a positive effect on the microbiota and intestinal health. Thus, the present study demonstrated that the consumption of chia flour associated with standard diet increases of acetic and butyric acids content, decreases the cecum pH, increases longitudinal muscle layer, circular muscle layer and crypt thickness and improves the mRNA gene expression of AP and SI. In addition, chia flour promotes some compositional changes in the gut microbiota.

The groups fed with high fat diet presented lower food intake (in grams per week) and higher weight gain, which corroborates previous reports that the higher amounts of fat and so, calories, can promote greater satiety and reduce consumption, while increasing the accumulation of body fat [14]. On the other hand, chia consumption improved some intestinal parameters, such as cecum content pH, increased the production of short-chain fatty acids and the expression of intestinal functionality biomarkers, which could be attributed to the higher amount of dietary fibers present in the food. The dietary fibers present in chia seeds are composed of soluble and insoluble fractions and can benefit intestinal health, since they are able to be fermented by colonic bacteria, thus producing short-chain fatty acids and consequently reducing intestinal pH, which may increase mineral solubility and therefore absorption [12]. In addition, the acidification of the intracolonic environment is considered important for the suppression of the production and the activity of a range of bacterial metabolites implicated in colonic diseases [33].

In this study, an increase was observed in crypt thickness, longitudinal muscle layer and circular muscle layer in the groups fed with chia in a standard diet. In *Gallus gallus* feeding, the digestive and absorptive capabilities of the brush border membrane may be directly related with morphometric parameters, such as villi height, crypt depth and the ratio between villi height and crypt depth [34]. These functional and morphological effects can be explained by the potential increased proliferation of intestinal cells due to the increased motility of the

digestive tract by the soluble fiber, which leads to hyperplasia and/or hypertrophy of intestinal muscle cells [15].

The gene expressions of aminopeptidase (AP), sodium-glucose transport protein 1 (SGLT1), sucrase isomaltase (SI) and peptide transporter 1 (PepT1) are used as biomarkers of brush border membrane (BBM) digestive and absorptive functions [12]. In the current study, the expression of BBM functional genes AP and SI was upregulated by chia consumption (STC) compared to the standard diet group (ST), and chia consumption (HFC) did not affect the gene expression of AP and PepT1 and decreased the expression of SGLT1 and SI in HFC group relative to the HF group. Furthermore, we observed an increase in longitudinal muscle layer and in crypt thickness in STC group compared to ST group. Thus, in diet normal conditions chia increased the intestinal BBM functionality, and morphology; however, it not happened in HFD condition. These results are benefic since AP is an exopeptidase that cleaves amino acids from the N-terminus of peptides, and SI is the major disaccharidase located on the duodenal BBM, which hydrolyzes disaccharides or oligosaccharides into monosaccharides for absorption [34]. Others studies observed that the intra-amniotic administration of different concentrations of chia soluble extract (5, 10, 25 and 50 mg/mL) [12], and black, carioca and white bean extracts (50 mg/mL) [35] did not affect the BBM functional gene expression. In the study of Wang *et al* [36] the wheat bran extract showed significant increases in the villus heights, goblet cell diameter and numbers. Thus, chia compared to other types of dietary fibers is a good source of this nutrient, which can improve the intestinal health.

Phytochemicals have been descripted as agents that exert effects similar to prebiotics. Phenolics compounds have been shown to be positive modulators of intestinal microbiota [37, 38]. This interaction entails microbial degradation of polyphenols and modulation of gut microbiota by polyphenols and their metabolites, which inhibits pathogenic bacteria and stimulates beneficial bacteria [39]. Chia phenolics demonstrated beneficial effects in gene expression modulation of gluconeogenic and glycolytic enzymes [40] and antioxidant and anti-inflammatory activity [37, 38]. Other important nutrient present in chia is alpha linolenic acid [11, 40, 41]. The alpha linolenic acid exerts effects on the intestinal microbiota, the host-microbiome interaction, and interactions between the host immune system and gut microbiota. Accordingly, the gut microbiota modulates the absorption and metabolism of omega-3 PUFAs and directly or indirectly modulates subsequent physiological and immune responses in the host [42]. Diets rich in alpha linolenic acid reduced the colonic abundance of potentially pathogenic bacteria and increased bacteria with beneficial potential. This eventually inhibits the host inflammatory responses [43]. Further, brown and golden flaxseeds with high content of

insoluble dietary fiber and alpha-linolenic acid demonstrated reduced intestinal permeability and endotoxemia in perimenopausal overweight women [44]. Thereby, the combination of dietary fiber, lipids, and polyphenol from chia in our study may explain some beneficial effects of chia on intestinal health.

In this study, the current sequencing depth could represent most of the bacterial community in the experimental groups. In alpha diversity, we found that the consumption of chia with a standard diet (STC x ST) increased the Chao index, improved richness, raised the number of different species and decreased the Simpson index, which reduced the dominance and indicated a greater number of different individuals in the community. Beta diversity analysis by the OTU's clustering showed difference between the ST and HF groups and between the ST and STC groups, which indicates a potential effect of the chia flour on the gut microbiome taxa.

Both the menopausal change and obesity were noted to enhance dysbiosis by reducing microbiome diversity and increasing the Firmicutes to Bacteroidetes ratio [6, 7]. This ratio has been used as a marker for intestinal homeostasis in animal studies [45, 46]. In our study, no difference was observed at the level of phylum and genus, but the Firmicutes to Bacteroidetes ratio was lower in the ST group, compared to the STC group. Findings related to the alterations in this ratio are still inconsistent, which highlights the complexity of the gut microbial ecosystem. The Firmicutes to Bacteroidetes ratio does not necessarily reflect a common pattern of all genera belonging to these phyla, since multiple genera of the same phylum can be found in higher or lower proportions [47]. Although we did not detect significant modifications in the taxonomy of the cecal microbiota, upregulation in the SCFA-producing bacteria in the group that fed chia was observed through LEfSe analysis.

LEfSe determines the features (organisms, clades, operational taxonomic units, genes or functions) most likely to explain differences between classes. Our LEfSe analysis suggested that *Bacteroides* genus and members of the Muribaculaceae and Lachnospiraceae families were the main bacteria that caused the difference in the structure of intestinal microbiota in the group fed with standard diet (STC X ST). *Bacteroides* genus includes species that produce acetate and propionate and promote goblet cell differentiation and the expression of mucin-related genes [48]. The Muribaculaceae family can degrade dietary carbohydrates and ferment polysaccharides into SCFAs [49–51]. The family Lachnospiraceae is also a SCFA producer, and its increased relative abundance is associated with increased production of SCFAs [52]. In the group HFC, the organisms most likely to explain differences, besides the Lachnospiraceae family, were *Faecalibaculum* and *Ruminococcus* genera, both producers of butyrate [48, 53].

The elevation in SCFA-producing bacterial populations justifies the increased SCFA concentration in the lumen. These SCFAs may lead to intestinal cellular proliferation, and this connection could explain the increased crypt thickness in chia fed group. Then, by favoring bacterial fermentation, chia seed affected the intestinal luminal pH [51]. In agreement with the Pearson correlation analysis we performed, the Lachnospiraceae family was positively correlated with butyric acid, and the concentration of butyric acid was negatively correlated with the pH of the cecal content. The study conducted by Guo *et al*., 2022 [52] demonstrated that inulin supplementation has beneficial effects on the intestinal microbiota, with an increased abundance of butyrate-producing bacteria from the Ruminococcaceae and Lachnospiraceae families, thus leading to increased production of SCFAs, which suggests that the beneficial effects of inulin on glucose and lipid metabolism can be partially attributed to the modulation of the gut microbiota and the signaling of SCFAs.

The gut microbiome data demonstrates how the specific microbial profile within a treatment group is linked to the physiological status of the host. The results reveal that chia consumption with a standard diet upregulated the pathways related to peptidoglycan and coenzyme A biosynthesis.

5. Conclusions

The consumption of chia by female ovariectomized *Wistar* rats positively affected gut health by increasing the production of short chain fatty acids, decreasing the pH of cecum content, improving the intestinal functionality and morphology, and promoting some compositional changes in the gut microbiota, mainly with the consumption of a standard diet. Thus, it was observed that chia, which is rich in fiber and phenolic compounds, demonstrated potential properties to improve intestinal health. Further studies are still needed to clarify the effects of chia on the intestinal health and microbiome and to make clearer what can be the implications to human population, whereas there is a just a few studies evaluating chia consumption and impacts on intestinal human health.

Statements and Declarations

Competing Interests

The authors declare no conflict of interest.

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9.3 Paper 3: Chia flour combined with a high fat diet increases propionic acid production and improves the microbial richness and diversity in female *Wistar* rats

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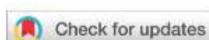

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Chia flour combined with a high fat diet increases propionic acid production and improves the microbial richness and diversity in female Wistar rats†

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Chia is a functional food because of its positive impact on reducing the risk of metabolic diseases. These benefits are due to its nutritional composition as a source of dietary fiber and bioactive compounds. In our previous study, chia consumption increased the richness of the microbiota and the production of short chain fatty acids (SCFAs) when consumed by male Wistar rats, so, the objective of this study was to assess the effects of the consumption of chia with a high fat diet on gut health in female Wistar rats. 32 adult female Wistar rats were allocated into four groups and received one of the following diets: standard diet (SD), standard diet + chia (SDC), high fat diet (HFD) or high fat diet + chia (HFDC) for 8 weeks. At the end of the study, the intestinal microbiota, SCFA content, cecum content pH, immunoglobulin A (IgA) quantification and brush border membrane functionality were evaluated. There was no difference in the relative abundance of the gut microbiota, but chia consumption increased the microbial richness and diversity, increased the production of acetic and butyric acids in the SDC group and propionic acid in the HFD group, and decreased the pH of cecal content. The HFDC group demonstrated a lower IgA concentration compared to the HFD group. The SDC group increased SI and AP gene expression and decreased SGLT1 and PepT1 compared to the SD group. The consumption of chia can be beneficial for the functionality of the microbiota, improving SCFAs and intestinal pH, and the effects of chia in the microbiota can be more pronounced in HFD.

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1. Introduction

The intestinal barrier is a complex network, composed of microbial, chemical, physical, immune, and vascular barriers, affects the absorption of nutrients, and is closely related to the health of the organism. In recent years, studies have found that many diseases are related to intestinal health, and the intestinal microbiota plays an important role in host health. 1,2

The expansion of pathogenic microorganisms and loss of beneficial microorganisms, triggering pro-inflammatory responses and immune dysregulation characterizes intestinal dysbiosis, which is recognized as one of the main hallmarks of obesity and other metabolic disorders.3,4 Therefore, the stability of the intestinal microbiota is an essential characteristic for the health of the host and several factors can influence the microbiome, such as sedentarism,

chronic intake of drugs, a lack of sleep, physiological or psychological stress, genetics, gender, age, use of antibiotics and diet. Among all these factors, the diet can cause significant changes.5

A high fat diet (HFD) consumption can cause intestinal dysbiosis by producing harmful compounds, increasing luminal pH and low-grade inflammation, potentially leading to metabolic disturbances. In contrast, after consumption of a diet rich in dietary fiber, the intestinal microbiota can ferment non digestible polysaccharides, produce short-chain fatty acids (SCFA) and lower luminal pH, which improves intestinal barrier function, intestinal microbiota composition and the inflammatory response.6 Thus, the consumption of a diet rich in dietary fiber may promote gut health through the proliferation of potentially beneficial microorganisms, regulating immune responses, and promoting the production of SCFA by intestinal bacteria. Consequently, SCFA promote benefits such as stimulating intestinal mucus secretion, regulating immune cell activity, increasing tight junction protein expression, and supporting intestinal peristalsis.7

Chia is considered a functional food due to its positive impact on reducing the risk of metabolic diseases. The consumption of chia by female *Wistar* rats was able to reduce the heart fat content, 8 improve the lipid profile, antioxidant activity, and reduce the inflammatory markers.9 These benefits are due to its nutritional composition, as a food source of dietary fiber and bioactive compounds.10–12 About gut health, chia may be a viable dietary ingredient that may improve gut health and contribute to intestinal mineral absorption. The intra-amniotic (*in ovo*) administration of chia seed soluble extracts improved the intestinal morphology, up-regulated Zn-related and iron-related proteins gene expression, and increased the *Bifidobacterium* and *Lactobacillus* relative abundance.13 Further, in our previous study, chia consumption increased the richness of microbiota and the production of SCFA when consumed by male *Wistar* rats.14

Therefore, the objective of this study was to assess the effects of chia flour consumption on gut health in adult female *Wistar* rats fed with a standard or a high fat diet. We hypothesized that the consumption of chia could improve intestinal bacterial populations, parameters related to intestinal health and intestinal brush border enzymes functionality in adult female *Wistar* rats. To explore this hypothesis, we designed an *in vivo* study investigating the effects of chia consumption on the microbiota composition, SCFA production, cecal content pH, immunoglobulin A (IgA) concentration and intestinal functionality in female *Wistar* rats.

2. Material and Methods

2.1. Sample material

For this study, chia seeds (*Salvia hispanica* L.) grown in the state of Rio Grande do Sul (Brazil) were used. The seeds were provided by the company Dubai Alimentos, from Ijuí, Rio Grande do Sul, Brazil. The characterization of the seeds was performed by our research group.11 The seeds showed brown pericarp and approximate diameter of 2 mm. The state of Rio Grande do Sul has temperate climate, with relative humidity ranging from 60 to 80%. The plantation was in January and the harvest in June. The soy type is clay soil, and the rainfall is regular. The seeds were packed and transported in cardboard boxes and then, were stored in hermetically sealed plastic bags, protected from light and frozen (−18 ± 1°C) until use. To obtain the flour, the seeds were ground up in three replicates, using a knife mill (Marconi Equipment, Brazil) to a particle size of 850 µm, immediately before being added to diets.

2.2. Experimental Design

All the experimental procedures with animals were performed in accordance with Directive 86/609/EEC of November 24, 1986, in compliance with the ethical principles for animal experimentation. The study protocol was approved by the Ethics Committee of the Federal University of Viçosa (Protocol 20/2017; date of approval: July 13th, 2017).

Thirty-two female *Wistar* rats (Rattus norvegicus, albinus variation), newly weaned, 21 days old, were obtained from the Central Animal Facility of the Center for Biological Sciences and Health at Federal University of Viçosa, Minas Gerais, Brazil. The animals were divided into 2 groups with 16 animals each, randomized by body weight, and then were distributed into individual stainless-steel cages in an environment with controlled temperature (22ºC ± 2ºC), automatically controlled light and dark cycles of 12 hours and 55% humidity. The animals received their respective diets based on the standard diet (17% fat, 19% protein and 64% carbohydrate) 15 or high fat diet (51% fat, 15% protein and 34% carbohydrate) (Research Diets, New Brunswick, NJ) with modifications, and deionized water ad libitum.

During the first 10 weeks, the animals received a standard diet (n=16) or high fat diet (n=16), according to their group. After this period, the animals were redistributed into 4 groups (n=8/group), and each group received one of the following diets for more 8 weeks: standard diet (SD); standard diet + chia (SDC); high fat diet (HFD) or high fat diet + chia (HFDC). The

amount of chia was based on a human consumption of 25 g of chia per day 16–18 and it was added in the diets considering its composition 11 (Table 1).

Table 1. Composition of experimental diets (g/kg of diet).

	First 10 weeks		**Experimental Diets**			
Ingredients	**SD**	**HFD**	**SD**	**SDC**	**HFD**	**HFDC**
Chia (g)*	-	-	-	232.60	-	232.60
Albumin (g)**	179.50	179.50	179.50	133.70	179.50	133.70
Dextrinized starch (g)	155.00	155.00	155.00	155.00	155.00	155.00
Sucrose (g)	100.00	100.00	100.00	100.00	100.00	100.00
Soybean oil (mL)	40.00	40.00	70.20	-	70.20	-
Lard (g)	-	240.00	-	-	195.00	195.00
Microcrystalline cellulose (g)	50.00	50.00	86.00	-	86.00	-
Mineral mix (g)	35.00	35.00	35.00	35.00	35.00	35.00
Vitamin mix (g)	10.00	10.00	10.00	10.00	10.00	10.00
L-cystine (g)	1.80	1.80	1.80	1.80	1.80	1.80
Choline bitartrate (g)	2.50	2.50	2.50	2.50	2.50	2.50
Corn starch (g)	420.00	178.45	347.50	319.40	151.00	122.90
Cholesterol	-	1.50	-	-	1.50	1.50
Nutritional composition						
Total calories (Kcal)	3778.00	4971.80	3759.80	3717.48	4728.80	4686.48
Caloric density (Kcal/g)	3.78	4.97	3.76	3.71	4.73	4.69

* Chia composition: lipids (32.16 g.100 g^{-1}), proteins (18.18 g 0.100 g^{-1}), total dietary fiber (33.37 g.100 g^{-1}), and carbohydrates (4.59 g. 100 g^{-1}). **Purity of 78%. SD: standard diet; SDC: standard diet + chia; HFD: high fat diet; HFDC: high fat diet + chia.

During the experimental period, the body weight and food intake were monitored weekly. After 18 weeks and 12 hours of fasting, the animals were anesthetized with isoflurane (3%, Isoforine, Cristália®) and euthanized by cardiac puncture. Cecum, cecal content and duodenum were collected, weighed and immediately stored at -80°C for analysis.

2.3. Cecal content pH

The determination of cecal content pH was performed according to Grancieri *et al.*19 Briefly, in duplicates, 1 g of the samples was homogenized in 10 mL of distilled water, with the aid of vortex glass spheres. Then, the glass electrode of the pHmeter was inserted and the pH was measure.

2.4. IgA quantification

The sIgA quantification was determined according to Vaz-Tostes *et al.* 20 Briefly, cecal content was defrosted on ice and the cecal content homogenates (1:5 (w/v)) were prepared according to standard procedures. 200 mg feces were added to 800 μL of phosphate-buffered saline and homogenizing using a vortex. The mucosal immunity was evaluated based on the cecal sIgA concentration, which was measured using an immunochron enzymelinked immunosorbent assay (ELISA).

2.5. Short chain fatty acids (SCFA) content

For analysis, 500 mg of cecal content was homogenized in 1 mL of Milli-Q water with the aid of vortex and centrifuged at 12000 g for 10 min. The supernatant was removed, and the other steps were performed according to Siegfried, Ruckemann, and Stumpf (1984).21 Then, the samples were analyzed by high performance liquid chromatography (HPLC), using a Dionex Ultimate 3000 Dual chromatograph coupled to a Shodex RI-101 refractive index (IR) detector using a Bio-Rad HPX-87H column, 300 mm x 4.6 mm maintained at 45 °C. Stock solutions of the standards were prepared using acetic, propionic and butyric acid.

2.6. Extraction of mRNA from intestinal tissue and cDNA synthesis

For total RNA extraction, duodenum was macerated under RNAse free conditions. Total RNA was extracted with the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and to synthesize the cDNA, it was used the M-MLV reverse transcription kit (Invitrogen Corp., Grand Island, NY). 22

2.6.1. Determination of gene expression of proteins involved in intestinal functionality by quatitative reverse transcriptase polymerase chain reaction (RT-qPCR)

The duodenum mRNA gene expression was analyzed by RT-qPCR. The SYBR Green PCR master mix from Applied Biosystems (Foster City, CA) was employed, and the analyses were performed at StepOne™ Real-Time PCR System (Thermo Fisher Scientific) involving SYBR-Green Fluorescence and Primer Express software (Applied Biosystems, Foster City, CA). Follow and reverse primer sequences (Choma Biotechnologies) were used to amplify aminopeptidase (AP) (ID: 301368687), sucrose isomaltase (SI) (ID: 301368688), peptide transporter 1 (PepT1) (ID: 301368693), and sodium-glucose transport protein 1 (SGLT1) (ID: 301368686). The relative expression levels of mRNA were normalized using ß actin. The sequences of the primers used in this work are summarized in Table 2. All the steps were performed under free RNase condition.

Table 2. Sequencing primers used in the RT-qPCR analysis.

Genes	Oligonucleotide (5'-3')	
	Forward	**Reverse**
ß actin	TTCGTTGCCGGTCCACACCC	GCTTTGCACATGCCGGAGCC
AP	CTCTCTCCTCAAACCACATGAA	AGTTCAGGGCCTTCTCATATTC
SI	CCTCCAGAACACAATCCCTATAC	GGAGAGGTGAGATGGGATTAGA
PepT1	CCTGGTCGTCTGCATCATATT	TTCTTCTCATCCTCATCGAACTG
SGLT1	CATCCAGTCCATCACCAGTTAC	CAATCAGGAAGCCGAGAATCA

AP: aminopeptidase; SI: sucrose isomaltase; PepT1: peptide transporter 1; SGLT1: sodium-glucose transport protein 1.

2.7. DNA extraction and sequencing

Genomic DNA was extracted from cecal stool samples by mechanical disruption and phenol/chloroform extraction protocol.23 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).24,25 PCR amplicon libraries targeting the hypervariable V4-region of the 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).24 Customized sequencing primers and procedures were used for sequencing amplicons on a 151bp x 12bp x 151bp MiSeq run.25 In this study the sequences obtained in each sample were submitted to Sequence Read Archive (SRA) on the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sra) under the accession number PRJNA806959 and the data analysis done by Mothur software, v.1.44.3.26 UCHIME was used to detected and removed Chimera sequences.27 The taxonomic classification and aligning the sequences with the 16S rRNA were done by *Si*LVA database v.138.1.28

The Operational Taxonomic Units (OTU) were grouped with a 97% sequence similarity cutoff. 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 from any sample. The standardized data table was used for calculating alpha and beta diversity, as well as for calculating the relative abundance of OTU. The Chao1, Shannon and Simpson indices were used for estimates the alpha-diversity. Beta-diversity between dietary groups was assessed by Principal Coordinate Analysis (PCoA) based on the Jaccard dissimilarity index.29 Metagenome functional predictive analysis was carried out using PICRUSt2 software.30 Normalized OTU abundance was identified, and the assigned functional traits were predicted based on reference genomes using the Kyoto Encyclopedia of Genes and Genomes (KEGG). The most abundant metabolic processes and significant fold-change differences in functional pathways among experimental groups were plotted. In addition, linear discriminant effect size analysis was performed to identify the functional microbial pathways that were differentially expressed in the experimental groups.

2.8. Statistical analysis

Data about murinometric variables were initially submitted to a Kolmogorov-Smirnov normality test and then submitted to one-way analysis of variance (ANOVA), followed by the post-hoc of Tukey or Tukey-Kramer test (according to the sample size) to compare all experimental groups. T-test was used to compare the same diet with or without chia.

Correlations among gut health markers and inflammatory parameters were assessed by Pearson's correlation test. Analysis was performed using GraphPad Prism version 9.0.

To evaluate the clustering of the gut microbiome samples, a PCoA plot, based on Jaccard dissimilarity metrics, was performed to show the distance in the bacterial communities of animals from different dietary groups. Nonparametric analysis of similarities (PERMANOVA, number of permutations = 10000) was performed using the Past software.31 Datasets were tested for homogeneity of variance by the Kolmogorov-Smirnov test and nonparametric and independent samples were submitted to Kruskal-Wallis with a Dunn's multiple comparison test. Data were corrected for multiple comparisons using false discovery rate (FDR) in the STAMP software. Statistical analysis was performed using SPSS software version 20.0 with Bonferroni correction. Data are presented as means ± standard deviation and statistical significance was established at $p < 0.05$.

3. Results

The final body weight and the weight gain were not different among the experimental groups. The total food intake was lower in both groups fed with HFD. The group fed with SDC showed a higher food intake than all other groups and higher energy intake compared to the group fed with SD. It is important to highlight that the consumption of a HFD (HFD or HFDC) did not alter the total energy intake. The cecal weight and cecal index was not different among the experimental groups (Table 3).

Table 3: Biometrical data of adult female *Wistar* rats during the experimental period.

Variables	**Groups**			
	SD	**SDC**	**HFD**	**HFDC**
Final body weight (g)	280.58 ± 12.62^{a}	293.99 ± 20.79^{a}	288.25 ± 21.06^{a}	295.20 ± 23.94^{a}
Weight gain (g)	190.24 ± 16.27^{a}	189.86 ± 24.73^{a}	194.40 ± 25.61^{a}	192.16 ± 26.50^{a}
Total food intake (g)	724.51 ± 41.31^{b}	800.58 ± 55.68^{a*}	626.32 ± 57.48^{c}	627.31 ± 45.54^{c}
Total energy intake (kcal)	2724.16 ± 155.36^{a}	2970.16 ± 206.58^{a*}	2962.49 ± 271.89^{a}	2942.10 ± 213.60^{a}
Cecum weight (g)	4.04 ± 1.20^{a}	4.49 ± 0.87^{a}	3.40 ± 0.63^{a}	4.18 ± 1.07^{a}
Cecal index (%)	1.44 ± 0.44^{a}	1.53 ± 0.32^{a}	1.18 ± 0.25^{a}	1.54 ± 0.41^{a}

Values represent means ± standard deviation. SD: standard diet; SDC: standard diet + chia;

HFD: high fat diet; HFDC: high fat diet + chia. Means followed by the same lowercase letter in the row do not differ significantly according to the Tukey or Tukey-Kramer test (according to the sample size) at the 5% threshold of probability. * Indicate differences between groups that received the same diet, but with or without chia (t-test, $p < 0.05$).

The HFD didn't change the cecal pH content compared to SD, but the chia consumption, regardless of the type of the diet consumed, decreased the cecal pH, when we made a comparison by pairs (SDC X SD and HFDC X HFD). Besides this, the HFD didn't change the cecal IgA concentration compared to SD, but also in a comparison by pairs, the group fed with a HFDC demonstrated lower IgA concentration compared to HFD group. Chia consumption increased the acetic acid and butyric acid in cecal content in SDC group in comparison to the SD group and increased the propionic acid concentration in cecal content in HFDC group in relation to the HFD. The content of propionic acid in the cecal content of animals that were fed with HFD was lower in relation to SD group, but when animals were fed with HFD associated with chia (HFDC), the propionic acid concentration was comparable to the SD group (Table 4).

Table 4. Cecal content pH, IgA concentration and short chain fatty acids content of adult female *Wistar* rats during the experimental period.

Variables	**Groups**			
	SD	**SDC**	**HFD**	**HFDC**
Cecal content pH	9.28 ± 0.24^{a}	$8.78 \pm 0.42^{ab*}$	9.04 ± 0.39^{a}	$8.42 \pm 0.39^{b*}$
IgA (ng)	628.00 ± 89.18^{a}	562.58 ± 227.32^{a}	$731.61 \pm 63.12^{a*}$	424.94 ± 126.16^{a}
Short Chain Fatty Acids (mM)				
Acetic Acid	9.71 ± 1.12^{ab}	$12.54 \pm 3.43^{a*}$	9.46 ± 2.33^{b}	9.29 ± 1.27^{b}
Butyric Acid	0.94 ± 0.28^{b}	$1.46 \pm 0.40^{a*}$	1.25 ± 0.25^{ab}	1.37 ± 0.24^{ab}
Propionic Acid	4.52 ± 2.81^{a}	2.85 ± 1.09^{ab}	1.50 ± 0.20^{b}	$2.85 \pm 0.53^{ab*}$

Values represent means ± standard deviation. SD: standard diet; SDC: standard diet + chia; HFD: high fat diet; HFDC: high fat diet + chia. Means followed by the same lowercase letter in the row do not differ significantly according to the Tukey or Tukey-Kramer test (according to the sample size) at the 5% threshold of probability. *Indicate differences between groups that received the same diet, but with or without chia (t-test, $p < 0.05$).

The HFD consumption reduced SGLT1 and PEPT1 gene expression compared to SD. Chia consumption with a standard diet (SDC) increased the SI and AP gene expression and decreased the SGLT1 and PepT1 gene expression, compared to SD group (Figure 1). Chia, when consumed associated with a high fat diet (HFDC), was able to increase the PepT1 and AP gene expression in relation to HFD group (Figure 1C and D). However, SI and SGLT1 gene expression did not differ between these groups (HFD X HFDC) (Figure 1A and B).

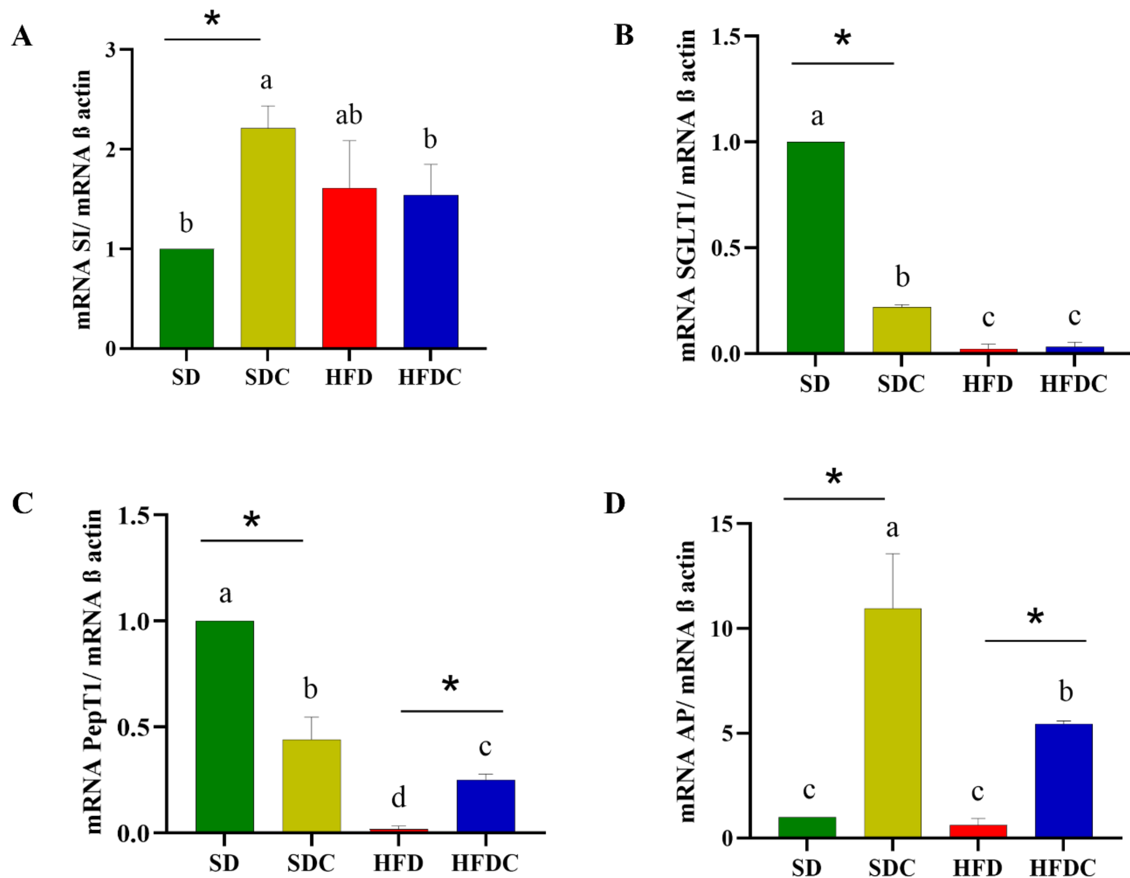


Figure 1. Effect of chia consumption on functional genes expression in the duodenum. RT-qPCR analysis. Values represent means ± standard deviation. (A) SI expression, (B) SGLT1 expression, (C) PepT1 expression and (D) AP expression. SD: standard diet; SDC: standard diet + chia; HFD: high fat diet; HFDC: high fat diet + chia; SI: sucrose isomaltase; SGLT1: sodium-glucose transport protein 1; PepT1: peptide transporter 1; AP: aminopeptidase. Means followed by the same lowercase letter did not differ significantly according to the Tukey or Tukey-Kramer test (according to the sample size) at 5% probability. *Indicate differences between groups that received the same diet, but with or without chia (t-test, $p < 0.05$).

The HFD didn't change the alpha diversity indexes of bacterial communities compared to SD. Chia consumption increased Chao1 index, regardless of the type of diet consumed (Figure 2A). Chia consumption increased Shannon index, regardless of the type of diet consumed when the groups were compared by pairs (Figure 2B). The Simpson index was not different among the experimental groups, in both of comparisons (Figure 2C).

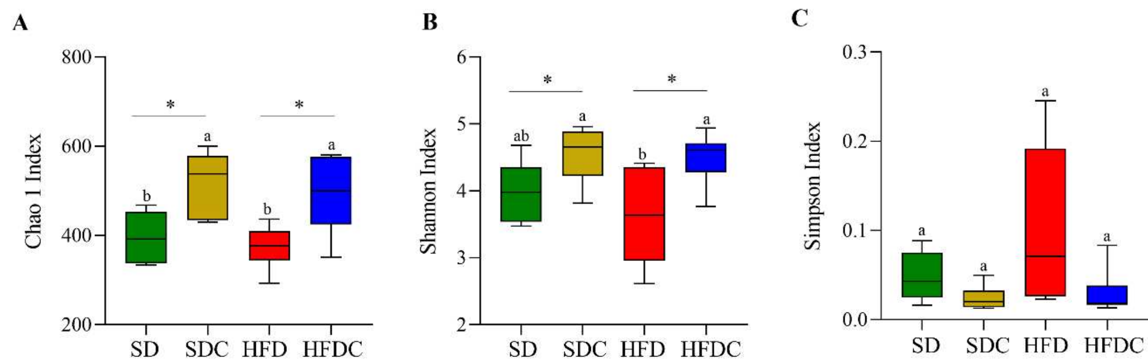


Figure 2: Alpha diversity metrics of bacterial communities in the cecal content of adult female *Wistar* rats according to each treatment group. (A) Chao Index; (B) Shannon Index; (C) Simpson Index. Data expressed as median; bars=minimum and maximum value. SD: standard diet; SDC: standard diet + chia; HFD: high fat diet; HFDC: high fat diet + chia; Means followed by the same lowercase letter did not differ significantly ($p<0.05$). *Indicate differences between groups that received the same diet, but with or without chia (t-test, $p < 0.05$).

When we evaluated the β-diversity of the cecal content microbiome, comparing all groups, the spatial ordination at the OTU level showed that groups fed with HFD (HFD and HFDC) were different from those that were fed with standard diet (SD and SDC), and the HFD and HFDC groups were different from each other (Figure 3A). At the phylum level, there was also a difference between the HFD and HFDC groups (Figure 3B). At the genus level, after Bonferroni correction, there was no difference between groups (Figure 3C). When we made the pairwise comparison, at the OTU level, the groups fed with chia were grouped differently compared to their respective controls (SD X SDC and HFD X HFDC) (Figure 3A). At the phylum and genus level, the HFD and HFDC clustered differently from each other (Figure 3B and C).

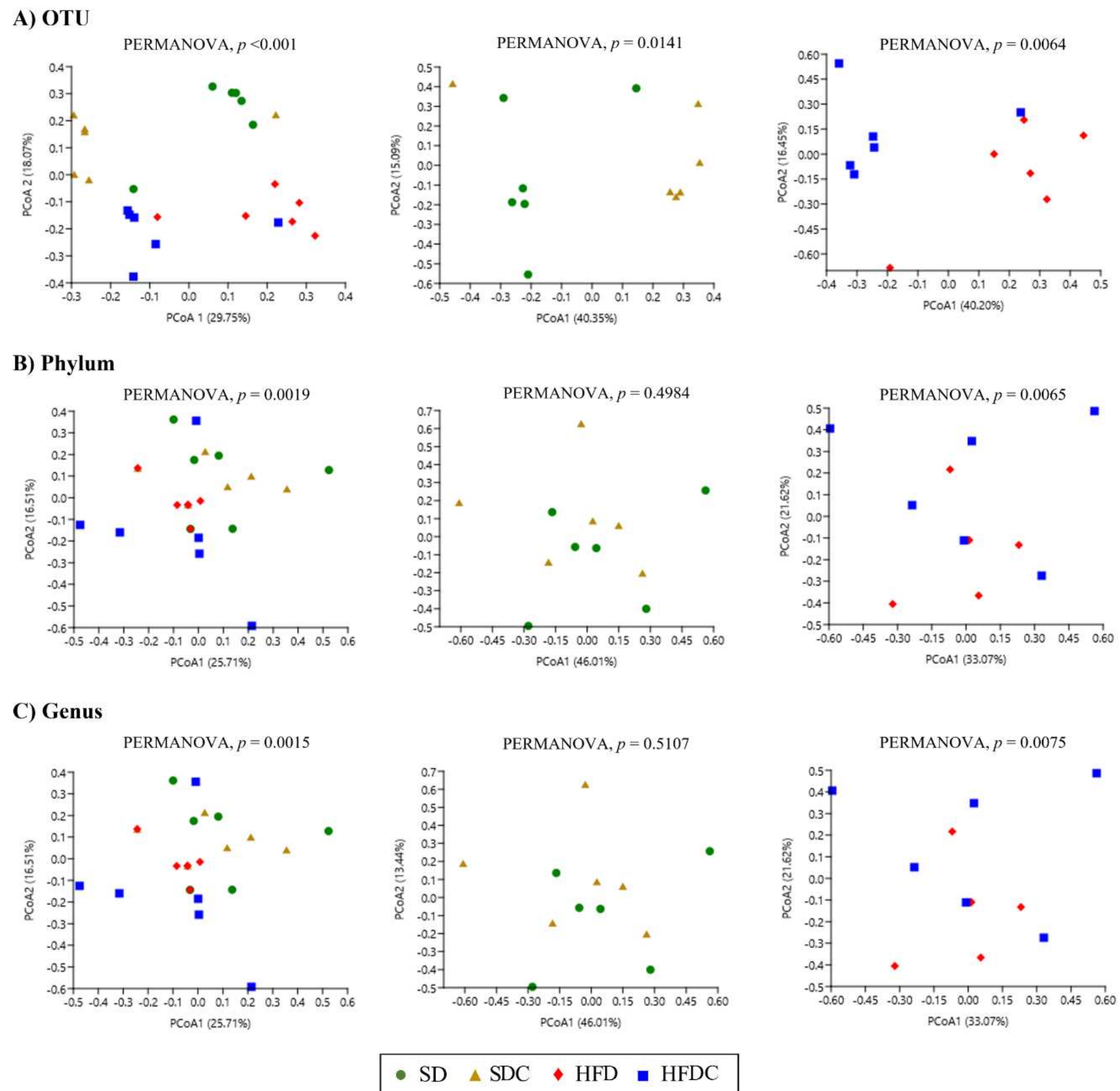


Figure 3: Changes in the β-diversity of the cecal content microbiome of adult female *Wistar* rats according to each treatment group. (A) Principal Coordinate Analysis (PCoA) based on the Jaccard distance at OTU level. (B) PCoA based on Jaccard distance at the Phylum level. (C) PCoA based on Jaccard distance at Genus level. SD: standard diet; SDC: standard diet + chia; HFD: high fat diet; HFDC: high fat diet + chia.

Comparing all the groups and comparing by pairs, there was no difference in the relative abundance of the phyla and genera identified in the samples (Figure 4A and B) and there was no difference in the Firmicutes to Bacteroidetes ratio (Figure 4C).

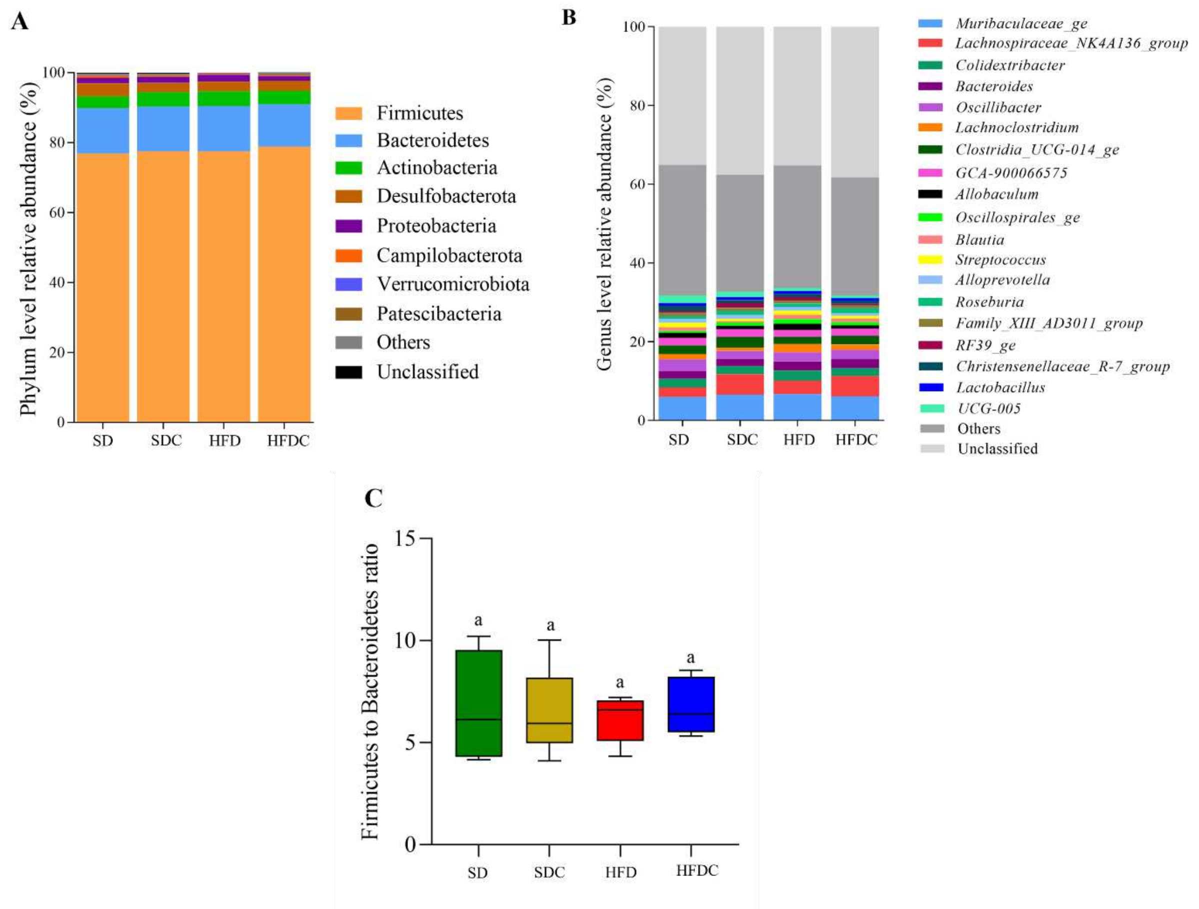


Figure 4: Relative abundances of bacterial microbiota composition at phylum and genera level of adult female *Wistar* rats. (A) Relative abundance of each identified phylum; (B) Genera relative abundance; (C) Firmicutes/Bacteroidetes ratio; Data expressed as median; bars=minimum and maximum value. SD: standard diet; SDC: standard diet + chia; HFD: high fat diet; HFDC: high fat diet + chia. Only phyla with abundance>0.2% and genera with abundance >1% in at least one group were displayed. Data were analyzed with an FDR correction. Means followed by the same lowercase letter did not differ significantly (p<0.05).

The linear discriminant analysis effect size method (LEfSe) was used to investigate the significant bacterial biomarkers that could identify differences in the intestinal microbiota among the treatment groups. LEfSe showed that 21 bacterial OTU had greater impact on the modification of the microbiota of the SDC group, while 9 taxa were enriched in the SD group (Figure 5A). Furthemore, 21 bacterial OTU had impact on the modification of the HFDC group microbiota, while 5 taxa were enriched in the HFD group (Figure 5B). In the SDC group, we observed that the most differentiated taxa were related to members of the Anaerobiospirillum genus and Romboutsia genus compared to SD group (Figure 5A). Comparing HFD and HFCD

groups, taxa related to members of Lachnospiraceae family were most differentiated in the HFDC group compared to HFD group (Figure 5B).

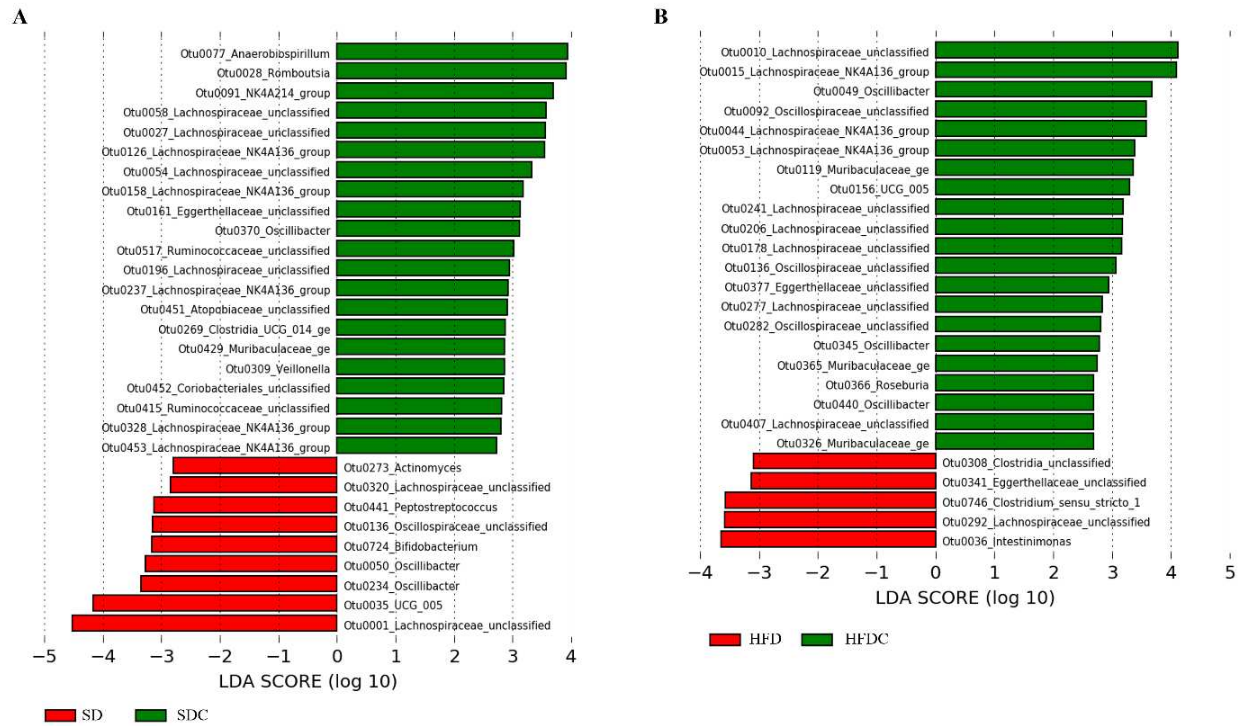


Figure 5: LEfSe method used to compute Linear Discriminant Analysis (LDA) scores of the relative abundance difference between the experimental groups. (A) LDA scores of the relative abundance difference between the SD group and the SDC group; (B) LDA scores of the relative abundance difference between the HFD group and the HFDC group. SD: standard diet; SDC: standard diet + chia; HFD: high fat diet; HFDC: high fat diet + chia.

There were no differences related to KEGG metabolic pathways between SDC and SD group (Figure 6A). The HFDC group demonstrated significant enrichment ($p<0.05$) of KEGG metabolic pathways related to teichoic acid (poly-glycerol) biosynthesis compared to the HFD group and a reduction of metabolic pathways related to pyrimidine nucleobases salvage and adenosine ribonucleotides de novo biosynthesis (Figure 6B).

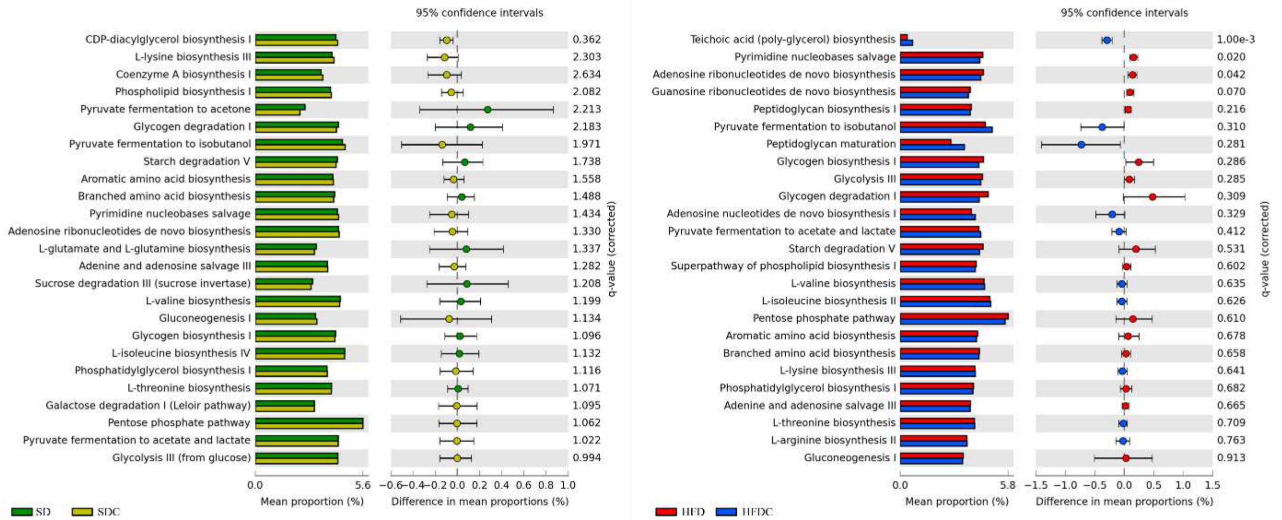
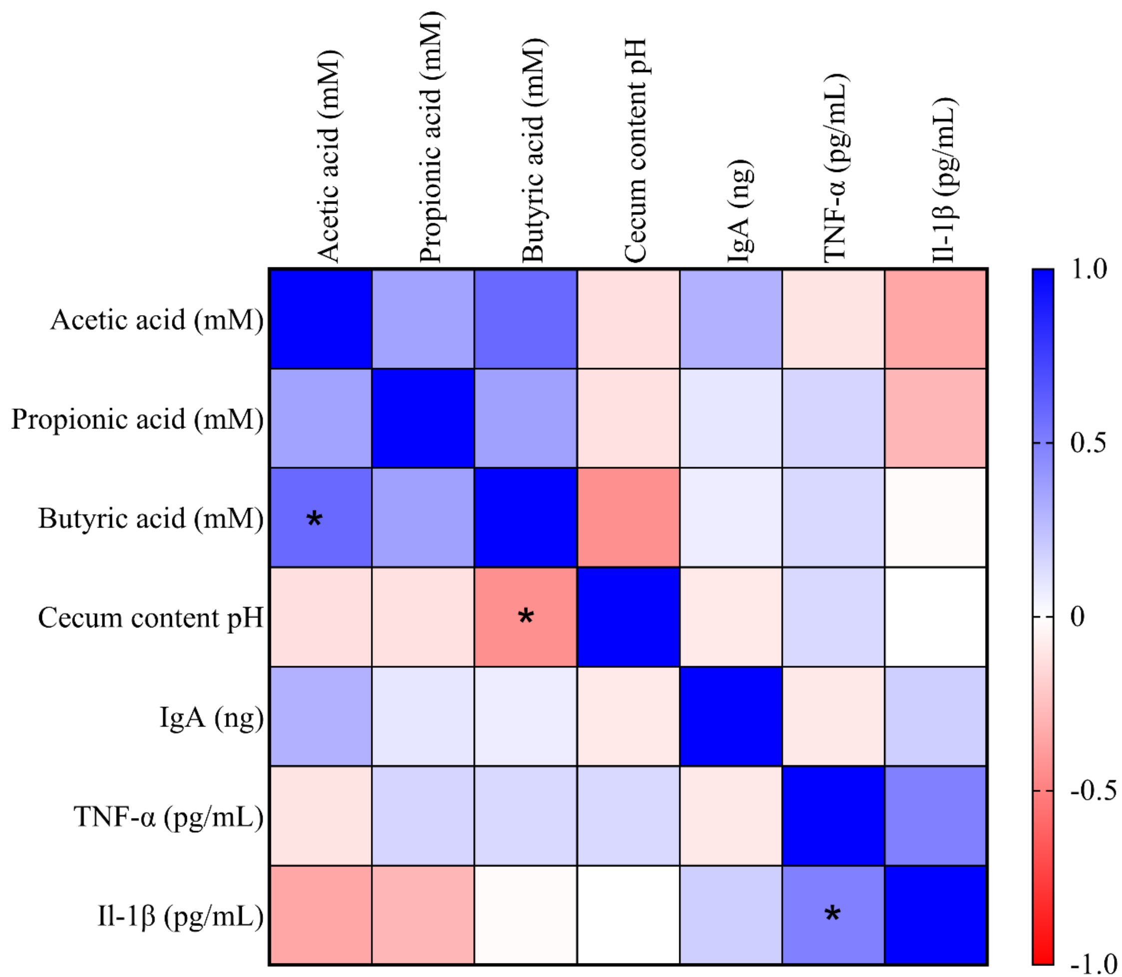


Figure 6: Difference in the relative abundance of the most abundant KEGG microbial metabolic pathways in the microbiota of adult female *Wistar* rats. (A) Enriched pathways between the SD and SDC treatment groups; (B) Enriched pathways between the HFD and HFDC treatment groups. Extended error bar plot was performed by bioinformatic software (STAMP) with FDR correction. Welch's two-sided test was used, and Welch's inverted was 0.95. SD: standard diet; SDC: standard diet + chia; HFD: high fat diet; HFDC: high fat diet + chia. Data expressed in 95% confidence interval.

According to correlation analysis, acetic acid was positively correlated with butyric acid ($r = 0.587$); butyric acid was negatively correlated with cecum content pH ($r = -0.437$). TNF-α was positively correlated with Il-1β ($r = 0.492$) (Supplementary Figure 1).



Supplementary Figure 1: The inflammatory markers were previously carried out (Da Silva *et al*. 2019). Heatmap of Pearson correlation analysis. IgA: immunoglobulin A, TNF-α: tumor necrosis factor α, Il-1β: Interleukin 1β. *Indicates statistically significant difference ($p<0.05$).

4. Discussion

Chia seeds are rich in dietary fiber, omega-3 polyunsaturated linolenic acids, high-quality protein and bioactive compounds.10,11 Chia seeds have many health-promoting properties and may be considered as a functional food ingredient.10,32 In our experiment, we investigated the effects of chia consumption on gut health in female *Wistar* rats. It was observed that there was no difference in the relative abundance of the microbiota, but chia consumption increased the richness and diversity, which probably contributed to the increased production of acetic and butyric acids in a standard diet and propionic acid in a high fat diet, and a consequently reduction in the pH of cecal content. Chia consumption increased the SI and AP

gene expression and decreased the SGLT1 and PepT1 gene expression compared to SD group. The HFDC group demonstrated lower IgA concentration compared to HFD group. Further, the correlation analysis showed that butyric acid and cecum content pH were negatively correlated.

The final body weight and weight gain were not different among the experimental groups. The total food intake was lower in both groups fed with HFD compared to groups fed with SD, but the consumption of a HFD did not alter total energy intake, that is why there was no difference in body weight. The HFD decreases food intake due the higher energy density and the increase in satiety, promoted by the fat, which corroborates with our previous reports.14,33 SDC group showed higher food intake and energy intake compared to SD group. The increased food and energy intake in SDC group, without increasing weight gain and final weight, may have occurred due to a decrease in nutrient absorption. Chia is rich in fiber, phenolic and bioactive compounds 13,32 and, has been shown to reduce the absorption of lipids and glucose.34

Chia consumption increased acetic and butyric production when consumed in a standard diet, and propionic acid in a HFD, reducing the intestinal pH. The result was supported by correlation analysis which showed negative correlation between butyric acid and cecum content pH. Chia is rich in dietary fibers which supports intestinal microbe-derived metabolites such as SCFA.35 Chia is also rich in α-linolenic acid,11 which is absorbed primarily in the intestine, where some microorganisms can directly utilize it and produce small molecules, such as SCFA.36 Our findings are accordingly with our previous results with male rats that also demonstrated that chia consumption can increase SCFA content 14 and reduce intestinal pH,33 without increasing the abundance of bacteria population.14,33 Although the consumption of chia did not change the abundance of the microbiota, metabolites produced by the microbiota were increased, representing an impact on the microbial activity, in a beneficial way.

We observed that there was no difference in IgA concentration between groups fed with standard diet. On the other hand, when chia was associated with HFD (HFDC group) the IgA concentration was reduced compared to HFD group. IgA is the main Ig isotype in the mucosa.37 Our results in this study were similar to our previous work with males, in which chia was not able to increase IgA production.14 We believe the result can be explained due to the type of dietary fiber most present in chia. IgA concentration seems to be more affected by soluble dietary fiber intake,38,39 and chia is rich in insoluble fiber.11 Even though the consumption of chia reduced the concentration of IgA in a HFD, another study has shown that the consumption

of chia associated with a HFD by female rats was able to reduce inflammation by reducing IL-1β.9

In the current study, we observed that chia consumption increased AP gene expression in a SD and a HFD, and SI gene expression was also increased in SDC group, in agreement with previous results.33 When chia was consumed associated with SD, PepT1 and SGLT1 gene expression was reduced. In our previous work, with males, we observed that HDF increased the expression of all intestinal functionality genes evaluated, and chia consumption decreased the expression of these genes, probably due to the fact that the organism is in homeostasis and does not require increase the expression of these genes to perform its function.14 Gene expression of these proteins are used as biomarkers of brush border membrane digestive and absorptive functions.13

Our α-diversity analyzes, which corresponds to each community evaluated individually, indicated that the groups that consumed chia had higher Chao1 index (estimates the richness of the bacterial community, number of different species) and higher Shannon index (indicates an increase in microbial diversity, how different the species are within the community). A richer and more diverse microbiota is associated with a healthier microbiota, because it can be more resistant to external factors.4 The analysis of β-diversity, which evaluate the degree of association or similarity between communities, revealed that groups fed with chia were grouped differently, mainly on a HFD, suggesting that their bacterial compositions were different. However, the relative abundance revealed that the composition of the intestinal microbiota was not different among the experimental groups. Although the groups fed with chia had a higher microbiota richness, the comparison of microbial taxon between the groups was not different, which is consistent with previous studies, including the study with male rats. 14,33 In addition, there was no difference in the Firmicutes to Bacteroidetes ratio. This ratio does not necessarily reflect a common pattern of all genera belonging to these phyla, since genera of the same phylum can be found in greater or lesser proportions.40 Thus, in the current study, changes in the bacterial community were probably not enough to reflect the Firmicutes to Bacteroidetes ratio. Our results suggest that the impact of chia consumption on the microbiota in female *Wistar* rats is mainly related to the functionality of the microbiota in comparison to the microbiota composition.

According to the predictive analysis related to KEGG microbial metabolic pathways, the HFDC upregulated pathways associated with teichoic acid (polyglycerol) biosynthesis compared to the HFD group, and downregulated metabolic pathways related to pyrimidine

nucleobases salvage and adenosine ribonucleotides de novo biosynthesis. The teichoic acids are found within the cell wall of most gram-positive bacteria and can be a target for IgA binds.41 Although this pathway was upregulated, IgA quantification was reduced in the HFDC group, which leads us to assume that even though this pathway was increased, it was not enough to increase the production of the teichoic acids and consequent increase in IgA.

The results described in this study suggest that chia consumption may be an effective strategy to improve intestinal health and capacity of intestinal absorption, by increasing the production of SCFA, which can impact in the pH of cecal content, and improving the microbial richness and diversity. The effects of chia in change the spatial organization of the microbiota (β diversity), at the OTU, genus and phylum level and impacting on pathways regulation was more pronounced when chia was associated with a HFD than with a SD in adult female *Wistar* rats. It has been shown that the microbiota of male and female can differ in the relative abundance of various genera,42,43 and the gut microbiome is not only associated with general obesity, but also with fat distribution, which is also sex-related. 43 Further, sex hormones were related to altered gastrointestinal functions and higher prevalence of irritable bowel syndrome was found in women.44 Thus, it is possible to assume that other parameters of intestinal health could be altered depending on sex. Our previous study with male *Wistar* rats, demonstrated that the intake of chia increased the production of SCFA, the diversity and abundance of intestinal bacteria were not affected, but the richness increased. Regarding intestinal functionality proteins, the results were similar to SGLT1 and PepT1, which were reduced by chia consumption in a standard diet. Thus, according to our results, chia consumption did not have different impacts depending on sex.

5. Conclusions

The present study provides evidence that chia consumption increased the richness and diversity of microbiota, increased the production of acetic and butyric acids in a SD and propionic acid in a HFD, decreased the pH of cecal content, and improve the brush border membrane functionality, although was not able to change the relative abundance of microbiota and showed lower IgA concentration in a HFD condition. Then, the consumption of chia, which is rich in insoluble dietary fiber and α-linolenic acid, can be beneficial for the functionality of microbiota improving the production of SCFA and intestinal pH and the effects of chia in the microbiota can be more pronounced in an HFD. And, according to our results, the effects of

chia flour consumption on SCFA production, the increased richness with no changes in relative abundance of microbiota and reduction in SGLT1 and PepT1 gene expression were similar between male and female *Wistar* rats.

Conflicts of interest

There are no conflicts of interest to declare.

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9.4 Paper 4: Effect of food derived bioactive peptides on gut health and inflammatory mediators *in vivo*: a systematic review

CRITICAL REVIEWS IN FOOD SCIENCE AND NUTRITION
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REVIEW

Effect of food derived bioactive peptides on gut health and inflammatory mediators in vivo: a systematic review

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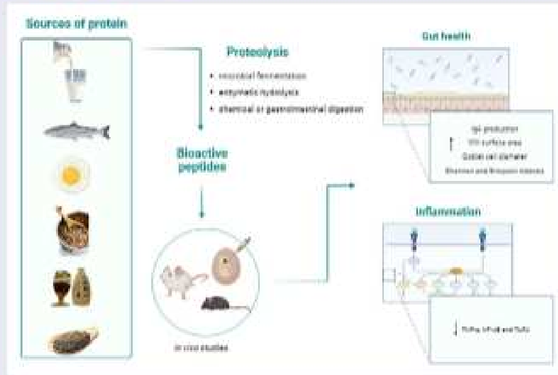
ABSTRACT
Dietary proteins serve as sources of exogenous peptides, after being released from the protein and absorbed, the bioactive peptides can perform several functions in the body. The objective of the current systematic review is to answer the question "How does food derived bioactive peptides can impact on gut health and inflammatory mediators in vivo?" The search was performed at PubMed, Cochrane, and Scopus databases for experimental studies, and the risk of bias was assessed by the SYRCLE tool. The data analysis was conducted following the PRISMA guidelines. Eleven studies performed in animal models evaluating bioactive peptides derived from animal and plant sources were included and evaluated for limitations in heterogeneity, methodologies, absence of information regarding the allocation process, and investigators' blinding. The bioactive peptides demonstrated potential positive effects on inflammation and gut health. The main results identified were a reduction in TNF-α, NF-κB, and TLR4, an improvement in IgA production and in intestinal morphology, with an increase in villi surface area and goblet cell diameter, and Shannon and Simpson indexes were also increased. However, more in vivo studies are still necessary to better elucidate the anti-inflammatory activity and mechanisms by which peptides regulate gut health. PROSPERO (CRD42023416680).

KEYWORDS
Peptides from food; intestinal health; anti-inflammatory mechanisms; inflammation; intestine; dietary protein

GRAPHICAL ABSTRACT



1. Introduction

Dietary proteins serve as sources of exogenous peptides that can perform regulatory activities comparable to those of endogenous peptides in our body. Exogenous peptides are called "bioactive peptides" due to their physiological relevance and nutritional importance (Bouglé e Bouhallab, 2017; Rabail *et al.*, 2021). These peptides are small fragments of proteins that usually contain 2 to 20 amino acid residues per molecule and remain inactive when bound

within the parent protein sequence. The peptides need to be activated by hydrolysis using enzymes, fermentation, chemical or gastrointestinal digestion, and the human body can produce these bioactive peptides from dietary proteins (Bouglé e Bouhallab, 2017; LeBlanc *et al.*, 2002; Rabail *et al.*, 2021; Rivera-Jiménez *et al.*, 2022).

Bioactive peptides have gained increasing attention in recent years. After being released from the protein and absorbed, they can perform several functions in the body. Some effects as antioxidant, anti-inflammatory, antihypertensive, hypoglycemic, antimicrobial, hypocholesterolemic, antithrombotic and immunomodulatory, adipogenesis regulation, increased absorption/bioavailability of minerals have already been reported (Cicero, Fogacci e Colletti, 2017; Grancieri, Stampini, Martino, Gonzalez, *et al.*, 2019; Grancieri, Martino e Mejia, 2019; Hou, Kolba, Glahn, Raymond P, *et al.*, 2017; LeBlanc *et al.*, 2002; Segura Campos *et al.*, 2013). Studies indicate that plant-derived bioactive peptides can resist to excessive inflammatory responses by modulating inflammatory signaling pathways and inhibiting the secretion of inflammatory factors (Liu, W. *et al.*, 2022). The bioactive peptides can produce anti-inflammatory effects via the immune system and then modulate the intestinal microbiome and homeostasis (Ren *et al.*, 2023), affecting intestinal brush border membrane barrier function, villous surface area, and intestinal microbiota (Hou, Kolba, Glahn, Raymond P, *et al.*, 2017; Jiao *et al.*, 2019; Mishima *et al.*, 2023; Requena *et al.*, 2017a). These properties might be linked to the type of dietary protein, their chemical composition, and molecular weight (Liu, W. *et al.*, 2022; Monteiro *et al.*, 2016; Ren *et al.*, 2023). Furthermore, the use of bioactive peptides is an alternative for using leftovers at an industrial level. Some industrial processes, such as the extrusion of seed oils, discard a relevant amount of proteins that can serve as a source for bioactive peptides, and generate value-added products in the food and medicinal industries (Rabail *et al.*, 2021).

Thus, the regular intake of bioactive peptides, which can be obtained from functional foods, whether by direct or indirect consumption, in the form of supplemented products or in nutraceutical formulations, could promote benefits improving disease biomarkers, as an alternative for the prevention and treatment of inflammatory and intestinal diseases (Rabail *et al.*, 2021; Rivera-Jiménez *et al.*, 2022). Therefore, the objective of the current systematic review is to answer the question "How does food derived bioactive peptides can impact on gut health and inflammatory mediators *in vivo*?". We reviewed the literature available to investigate the effects of food derived bioactive peptides (from animal and plant sources) on several parameters of gut health and inflammation. It is hypothesized that food derived bioactive peptides can

promotes beneficial changes to inflammation with the decrease in production of pro-inflammatory parameters and promotes beneficial changes to the gut health with increased production of metabolites associated with intestinal barrier improvement.

2. Materials and Methods

2.1. Eligibility Criteria

The eligibility criteria were based on the PICOS (population, intervention, comparison, outcomes, and study design) model strategy. Duplicate studies were excluded, and the search and screening for titles and abstracts were carried out independently by the authors according to the inclusion and exclusion criteria (Table 1).

Table 1. PICOS criteria for inclusion and exclusion of studies.

Parameter	**Inclusion criteria**	**Exclusion criteria**
Population	*In vivo* animal studies, adults	*In vitro* studies, clinical studies, preterm animals, intestinal diseases
Intervention	Intake or administration of food derived peptides	Food derived peptides associated with other foods/compound, peptides in the whole food (not isolated), peptides not derived from food (e.g.: synthetic peptides), interventions different from diet (e.g.: surgery, drug administration)
Comparator	Control group without the intervention/ diet with no food derived peptides	No control group
Outcomes	Changes in gut microbiota composition, intraluminal pH, short chain fatty acids, histological intestinal parameters, gene expression of tight junction's proteins and/or intestinal brush border membrane proteins, integrity of intestinal barrier and permeability, and inflammatory mediators (e.g.:cytokines of the	

	interleukin-family [IL's] such as IL-6, IL-1β, IL-8 and IL-10, interferons such as interferon-γ, tumor necrosis factors such as tumor necrosis factor-α, toll-like receptor 4 [TLR4], nuclear factor-κB [NF-κB])	
Study design	Placebo-controlled studies	*In vitro* studies, studies without a control group, review articles, theses, dissertations, book chapters, and studies published in other languages than English.

2.2. Information Source

Two researchers independently searched for original articles. PubMed, Cochrane and Scopus were used to search studies performed with animal models that evaluated the effects of food derived bioactive peptides on gut health and inflammation. No time restriction was used.

2.3. Search Strategy

The following English search terms were used: (food-derived bioactive peptide OR food derived bioactive peptide OR bioactive peptide) AND (intestine OR gut OR inflammation). Only articles published in English were considered in this review. The last search was performed on 20 February 2023. The first selection of the studies was based on the title and abstract. We excluded review articles, *in vitro* experiments and studies published in other languages than English. Further, we excluded studies in which the intake/administration of food derived peptides was associated with other foods/compound or diseases, the peptides were intake in the whole food (not isolated), the peptides were not derived from food (e.g.: synthetic peptides) or interventions different from diet (e.g.: ovariectomy, drug administration). Studies were eligible for inclusion if they fulfilled the following criteria: (a) studies conducted with adult animals; (b) the intervention was the intake/administration of food derived peptides (c) the comparator was the negative control (without the intervention); (d) the outcomes searched were changes related to the intestinal health, mainly: changes in the gut microbiota composition, intraluminal pH, short chain fatty acids, histological parameters of small and large intestine,

gene expression of tight junction's proteins, gene expression of intestinal brush border membrane, integrity of intestinal barrier and intestinal permeability; (e) the outcomes searched were changes related to the inflammatory mediators, mainly: cytokines of the interleukin-family (IL's) such as IL-6, IL-1β, IL-8 and IL-10, interferons such as interferon-γ, tumor necrosis factors such as tumor necrosis factor-α, toll-like receptor 4 (TLR4), nuclear factor-κB (NF-κB).

2.4. Selection, Data Collection Process and Data Items

Titles of studies retrieved using the search strategy and those from additional sources were screened independently by two review authors to identify studies that potentially meet the inclusion criteria outlined above. The screening was performed independently by the authors, using the Rayyan app. Subsequently, the authors in pairs, read the abstract and then the potentially eligible articles in full. The full text of these potentially eligible studies was retrieved and independently assessed for eligibility by two review team members. After reading and reviewing the selected research articles in full, the data were compared to ensure integrity and reliability. Divergent decisions were settled by consensus.

The eligible outcomes evaluated were broadly categorized as follows:

- Gut microbiota: short chain fatty acids (cecal, fecal or in the serum); intraluminal pH (ileal, cecal or feces); microbial quantification; secretory immunoglobulin A (sIgA);

- Epithelial physical barrier: tight junction proteins; proteins of intestinal brush border membrane; intestinal permeability; plasm endotoxin;

- Intestinal morphology: number of goblet cells; length, height and depth of villi and crypts; mucin secretion; antimicrobial peptides.

- Inflammatory mediators: cytokines of the interleukin-family (ILs) such as IL-6, IL-1β, IL-8 and IL-10, interferons (IFNs) like interferon-γ (INF-γ), tumor necrosis factors (TNFs) like tumor necrosis factor-α (TNF-α), toll-like receptor 4 (TLR4), nuclear factor-κB (NF-κB).

Any measure and methodology of these outcomes was eligible for inclusion. Further, for each experimental study included, we reported relevant information related to the authors, publication year, country of publication and experimental model, age, sex, number of groups and sample per group. To access the research methods, we extracted specific information related to the groups such as type of food intervention, and control group. For the control test of food intake, we extracted information related to the method of administration/intake that was used

in the intervention, the duration of the intervention, how was made the proteolysis, the molecular weight of peptides, the dosage of food derived bioactive peptide and main results (control x intervention).

We screened the reference lists of included studies for additional eligible studies not retrieved by our search. For this review, data from the eligible studies are expressed in tables and figures. We provided a narrative synthesis of the results according to the main characteristics and results.

2.5. Study Risk-of-Bias Assessment

The methodological quality of the included studies was assessed, and the risk of bias was verified using the Systematic Review Centre for Laboratory Animal Experimentation Risk of Bias (SYRCLE RoB) tool (Hooijmans *et al.*, 2014), which is responsible for identifying study quality and measuring the bias in research involving animal studies. The SYRCLE RoB toll considers ten entries that are related to six types of bias: selection bias, performance bias, detection bias, attrition bias, reporting bias and other. For each included study, the bias were classified as "high" (−; red), "low" (+; green) or "unclear" (?; yellow).

3. Results

3.1. Study Selection

The flow diagram of the literature search and selection process was built in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guideline (Figure 1). After the search in the selected databases, we identified 5946 articles (Pubmed = 5825; Cochrane = 43 and Scopus = 78). From these, 5924 were excluded for the following reasons: duplicate studies (n = 82), title and abstract not suited to the topic (n = 5726), *in vitro* studies (n = 53), review articles (n = 43), wrong population/ intervention (n =18), food derived peptides associated with other foods/compounds (n = 1), and other languages than English used (n = 1). After, 22 articles were read fully. From these, we excluded 15 articles: food derived peptides associated with intestinal disease (n = 9); food derived peptides associated with interventions different from diet (n= 2); synthetic peptide (n= 4). We identified 5 articles by other methods (Citation searching = 3; Organizations = 2). From these, 1 article was excluded because utilized synthetic peptide. Therefore, 11 studies were included in this systematic review.

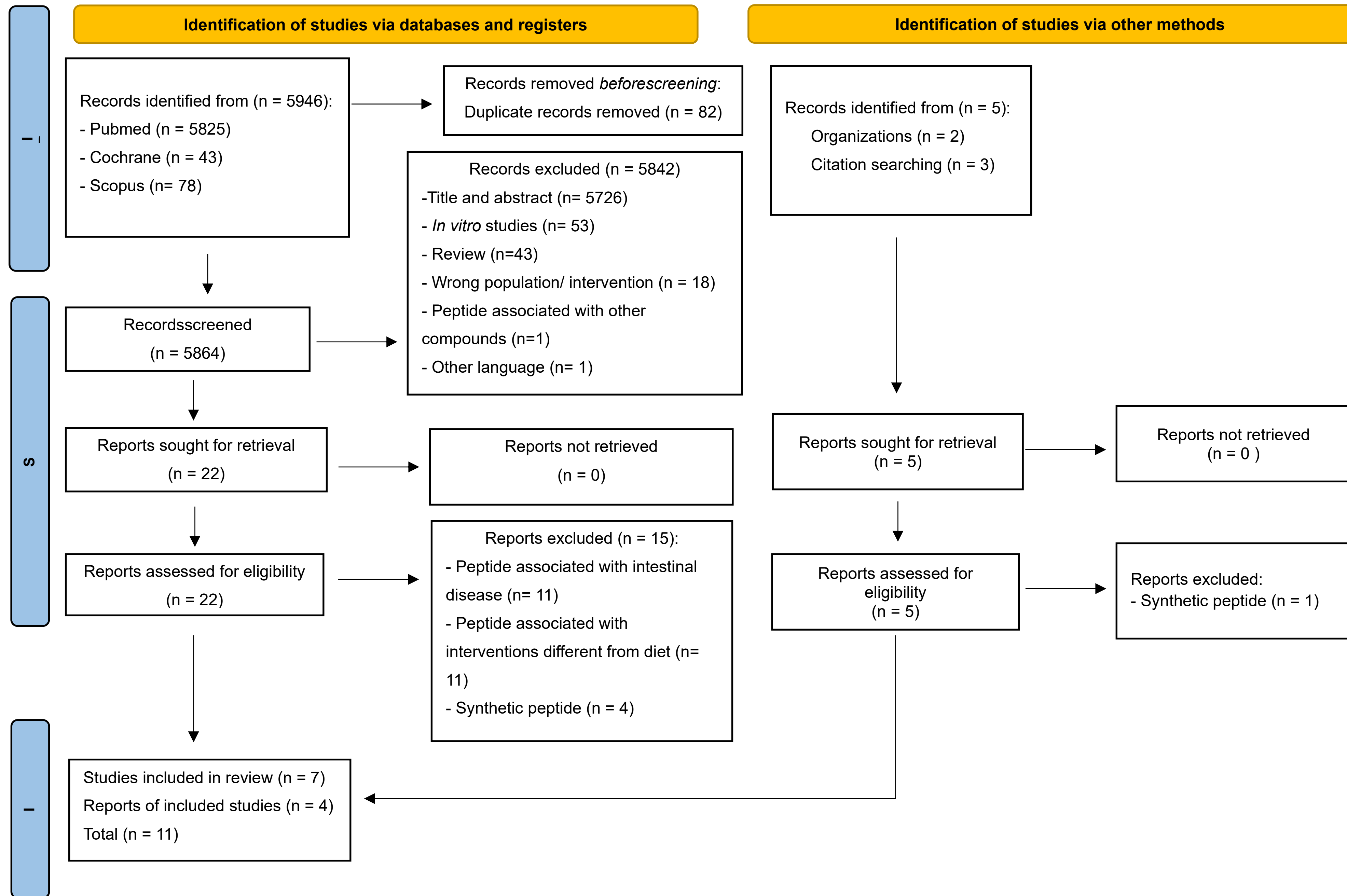

Identification of studies via databases and registers
Identification of studies via other methods
Records identified from (n = 5946):
- Pubmed (n = 5825)
- Cochrane (n = 43)
- Scopus (n= 78)
Records removed beforescreening:
Duplicate records removed (n = 82)
Records identified from (n = 5):
Organizations (n = 2)
Citation searching (n = 3)
Records excluded (n = 5842)
-Title and abstract (n= 5726)
- In vitro studies (n= 53)
- Review (n=43)
- Wrong population/ intervention (n = 18)
- Peptide associated with other compounds (n=1)
- Other language (n= 1)
Recordsscreened
(n = 5864)
Reports sought for retrieval
(n = 22)
Reports not retrieved
(n = 0)
Reports sought for retrieval
(n = 5)
Reports not retrieved
(n = 0)
Reports assessed for eligibility
(n = 22)
Reports excluded (n = 15):
- Peptide associated with intestinal disease (n= 11)
- Peptide associated with interventions different from diet (n= 11)
- Synthetic peptide (n = 4)
Reports assessed for eligibility
(n = 5)
Reports excluded:
- Synthetic peptide (n = 1)
Studies included in review (n = 7)
Reports of included studies (n = 4)
Total (n = 11)

Figure 1: Flowchart of the search for articles included in the systematic review, according to PRISMA (2020) recommendation.

3.2. Study Characteristics

The included studies (n = 11) were performed in five different countries, three of them were conducted in China (Guo *et al.*, 2021; Ren *et al.*, 2023; Shi *et al.*, 2021), two in Unites States of America (Hou, Kolba, Glahn, Raymond P, *et al.*, 2017; Mishima *et al.*, 2023), two in Canada(Duarte *et al.*, 2006; LeBlanc *et al.*, 2002), two in Spain (Garcés-Rimón *et al.*, 2016; Requena *et al.*, 2017b) and two in Brazil (Grancieri *et al.*, 2022; Monteiro *et al.*, 2016). Regarding the population, six studies were performed with mice, four studies with C57BL/6 (Grancieri *et al.*, 2022; Monteiro *et al.*, 2016; Ren *et al.*, 2023; Shi *et al.*, 2021) and two with BALB/c (Duarte *et al.*, 2006; LeBlanc *et al.*, 2002), three with rats, two with Zucker fatty rats (Garcés-Rimón *et al.*, 2016; Requena *et al.*, 2017b) and one with Spontaneously Hypertensive Rat (SHR) (Guo *et al.*, 2021), and two studies with chickens (*Gallus gallus*) (Hou, Kolba, Glahn, Raymond P., *et al.*, 2017; Mishima *et al.*, 2023). Six studies used male animals (Garcés-Rimón *et al.*, 2016; Grancieri *et al.*, 2022; Guo *et al.*, 2021; Monteiro *et al.*, 2016; Requena *et al.*, 2017b; Shi *et al.*, 2021), one used female animals (Duarte *et al.*, 2006), the studies with chickens used both male and female samples (Hou, Kolba, Glahn, Raymond P., *et al.*, 2017; Mishima *et al.*, 2023). Two studies did not describe animals' sex (LeBlanc *et al.*, 2002; Ren *et al.*, 2023). The age of the animals ranged from 5 to 10 weeks, although two studies did not mention this information (LeBlanc *et al.*, 2002; Ren *et al.*, 2023), one study only mention "young adult" (Monteiro *et al.*, 2016) and the studies with chickens conducted an intra amniotic administration of the peptides on day 17th on incubation (Hou, Kolba, Glahn, Raymond P., *et al.*, 2017; Mishima *et al.*, 2023). The number of samples per group ranged from 5 to 11. The studies' main characteristics were chronologically organized by the publication year, starting with the first published (Table 2).

Table 2: Characteristics of studies on the effects of bioactive peptides on intestinal health and inflammation.

Authors, publication year	Country of publication	Experimental model	Age	Sex	Number of groups and sample per group	Type of intervention
LeBlanc *et al*., 2002	Canada	BALB/c mice	NS	NS	3 groups (n = minimum of 5 mice/group)	Three peptidic fractions from milk fermentation
Duarte *et al*., 2006	Canada	BALB/c mice	6–8-week-old	Female	19 groups (n = 5 mice/group)	Fermented fish protein concentrate (*Merluccius productus*) (FPC)
Garcés-Rimón *et al*., 2016	Spain	Zucker fatty rats	8-week-old	Male	3 groups (n= 10 animals)	Egg white hydrolyzed

Monteiro *et al*., 2016	Brazil	C57BL/6	young adult	Male	4 groups (n= 8-10 animals)	Hydrolyzed whey protein
Hou *et al*., 2017	USA	Chicken embryos (*Gallus gallus*)	Day 17th of embryonic incubation	Male and female	11 groups (n = 10 chickens/group)	Duck Egg White Peptides (DPs)
Requena *et al*., 2017	Spain	Zucker fatty (fa/fa) rats	8-week-old	Male	3 groups (n = 10 rats/group)	Egg white hydrolyzed with pepsin (EWH)
Guo *et al*., 2021	China	Spontaneously hypertensive rat (SHR)	10-week-old	Male	4 groups (n = 7 rats/group)	Quinoa protein

Shi *et al*., 2021	China	C57Bl/6 mice	5-week-old	Male	6 groups (n = 7–10 mice/group)	Three peptides from huangjiu (Chinese rice wine)
Grancieri *et al*., 2022	Brazil	C57Bl-6 mice	8-week-old	Male	4 groups (n = 11 mice/group)	Proteins from chia seeds (*Salvia hispanica*)
Mishima *et al*., 2023	USA	Chicken embryos (*Gallus gallus*)	Day 17th of embryonic incubation	Male and female	5 groups (n = 7-9 chickens/group)	Proteins from chia seeds (*Salvia hispanica*)
Ren *et al*., 2023	China	C57BL/6J mice	NS	NS	4 groups (n = 8 mice/group)	*Lophius litulon* (monkfish) peptides (LPs)

NS: Not specified; USA: United States of America; n: number of animals

The intervention with bioactive peptides varied by the protein source that was used. The foods used as a source of peptides were egg white (Garcés-Rimón *et al.*, 2016; Hou, Kolba, Glahn, Raymond P, *et al.*, 2017; Requena *et al.*, 2017a), fish (Duarte *et al.*, 2006; Ren *et al.*, 2023), chia seed (Grancieri *et al.*, 2022; Mishima *et al.*, 2023), milk (LeBlanc *et al.*, 2002), quinoa (Guo *et al.*, 2021), whey (Monteiro *et al.*, 2016) and huangjiu (Chinese rice wine) (Shi *et al.*, 2021). The proteolysis to obtain the peptides was made by microbial fermentation (Duarte *et al.*, 2006; LeBlanc *et al.*, 2002; Shi *et al.*, 2021) or enzymatic hydrolysis (Garcés-Rimón *et al.*, 2016; Grancieri *et al.*, 2022; Guo *et al.*, 2021; Hou, Kolba, Glahn, Raymond P, *et al.*, 2017; Mishima *et al.*, 2023; Ren *et al.*, 2023; Requena *et al.*, 2017a) and one study did not describe how was made the proteolysis (Monteiro *et al.*, 2016). The peptides were administered dissolved in water (Duarte *et al.*, 2006; Garcés-Rimón *et al.*, 2016; LeBlanc *et al.*, 2002; Requena *et al.*, 2017a) or a saline solution (Guo *et al.*, 2021), in the diet in powder (Monteiro *et al.*, 2016), in the diet in pellets (Grancieri *et al.*, 2022), via gavage (Ren *et al.*, 2023; Shi *et al.*, 2021) or via intra amniotic administration (Hou, Kolba, Glahn, Raymond P, *et al.*, 2017; Mishima *et al.*, 2023). The duration of intervention ranged 2 days to 12 weeks, and 2 studies evaluated the intra amniotic administration (17th day).

Five studies presented the molecular weight of the peptides (Grancieri *et al.*, 2022; Hou, Kolba, Glahn, Raymond P, *et al.*, 2017; Mishima *et al.*, 2023; Ren *et al.*, 2023; Shi *et al.*, 2021). The molecular weight of the peptides ranged from 425.23Da to <5kDa. Regarding the peptides dosage, the studies that did the administration in a solution, gavage or pellets, the doses varied from 50 µg (0.05mg) of peptide/day (LeBlanc *et al.*, 2002) to 750mg/kg body weight/day (Garcés-Rimón *et al.*, 2016; Requena *et al.*, 2017a)or 0.20mg/mL to 0.30mg/mL (Duarte *et al.*, 2006), in the intra amniotic administration studies the dosage varied from 10mg/mL/egg (Mishima *et al.*, 2023) to 40mg/mL/ egg (Hou, Kolba, Glahn, Raymond P, *et al.*, 2017), and one study added the peptides in the diet in the dose of 18.03g/100g of diet (Monteiro *et al.*, 2016) (Table 3).

Table 3: Main findings in studies on the effects of food derived bioactive peptides on intestinal health and inflammation.

Reference	Intervention group	Control group	Method of administration/ intake	Duration of the intervention	Proteolysis	Molecular weight of peptides	Dosage of peptides	Main Results (control x intervention)
LeBlanc *et al.*, 2002	Peptidic fractions from milk fermentation: - Fraction I = large peptides - Fraction II = medium peptides - Fraction III = small peptides	Water	Administered orally (one of the three peptidic fractions resuspended in 50 µL of water)	2, 5, or 7 days	Microbial fermentation	NS	50 µg (0.05mg) of peptide/day	- Fraction III: ↑ number of IgA+ B cells after all three feeding periods (2, 5, and 7 days); - Fraction II: ↑ number of IgA+ B cells after 2 and 7 days of feeding; and a double dose of this peptidic fraction, for 7 days, ↑ number of IgA+ B cells versus control mice and mice fed with normal concentration of Fraction II.
Duarte *et al.*, 2006	Fermented fish protein concentrate (*Merluccius productus*) (FPC)	Water	Aqueous solution of FPC	2, 5 or 7 days	Microbial fermentation	NS	-0.20 mg of FPC/ml -0.25 mg of FPC /ml -0.30 mg of FPC /ml Daily water intake: 3.1±0.3 ml/day/mouse	- ↔ morphological architecture of the small intestine; FPC (0.3 mg/ml) for 7 days: - ↑ number of IgA+ cells and the content of S-IgA in the small intestine - ↑ number of IL-4+, IL-10+ and IL-6+ cells; FPC (0.3 mg/ml) for 5 and 7 days: - ↑ IFNγ+ and TNF-α + cells.
Garcés-Rimón *et al.*, 2016	Obese Zucker rats + - Egg white hydrolyzed with pepsin + obese - Egg white hydrolyzed with aminopeptidase	Obese Zucker rats + water	Dissolved in tap water	12 weeks	Enzymatic hydrolysis	NS	750 mg of peptides / kg body weight / day	Egg white hydrolyzed with pepsin: - ↓ TNF-α

Monteiro *et al*., 2016	Hydrolyzed whey protein + HFD (HFWPH)	HFD	In the diet	9 weeks	NS	NS	18.03g/100g of diet	- HFWPH helped to preserve the Bacteroidetes; - ↓Verrucomicrobia - ↑Proteobacteria - ↓ LPS - ↓ TLR4
Hou *et al*., 2017	Duck Egg White Peptides (DPs)	- non-injected (NI) - 18 MΩ H_2O	Intra-amniotic administration (1 mL per egg)	17th day to 21st day	Enzymatic hydrolysis	<5 kDa	40mg of DP/mL/egg	- ↑ cecum weight X NI and 18 MΩ H_2O; - ↓ *Bifidobacterium*, *Clostridium* and *E. coli* X 18 MΩ H_2O; - ↑ villus surface area and diameter of goblet cells X NI and 18 MΩ H_2O; - ↔ BBM functional genes X NI and 18 MΩ H_2O.
Requena *et al*., 2017	Obese Zucker rats + Egg white hydrolyzed with pepsin (EWH)	Obese Zucker rats + water	Dissolved in tap water	12 weeks	Enzymatic hydrolysis	NS	750 mg of EWH / kg body weight/day	- ↓ *Lactobacillus /Enterococcus*, *C. leptum* and total bacteria - ↔ SCFA
Guo *et al*., 2021	Rat SHR + quinoa protein (SHR-Q)	Rat SHR + saline solution	Oral administration, using syringes, dissolved in saline solution	5 weeks	Enzymatic hydrolysis	NS	200 mg of quinoa protein/kg body weight	- SHR–Q: ↑ Shannon and Simpson indexes; - ↑ Staphylococcus, *Allobaculum* and *Turibacter*; -The community structure of the microbiota has undergone significant alternation by Bray–Curtis and unweighted UniFrac distances.
Shi *et al*., 2021	Peptides from huangjiu + HFD: - HpT1 (Tyr-Val-Lys-Val) - HpT2 (Leu-Phe-Trp)	HFD	Gavage (daily)	8 weeks	Microbial fermentation	- HpT1 (507,30 Da) - HpT2 (464,24 Da) - HpT3 (425,23 Da)	0.3 g kg^{-1} (300 mg) of peptide solution/kg body weight	- HpT2: ↑ Shannon and Simpson indexes; - HpT3: ↓ Shannon index; - HpT1 and HpT2 reversed the gut microbiota dysbiosis induced by HFD.

	- HpT3 (Phe-Leu-Phe)							
Grancieri *et al.*, 2022	Digested proteins from chia seeds (*Salvia hispanica*) + HFD (HFDP)	HFD	Diet pellets	9 weeks	Enzymatic hydrolysis	>500Da	400 mg of DP/kg of body weight	<u>HFDP X HFD:</u> ↓ NF-κB p65 ↓ PPAR-γ ↓ TNF-α
	Digested proteins from chia seeds (*Salvia hispanica*) (NDDP)	Normal diet (ND)						<u>NDDP X ND:</u> ↔ NF-κB p65 ↔ PPAR-γ ↔TNF-α
Mishima *et al.*, 2023	Hydrolyzed chia protein (CP)	- non-injected (NI) - 18 MΩ H_2O	Intra-amniotic administration (1 mL per egg)	17th day to 21st day	Enzymatic hydrolysis	>500Da	10 mg of CP/mL/egg	↓ TNF-α X NI; ↑ OCLN X NI and 18 MΩ H_2O; ↑ MUC2 X 18 MΩ H_2O; ↑ AP X NI and 18 MΩ H_2O; ↓ *Bifidobacterium* X NI and 18 MΩ H_2O; ↑ *Lactobacillus* X NI; ↑ depth crypt, villi height and villus surface area X NI and 18 MΩ H_2O; ↑ Paneth cell number X NI and 18 MΩ H_2O; ↓ villi goblet cell number X 18 MΩ H_2O; ↑ villi goblet cell diameter X 18 MΩ H_2O; ↑ crypt goblet cell diameter X NI; ↓ crypt goblet cell number X NI;

Ren *et al.*, 2023	*Lophius litulon* (monkfish) peptides (LPs) + HFD	HFD	Gavage (daily)	4 weeks	Enzymatic hydrolysis	<1 kDa	- 100 mg of LP/kg body weight - 200 mg of LP/kg body weight	- 100 and 200 mg of LP/kg: ↓ IL-1β, IL-6, TNF-α and TLR4 in the kidney; - 200 mg of LP/kg: ↓ phospho-NF-κB (p-p65); - 100 and 200 mg of LP/kg: ↑ OTU, Chao and Ace indexes; - 200 mg of LP/kg: ↑Shannon index; - 100 and 200 mg of LP/kg: ↑ Saccharibacteria and ↓ Tenericutes.

↑: increase; ↓: decrease; ↔: no difference; 18 MΩ H_2O: Ultrapure Water; *Ap*: aminopeptidase; BBM: Brush border membrane; HFD: high fat diet; IFNγ: Interferon γ; IgA: Immunoglobulin A; IL: Interleucin; LPS: Lipopolysaccharides; MUC2: Mucin2; NF-κB: Nuclear Factor kappa-B; NS: Not specified; OCL: Ocludin; OUT: Operacional taxonomic units; PPARγ: Peroxisome Proliferator-Activated Receptor gamma; SCFA: Short chain fatty acid; SHR: Spontaneously hypertensive rat; S-IgA: secretory-IgA; TLR4: Toll like receptor 4; TNF-α: Tumor necrosis factor-alpha.

3.3. Main Findings

The reviewed studies demonstrated that the food derived bioactive peptides intake/administration potentially provided beneficial effects to intestinal health and inflammatory mediators. We found a reduction in TNF-α, NF-κB and TLR4, an improvement in IgA production and in intestinal morphology, with an increase in villi surface area and goblet cell diameter, and the Shannon and Simpson indexes were increased (Table 3).

The inflammatory mediators were evaluated in five studies, four of these studies observed a reduction in TNF-α (Garcés-Rimón *et al.*, 2016; Grancieri *et al.*, 2022; Mishima *et al.*, 2023; Ren *et al.*, 2023) and one study observed an increase (Duarte *et al.*, 2006). NF-κB was evaluated in two studies (Grancieri *et al.*, 2022; Ren *et al.*, 2023) and both observed a reduction in NF-κB, in both of these studies the peptides were intake with a high fat diet and in a normal diet there was no difference (Grancieri *et al.*, 2022). TLR4 was evaluated and reduced in two studies (Monteiro *et al.*, 2016; Ren *et al.*, 2023). PPAR-γ and IFNγ were evaluated only in one study, PPAR-γ (Grancieri *et al.*, 2022) was reduced, and IFNγ was increased (Duarte *et al.*, 2006). Interleukins were evaluated in two studies, one study observed that the number of IL-4+, IL-10+ and IL-6+ cells was increased (Duarte *et al.*, 2006) and other study observed that IL-1β and IL-6 decreased (Ren *et al.*, 2023).

Two studies evaluated the IgA production, in both of these studies the IgA production was increased (Duarte *et al.*, 2006; LeBlanc *et al.*, 2002) and seven days were enough to that result. The intestinal morphology was evaluated in three studies. In one of them no differences in morphological architecture of the small intestine were observed, but it does not clearly define which parameters were evaluated (Duarte *et al.*, 2006). Two studies observed an increase in villus surface area and diameter of goblet cells (Hou, Kolba, Glahn, Raymond P, *et al.*, 2017; Mishima *et al.*, 2023). One study observed an increase in depth crypt, villi height and Paneth cell number, and a decrease in goblet cell number (Mishima *et al.*, 2023).

The microbiota was evaluated in six studies that evaluated different parameters (Guo *et al.*, 2021; Hou, Kolba, Glahn, Raymond P, *et al.*, 2017; Mishima *et al.*, 2023; Monteiro *et al.*, 2016; Requena *et al.*, 2017a; Shi *et al.*, 2021). The Shannon index was increased in three studies (Guo *et al.*, 2021; Ren *et al.*, 2023; Shi *et al.*, 2021), in one of these studies, the Shannon index was decreased accordingly to the molecular weight of the peptides (Shi *et al.*, 2021), the Simpson index was increased in two studies (Guo *et al.*, 2021; Shi *et al.*, 2021). Further, alterations in specific species, genus and phyla were observed in some of the studies.

Two studies evaluated the gene expression of proteins for intestinal functionality, one study found an increase in aminopeptidase gene expression (Mishima *et al.*, 2023), but in other study there was no difference in this protein (Hou, Kolba, Glahn, Raymond P, *et al.*, 2017). The following results were evaluated for just one study. The short chain fatty acids (SCFA) presented no difference (Requena *et al.*, 2017a). The lipopolysaccharide (LPS) concentration was decreased (Monteiro *et al.*, 2016). The gene expression of intestinal barrier proteins ocludin (OCLN) and mucin (MUC2) were increased (Mishima *et al.*, 2023). And the cecum weight was increased (Hou, Kolba, Glahn, Raymond P, *et al.*, 2017).

3.4. Risk of Bias

In most of the studies, the allocation of animals was not described in detail, since there was no information about the randomization process, only two studies described that the randomization process was made based on the weight (Hou, Kolba, Glahn, Raymond P., *et al.*, 2017; Mishima *et al.*, 2023), and four studies did not mention if the animal allocation to treatment groups was performed randomly (Duarte *et al.*, 2006; Grancieri *et al.*, 2022; Monteiro *et al.*, 2016; Shi *et al.*, 2021). From all the studies that were included in the current systematic review (n = 11), the baseline characteristics sex and age were complete in six studies (Duarte *et al.*, 2006; Garcés-Rimón *et al.*, 2016; Grancieri *et al.*, 2022; Guo *et al.*, 2021; Requena *et al.*, 2017a; Shi *et al.*, 2021), some of them also presented the initial weights of the animals, for the studies with intra amniotic administration, the authors mentioned the age and that the eggs were weighed before the distribution (Hou, Kolba, Glahn, Raymond P., *et al.*, 2017; Mishima *et al.*, 2023). None of the studies reported about blinding the investigators involved in the research, about the randomized outcome assessment and blinding the outcome assessor. Three studies did not include all animals in the analysis, and the exclusion criteria were not reported (Hou, Kolba, Glahn, Raymond P, *et al.*, 2017; Mishima *et al.*, 2023; Shi *et al.*, 2021) (Figure 2).

	Sequence generation (selection bias)	Baseline characteristics (selection bias)	Allocation concealment (selection bias)	Random housing (performance bias)	Blinding (performance bias)	Random outcome assessment (detection bias)	Blinding (detection bias)	Incomplete outcome data (attrition bias): All outcomes	Selective outcome reporting (reporting bias)	Other bias
Duarte et al., 2006	−	+	?	+	?	?	?	?	+	+
Garcés-Rimón et al., 2016	?	+	?	?	?	?	?	+	+	+
Grancieri et al., 2022	−	+	?	?	?	?	?	+	+	+
Guo et al., 2021	?	+	?	?	?	?	?	?	+	+
Hou et al., 2017	+	+	?	+	?	?	?	−	+	+
LeBlanc et al., 2002	?	−	?	?	?	?	?	+	+	+
Mishima et al., 2023	+	+	?	+	?	?	?	−	+	+
Monteiro et al., 2016	−	−	?	?	?	?	?	?	+	+
Ren et al., 2023	?	−	?	?	?	?	?	?	+	+
Requena et al., 2017	?	+	?	+	?	?	?	?	+	+
Shi et al., 2021	−	+	?	?	?	?	?	−	+	+

Figure 2: Risk of bias of the studies, + (red): "high" (+), - (green): "low" (−) or ? (yellow): "unclear" (?).

4. Discussion

In this systematic review, we reviewed studies available in the literature, in animal models, to investigate how food derived bioactive peptides, from animal and plant sources, can impact on several parameters of gut health and inflammatory mediators. The reviewed studies demonstrated that the intake/administration of food derived bioactive peptides potentially

provided beneficial effects to intestinal health and inflammatory mediators. As more consistence results, we found a reduction in TNF-α, NF-κB and TLR4, an improvement in IgA production and in intestinal morphology, with an increase in villi surface area and goblet cell diameter, and an increase in the Shannon and Simpson indexes (Table 3).

A small number of studies were included, and these studies evaluated different parameters about intestinal health and inflammation. The result we observed that was most repeated was the reduction of TNF-α. This effect was already verified in *in vitro* studies (Galdino Alves *et al.*, 2016; Grancieri, Martino e Mejia, 2019; He *et al.*, 2022). NF-κβ, which was also reduced in two studies (Grancieri *et al.*, 2022; Ren *et al.*, 2023), is translocated to the cytosol, and into the nucleus is responsible for inducing cytokine gene expression to control inflammatory and immune responses, initiating the pathway to drive the expression of target genes such as TNF-α, IL1β, IL6, and IL10 (Yu *et al.*, 2020). TNF-α is a pro-inflammatory cytokine that plays a key role in the inflammatory cascade that can causes increased intestinal permeability and then leads to chronic intestinal inflammation (Kaminsky, Al-sadi e Ma, 2021; Pedersen *et al.*, 2014). The studies evaluated different types of peptides that were able to reduce the expression of TNF-α, both peptides derived from plant-based proteins and from animal-based proteins presented this anti-inflammatory effect. The characteristics of these peptides may be responsible for their effects. Peptides with hydrophobic amino acids can block free radicals, because of the ionizable groups, which can increase antioxidant activity. Also, peptides with fewer than 20 amino acid residues per molecule, can present this effect, since small peptides are better able to cross the intestinal barrier and exert their biological effects (Brandelli, Daroit e Corrêa, 2015; Kou *et al.*, 2013). Further, peptides with hydrophobicity ≤20 kcal mol^{-1} are more effective at penetrating the cell membrane (Sacks *et al.*, 2017). The anti-inflammatory effect of a bioactive peptide is influenced by its molecular weight. Peptides with a low molecular weight (less than 1kDa) tend to have the highest levels of anti-inflammatory activity, and peptides with molecular weights of approximately 500Da have the strongest anti-inflammatory activity (Liu, W. *et al.*, 2022). The range of molecular weight of the peptides related in the studies included in this review that observed the anti-inflammatory effect was from 500Da to 1kDa.

Two studies evaluated the Immunoglobulin A (IgA) production, in both studies the intake of peptides increased the IgA production and around seven days was enough to this change. The studies that verify the increase in IgA production were carried out with peptides of animal origin derived from milk and fish, both with proteolysis made by microbial

fermentation, however with a relevant difference in the dose of peptides in the two studies, the milk peptide study used 0.05mg/day, and the fish peptide was 0.20 mg/ml to 0.30mg/ml. IgA is the predominant antibody produced by mucosal secretions, whose primary function is to defend the surfaces of these regions. IgA provides dual humoral responses that create a symbiotic environment for the resident gut microbiota and prevent the invasion of enteric pathogens (Abokor *et al.*, 2021). IgA can act eliminating pathogens, restricting the colonization or expansion of intestinal microorganisms, and reinforcing the beneficial microbiota, increasing colonization, stability, and resilience. In the intestinal lumen, secretory IgA antibodies bind to the surface of commensal bacteria to maintain their homeostatic existence with the intestinal tissues. Molecules that are essential for the intestinal immune system are provided through the diet and the microbiota, thus, dietary antigens can regulate the production of IgA (Kim *et al.*, 2016; Takeuchi e Ohno, 2021).

Two studies, evaluating duck egg white peptides and protein of chia seed, observed that the surface of the villi was increased as well as the diameter of goblet cells (Hou, Kolba, Glahn, Raymond P., *et al.*, 2017; Mishima *et al.*, 2023). The villus length improvement can resultant in an increase in absorptive surface area, expression of brush border enzymes, transport of nutrients and then the digestive and absorptive activities of the small intestine can increase (Hou e Tako, 2018; Mishima *et al.*, 2023; Mousa *et al.*, 2023; Silva, Da, Martino e Tako, 2021). The primary and main function of proteins is tissue formation, thus it has been suggested that protein is critical among other macronutrients for the development of intestinal villi and epithelial cells (Incharoen *et al.*, 2010; Mousa *et al.*, 2023). Only one study evaluated the gene expression of proteins related to intestinal barrier, it was observed that the gene expression of ocludin and mucin were increased, by the protein of chia seed. The same study observed a decrease in inflammation by regulation of gene expression of TNF-α, then the improvement in intestinal barrier was attributed the reducing inflammatory factors such as TNF-α (Mishima *et al.*, 2023).

Most of the studies included in this systematic review evaluated, in some way, the influence of food derived peptides on the microbiota. Similarly, as in humans, Bacteroidetes and Firmicutes are the two main phyla in rodents' gastrointestinal tract. The *Gallus gallus* model also presents an intestinal microbiota similar to that of humans, with predominance of the Firmicutes, Proteobacteria and Actinobacteria phyla (Hou e Tako, 2018). In this sense, these experimental models are used to analyze interventions that evaluate the effects of the diet on the gut microbiota, health, and disease interaction. In this review, some studies observed an increase in Shannon and Simpson indexes, representing an increase in diversity and dominance,

which is correlated with a healthier microbiota. And some of the included studies also found that the ingestion of peptides reversed the deleterious effects of high-fat diets on the microbiota. Different phyla, genera and species of bacteria were evaluated in different studies and given the bacterial variety and small number of studies it is not possible to establish a consensus on the modulation of relative abundance of some specific bacteria.

The biological and functional properties of peptides are usually determined by their amino acid sequence, relative proportion of specific amino acids, their hydrophobicity, molecular weight and net charge. However, limited information on the relationship between structure and anti-inflammatory activity of bioactive peptides is available (Rivera-Jiménez *et al.*, 2022). We observed that many studies involving bioactive peptides were performed *in vitro,* and most *in vivo* studies induced diseases, such as colitis. Furthermore, many studies are performed with peptides that are not from food sources such as plant peptides, or even synthetic peptides. Moreover, few studies have evaluated intestinal health parameters different than the microbiota, such as histology, SCFA production, intestinal barrier (tight junctions) and brush border transporters (functionality). The vast majority only assess the intestinal microbiota, and intestinal health is more than just the microbiota composition. In addition, the composition of the microbiota is just one way of evaluating changes in the microbiome, the evaluation of SCFA, for example, is also a way of evaluating the functionality of the microbiota.

Only two studies included in this systematic review evaluated different times of intervention and found that some of the markers were produced only after a few days of peptide ingestion. Therefore, we can believe that the intervention time is one of the factors that interfere with the performance of peptides on intestinal health, but also other important factors such as the characteristics of the peptides (amino acid sequence, hydrophobicity, molecular weight, charge) and dosage. All studies included in the review were conducted with young adult rats, all weaned. The age of the animals may be a determining factor for the metabolism of these peptides, but we believe that since they were administered/ingested in the form of peptides and not proteins, perhaps the absorptive state of the intestines of these animals interferes more in the absorption than the age itself.

The risk of bias was assessed according to the SYRCLE RoB tool. The absence of some baseline characteristics and sequence generation probably did not influence the main conclusion of this review, as these characteristics were not comparable across studies. The risk of bias related to performance, detection and outcome may have influenced the results in each study, however, these biases may not represent a major impact on the main conclusions, considering

the methodological rigor that this review followed. The number of studies included in this review may be a limitation to directly demonstrate how food derived bioactive peptides can influence in intestinal health and inflammatory markers. Further, no animal model provides physiological responses that can be extrapolated to the humans. In *in vivo* studies in general, animals consume a specific diet in a controlled environment and specific study design, which is different from humans. The results found in this review serve as a guide for the development of more *in vivo* studies with food derived bioactive peptides and future clinical trials to investigate the effects on intestinal health and inflammatory parameters. Moreover, the identification of food derived bioactive peptides and potentially beneficial peptide sequences, mainly produced by enzymatic hydrolysis, has served to design and produce new synthetic anti-inflammatory peptides (Rivera-Jiménez *et al.*, 2022). In this sense, future research on bioactive peptides should be directed towards advancing knowledge about results that have been little evaluated but are promising, such as the effects of bioactive peptides on intestinal barrier and even on other parameters of intestinal health such as the expression of enzyme markers of intestinal functionality, morphology, and IgA production. We also suggest that research performed with animals follows the SYRCLE protocol to avoid a lack of information in the studies. More studies are still needed to try to answer questions such as: if there are differences between the performance of peptides of animal and plant origin, whether the method of extraction can alter the performance of bioactive peptides and how are the gastrointestinal interactions with the food matrix of the peptides. Better elucidating these questions is an important step before indicating the use of bioactive peptides as potential agents in the prevention and treatment of chronic inflammatory diseases.

5. Conclusion

The evidence from the reviewed *in vivo* studies demonstrates that the intake of bioactive peptides derived from food can potentially decrease the inflammation via reduced gene expression of TNF-α and can improve intestinal health, not only by modulating inflammation, but also by the increase of IgA, immunoglobulin of mucosal defense, and the intestinal morphology. These intestinal changes, in association, may be the mechanisms by which the bioactive peptides intake exert beneficial effects on intestinal health. The intake ranged from 2 days to 12 weeks, and since day 7 it was already possible to notice changes in IgA. The data found in this review are heterogeneous, since different animal models, methodologies and large ranges of time and dose of peptides were observed, so it is not adequate to establish a specific dose and a specific time to achieve the effects but could be used as guidelines for future

researchers. However, more *in vivo* studies are still necessary to better elucidate the anti-inflammatory activity and mechanisms by which peptides regulate gut health.

Registration and Protocol

This systematic review was realized according to the protocol: Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement 2020 (Page *et al.*, 2021). The review is registered in the PROSPERO under the number CRD42023416680 (Centre for Reviews and Dissemination, University of York).

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Conflicts of Interest:

The authors declare no conflict of interest.

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9.5 Paper 5: Effects of Intra-Amniotic Administration of the Hydrolyzed Protein of Chia (*Salvia hispanica* L.) and *Lacticaseibacillus paracasei* on Intestinal Functionality, Morphology, and Bacterial Populations, *In vivo* (*Gallus gallus*)

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Effects of Intra-Amniotic Administration of the Hydrolyzed Protein of Chia (*Salvia hispanica* L.) and *Lacticaseibacillus paracasei* on Intestinal Functionality, Morphology, and Bacterial Populations, In Vivo (*Gallus gallus*)

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Abstract: As a protein source, chia contains high concentrations of bioactive peptides. Probiotics support a healthy digestive tract and immune system. Our study evaluated the effects of the intra-amniotic administration of the hydrolyzed chia protein and the probiotic *Lacticaseibacillus paracasei* on intestinal bacterial populations, the intestinal barrier, the inflammatory response, and brush border membrane functionality *in ovo* (*Gallus gallus*). Fertile broiler (*Gallus gallus*) eggs ($n = 9$/group) were divided into 5 groups: (NI) non-injected; (H_2O) 18 MΩ H_2O; (CP) 10 mg/mL hydrolyzed chia protein; (CPP) 10 mg/mL hydrolyzed chia protein + 10^6 colony-forming unit (CFU) *L. paracasei*; (P) 10^6 CFU *L. paracasei*. The intra-amniotic administration was performed on day 17 of incubation. At hatching (day 21), the animals were euthanized, and the duodenum and cecum content were collected. The probiotic downregulated the gene expression of NF-κβ, increased *Lactobacillus* and *E. coli*, and reduced *Clostridium* populations. The hydrolyzed chia protein downregulated the gene expression of TNF-α, increased OCLN, MUC2, and aminopeptidase, reduced *Bifidobacterium*, and increased *Lactobacillus*. The three experimental groups improved in terms of intestinal morphology. The current results suggest that the intra-amniotic administration of the hydrolyzed chia protein or a probiotic promoted positive changes in terms of the intestinal inflammation, barrier, and morphology, improving intestinal health.

Keywords: gut health; microbiota; intestinal barrier; inflammation; chia seed; bioactive peptides; in vivo; probiotic

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1. Introduction

Chia (*Salvia hispanica* L.) is considered a potentially bioactive food source, since it has multiple demonstrated health benefits, as decrease in adiposity, modulation of glucose metabolism [1], hypoglycemic effect, reduction of hepatic fat deposition [2], improved lipid profile [2–5], reduction of inflammatory processes [4–6], antioxidant protection [4,7] and reduced fat content of the heart [7]. Regarding intestinal health, chia seed showed to improve

intestinal brush border membrane and favored its functionality [2,8–10], increased production of short chain fatty acids and increased the richness of microbiota [9,10].

Chia seed has high concentrations of lipids (30.17 g.100g-1), proteins (19.72g.100g-1), total dietary fiber (37.18 g.100g-1) and bioactive compounds as phenolic compounds, tocopherols and tocotrienols [11]. As a protein source, hydrolyzed protein of chia seed contains high concentration of bioactive peptides with promising composition and sequence. The amino acids sequences of chia seed proteins have been identified, these proteins were associated with hypoglycemic and hypotensive activity, antioxidant effect and glucose uptake stimulating peptide [12]. Further, the hydrolyzed protein of chia demonstrated these antioxidant, antihypertensive and hypoglycemic properties *in vitro* [13], and also improved biochemical profile, reduced the TNF-α expression, reduced the production of activated NF-κβ and affected adipogenesis, with antilipidemic and antiadipogenic action, *in vivo* [14]. Bioactive peptides have diverse functions, including modulating intestinal homeostasis, affecting barrier function, villus surface area, mucosal immune responses, inflammation, and the gut microbiota [15–18]. Studies are still needed to understand the effects of the intestinal environment on the bioavailability of bioactive peptides, its interaction with other compounds, and fundamental questions regarding the effectiveness of hydrolyzed protein as a source of bioactive peptides on intestinal health and their interactions between intestinal barrier function, microbiota and immune system [19].

Probiotics are live microorganisms that, when administered in adequate amounts, confer benefits to the health of the host. Using probiotics supports a healthy digestive tract and a healthy immune system, so the overall benefit of probiotics on the gut microbiota derives from creating a favorable gut environment [20–22]. Probiotic food products represent a market trend, associated with the increasing consumer's awareness regarding the link between diet and well-being, and especially gut health. Strains pertaining to several Lactobacilli were shown to exhibit probiotic properties, particularly within the *Lacticaseibacillus* spp [23–25]. Lactobacilli are considered autochthonous residents in the gastrointestinal tract of animals as chicken, rodents and humans [24,25]. They may promote host intestinal health and immune function in different ways, such as by strengthening the epithelial barrier, competitively rejecting pathogenic microorganisms, producing antimicrobial substances, and interacting with immune cells by stimulating pattern recognition receptors [24]. Studies showed that *Lacticaseibacillus* paracasei upregulated the expression of tight junction proteins, downregulated the production of pro-inflammatory cytokines and altered the structure of the intestinal microbiota [26].

The *Gallus gallus* is an animal model that presents a complex gut microbiota with high homology of gene sequences, at the phylum level, to humans [27]. The intra-amniotic feeding model is widely used as an *in vivo* method to assess bioactive compounds with potential nutritional, specially prebiotic, effects. The intra-amniotic administration is conducted on day 17 of embryonic development, prior to the embryo's oral consumption of the amniotic fluid [28]. The intra-amniotic administration of proteins can stimulate the maturation and functionality of the small intestine by promoting cell proliferation and differentiation, and expansion of the absorptive surface area [29]. Intra-amniotic administration of duck egg white peptides was able to increase the villus surface area and goblet cells diameter by promoting the proliferation of enterocytes, promotion of beneficial bacterial populations and limitation of potentially pathogenic bacterial populations, and promote and contribute to calcium uptake [15]. The use of probiotics in poultry nutrition has shown several benefits such as improvement in the microbiological homeostasis of the intestine, immune response and growth, and the variation in these benefits can be given by differences, for example, in the strains of probiotics and in the dose of probiotics [30]. Intra-amniotic administration of a probiotic can upregulate the mRNA expression of intestinal function-related genes and nutrient transporter-related genes [31].

To date, no studies have explored the combined effects of the intra-amniotic application of hydrolyzed protein of chia and the probiotic L. paracasei. Then, the objective of this study was to assess the effects of intra-amniotic administration of the hydrolyzed protein of chia and the probiotic L. paracasei on intestinal bacterial populations, intestinal barrier, inflammatory response, and brush border membrane (BBM) functionality, *in vivo*. As bioactive peptides might have functions that affect intestinal homeostasis, in addition to probiotics that were demonstrated to have a supporting role in the digestive tract health and function, we therefore hypothesized that the hydrolyzed protein of chia, combined with probiotic will increase the abundance of beneficial bacterial populations. This effect will further improve the intestinal BBM functionality, via regulation of gene expression of key proteins that are required for tight junction development and inflammatory response.

2. Materials and Methods

2.1. Sample material

2.1.1. Hydrolyzed protein of chia

Chia seeds grown in Rio Grande do Sul/Brazil were prepared according to Orona-Tamayo (2015) [32], the total digested protein was produced and analyzed in a Laboratory at the University of Illinois, USA as detailed by Grancieri (2019) [13]. Briefly, for mucilage formation, the seeds were immersed for 1h in distilled water (1g/20mL), frozen overnight (-80°C) and freeze-dried (Labconco Freeze Dryer 4.5; Kansas, MO, USA). The mucilage was removed from the seeds using a sieve (500 µm per 35 mesh). Using a coffee grinder (Mr. Coffee) the mucilage-free seeds were ground, sieved (500 µm per 35 mesh), and degreased using hexane (1g/10mL) at 60°C for 2h under constant stirring. The mixture was centrifuged (6000 × g, 15 min, 4°C) and the resulted flour was left overnight under a hood and stored at 4°C until use. Then, deionized water was added to the mucilage and fat-free chia flour (1g/20 mL), the pH was adjusted to 8.0 and placed under constant stirring (35°C per 1h). The mixture was centrifuged (5000 × g; 15 min; 25°C) and the supernatant collected, freeze-dried, and stored at −20°C. This fraction is referred as "total proteins".

For the digestion of the extracted protein, a previously described technique was used [33] in which gastrointestinal digestion is simulated. Briefly, the extracted protein was suspended in deionized water (1g/20mL), the pH was adjusted to 2.0, pepsin added at a concentration of 1:20 (enzyme: protein) and kept under stirring for 2h at 37°C. Afterwards, the pH was adjusted to 7.5, pancreatin 1:20 (enzyme: protein) was added and then the digestion was carried out as above. At the end, the simulated digestion was stopped by placing the mixture in a water bath (75°C, 20 min) and then centrifuged twice at 20.000g (4°C, 15 min). The supernatant was collected (total protein digested) and dialyzed using a 100-500Da molecular weight exclusion membrane (Spectra/Por®, Biotech CE Membrane), lyophilized, and stored at -20°C until use.

2.1.2. Probiotic

Freeze-dried *L. paracasei* (strain TRA038563) was provided by the Brazilian Agriculture Research Corporation (EMBR*Ap*A).

2.2. The intra-amniotic administration

Cornish-cross fertile broiler eggs (n = 50), obtained from a commercial hatchery (Moyer's chicks, Quakertown, PA, USA), were incubated under optimal conditions (37 ± 2º C and 89.6 ± 2% humidity) at the Cornell University Animal Science poultry farm incubator. All animal protocols were approved by Cornell University Institutional Animal Care and Use Committee (protocol code: 2020–0077).

The hydrolyzed protein of chia in powder form were diluted in 18 MΩ H2O. The osmolarity of the solution was tested to determine the concentration necessary to maintain an osmolarity value (Osm) of less than 320 Osm, and ensure that the chicken embryos would not be dehydrated upon injection of the solution. The intra-amniotic administration followed the methodology previously described [34–36].

At 17 days of embryonic incubation, eggs were candled to discard those that presented as infertile, cracked, contaminated, or early dead embryos. Eggs containing viable embryos were weighed and randomly allocated into 5 groups with a similar weight frequency distribution (n = 10/group). Briefly, all eggs were disinfected with 70% ethanol. Each group was then injected with the specified solution (1 mL per egg) with a 19mm gauge needle, vertically inserted, into the amniotic fluid, which was identified by candling.

The 5 groups were assigned as follows: (NI) non-injected; (H_2O) 18 MΩ H2O; (CP) 10mg/mL (1%) hydrolyzed protein of chia; (CPP) 10mg/mL (1%) hydrolyzed protein of chia + 106 colony-forming unit (CFU) *Lacticaseibacillus paracasei* (800µL of hydrolyzed protein of chia + 200µL of probiotic/egg); (P) 106 CFU *Lacticaseibacillus paracasei*.

After the injections, the injection holes were sealed with cellophane tape and the eggs placed in hatching baskets. Immediately after hatch (21 days) and from each treatment group, chicks were weighed and then euthanized by CO_2 exposure. Their duodenum, cecum and cecum content were collected.

2.3. Extraction of the total RNA from the duodenum tissue samples

According to the manufacture's protocol (Rneasy Mini Kit, Qiagen Inc., Valencia, CA, USA) the total RNA was extracted from 30mg of the duodenum (n = 5 animals/group) [35–37]. The total RNA was eluted in 50 µL of Rnase-free water. All steps were carried out under Rnase-free conditions. The RNA was quantified by absorbance at 260/280 and the integrity of the 18S ribosomal RNAs was verified by 1.5% agarose gel electrophoresis, followed by ethidium bromide staining. The samples were stored at −80 ºC until analysis.

2.4. Real-time polymerase chain reaction (RT-PCR) and prime design

The cDNA was created using a total of 20 µL reverse transcriptase (RT) reaction in a BioRad C1000 touch thermocycler using the Improm-II Reverse Transcriptase Kit (Catalog #A1250; Promega, Madison, WI, USA). The concentration of cDNA obtained was determined by measuring the absorbance at 260 nm and 280 nm using an extinction coefficient of 33 (for

single-stranded DNA). The gene expression of the duodenum was determined by real-time polymerase chain reaction (RT-PCR).

The primers used in the real-time qPCR (Table 1) were designed based on gene sequences from the GenBank database using Real-Time Primer Design Tool software (IDT DNA, Coralvilla, IA, USA) [36,38]. The specificity of the primers was tested by performing a BLAST search against the genomic National Center for Biotechnology Information (NCBI) database. The *Gallus gallus* primer 18S rRNA was designed as a reference gene.

Table 1. Sequence of experimental primers used in this study.

Gene	**Oligonucleotides (5'- 3')**	
	Forward Primer (5′-3′)	**Reverse Primer (5′-3′)**
BBM functionality		
AP	CGTCAGCCAGTTTGACTATGTA	CTCTCAAAGAAGCTGAGGATGG
SI	CCAGCAATGCCAGCATATTG	CGGTTTCTCCTTACCACTTCTT
18S rRNA	GCAAGACGAACTAAAGCGAAAG	TCGGAACTACGACGGTATCT
Inflammation		
TNF-α	GACAGCCTATGCCAACAAGTA	TTACAGGAAGGGCAACTCATC
NF-κβ1	CACAGCTGGAGGGAAGTAAAT	TTGAGTAAGGAAGTGAGGTTGAG
Intestinal Barrier		
MUC2	CCTGCTGCAAGGAAGTAGAA	GGAAGATCAGAGTGGTGCATAG
OCLN	GTCTGTGGGTTCCTCATCGT	GTTCTTCACCCACTCCTCCA

BBM: brush border membrane; AP: aminopeptidase; SI: sucrose isomaltase; TNF-α: tumor necrosis factor-alpha; NF-κβ1: nuclear factor kappa beta; MUC2: mucin 2; OCLN: occludin.

2.5. Real-time qPCR design

All procedures were performed as previously described [8,34,36,39]. Briefly, cDNA was used for each 10 μL reaction containing 2 × BioRad SSO Advanced Universal SYBR Green Supermix (Hercules, CA, USA). For each reaction, 8 μL of the master mix and 2 μL cDNA were pipetted into a 96-well plate, and for the standard curve, seven points were evaluated in duplicate. A "no template" control of nuclease-free water was included to exclude DNA contamination in the PCR mix. Table 1 shows the primers used in this study The double-

stranded DNA was amplified in the Bio-Rad CFX96 Touch (Hercules, CA, USA) using the following PCR conditions: initial denaturing at 95 ºC for 30 s, 40 cycles of denaturing at 95 ºC for 15 s, various annealing temperatures according to Integrated DNA Technologies (IDT) for 30 s and elongating at 60 ºC for 30 s.

The data on the expression levels of the genes were obtained as Cp values based on the "second derivative maximum" (automated method) as computed by Bio-Rad CFX Maestro 1.1 (Version 4.1.2433.1219, Hercules, CA, USA). The assays were quantified by including a standard curve in the real-time qPCR analysis, and a standard curve with four points was prepared by a 1:10 dilution (duplicates). The software produced a Cp vs. log 10 concentrations graph, and the efficiencies were calculated as 10 (1/slope). The specificity of the amplified real-time RT-PCR products was verified by melting curve analysis (60–95 ºC) after 40 cycles, resulting in several different specific products with specific melting temperatures.

2.6. Collection of microbial samples and intestinal contents DNA extraction

The cecum was sterilely removed and treated as described previously [15,34]. Briefly, the cecum contents were homogenized using a vortex and glass beads. Debris were removed and the supernatant was collected and centrifuged. The pellet was washed and stored at −20 ºC until DNA extraction. Then the pellet was resuspended in ethylenediaminetetraacetic acid (EDTA) and treated with lysozyme (Sigma Aldrich CO., St. Louis, MO, USA; final concentration of 10 mg/mL). A Wizard Genomic DNA purification kit (Promega Corp., Madison, WI, USA) was used to isolate the bacterial genomic DNA.

2.7. Primer's design and PCR amplification of bacterial 16S rDNA

Primers for *Bifidobacterium*, *Lactobacillus*, *Escherichia coli* and *Clostridium* were used [40]. The universal primers were designed with the invariant region in the 16S rRNA of bacteria and were used as internal standards. The relative abundance of each examined bacterium was evaluated as previously described [38,41]. Briefly, the PCR products were quantified using 2% agarose gel, stained with ethidium bromide. All products were expressed relative to the content of the universal 16S rRNA primer product and proportions of each examined bacterial product.

2.8. Morphological examination of duodenal tissue

Intestinal morphology was performed as previously described [8,36]. Briefly, the duodenum samples were fixed in fresh 4% (v/v) buffered formaldehyde, numerous sections were cut and placed on glass slides. The sections were deparaffinized in xylene and rehydrated

in ethanol. After, the slides were stained with Alcian Blue/Periodic acid-Schiff and examined by light microscopy (CellSens Standard software, Olympus, Waltham, MA, USA). The following morphometric measurements were evaluated: villus height (µM), villus surface area (µM), depth of crypts (µM), Paneth cell number and diameter (µM), goblet cell number and goblet cell diameter (µM) in the villi and crypts, and goblet cell type (acidic, neutral and mixed). Four segments for each biological sample (n = 3/treatment group) were performed and ten randomly selected villi and crypts were analyzed per segment (40 replicates per biological sample). For the Alcian Blue and Periodic acid-Schiff stain, the segments were only counted for the type of goblet cells (acid, neutral or mixed) in the villi epithelium and in the crypts. The goblet cells were enumerated at 10 villi or crypts/sample, and the means were calculated for statistical analysis. Villus surface area was obtained by the equation:

Villus surface area=2 VW/2 x VL

Where VW = villus width average of three measurements, and VL = villus length.

A representative image of the histological cross-section of the duodenum from each experimental group indicates the villus surface area measurements.

2.9. Statistical analysis

Experimental treatments for the intra-amniotic administration procedure were arranged in a completely randomized design. All the results were expressed as means ± standard error deviation (SED) from seven to nine biological samples per treatment group (according to hatching). Differences were considered significant when $p < 0.05$.

The Shapiro–Wilk normality test was used to evaluated values for normal distribution and variance homogeneity. Normally distributed results were analyzed using a one-way Analysis of Variance (ANOVA). For significant "p-value," the post hoc Duncan test was used for those with a normal distribution. The means without normal distribution were analyzed using Kruskal–Wallis and a post-hoc Dunn's test. The statistical analyses were performed using the statistical software IBM SPSS Statistics®, version 25.

The correlation between biomarkers of intestinal health, bacterial population, and histological parameters was analyzed by Spearman's rank correlation coefficient. GraphPad Prism® version 9.0 software packages (GraphPad Software Inc., San Diego, CA, USA) were used for graphics.

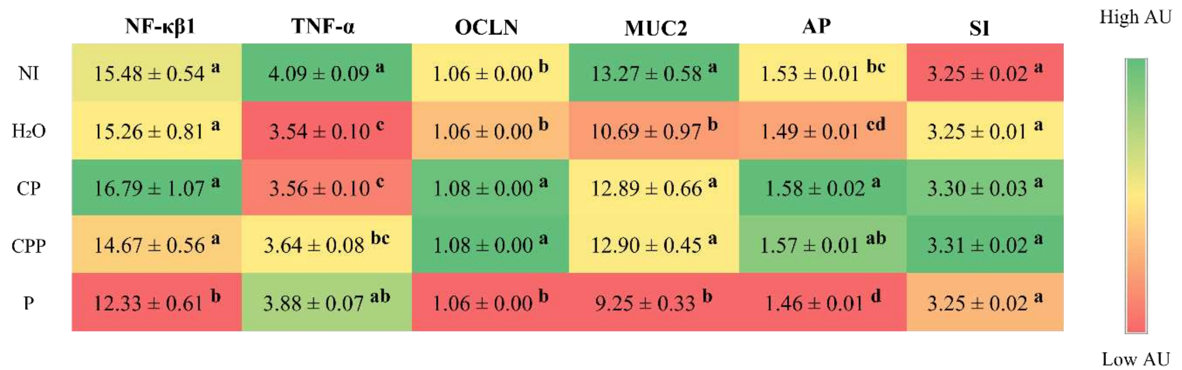
3. Results

3.1. Body weight

The body weight was similar among all the groups compared according to one way ANOVA followed by post hoc Duncan test. Non-injected (35.00 ± 0.82), 18 MΩ H_2O (35.13 ± 1.09), hydrolyzed protein of chia (36.56 ± 0.65), hydrolyzed protein of chia + *Lacticaseibacillus paracasei* (34.38 ± 1.28) and probiotic (*Lacticaseibacillus paracasei*) (34.78 ± 0.95).

3.2 Effect of chia protein and/or probiotic on gene expression of intestinal inflammation, intestinal barrier proteins and brush border membrane functional proteins

In the duodenum, the expression of tumor necrosis factor-alpha (TNF-α) decreased in the groups that received the hydrolyzed protein of chia and hydrolyzed protein of chia + probiotic compared to the non-injected group, but was similar to the group injected with H_2O. Further, the probiotic group was not able to reduce the expression of TNF-α when compared to both control groups (NI and H_2O), however it decreased expression of nuclear factor kappa beta (NF-κβ1) relative to all other groups. Regarding intestinal barrier proteins, the groups that received the hydrolyzed protein of chia or the hydrolyzed protein of chia + probiotic showed a higher expression of occluding (OCLN) compared to both control groups (NI and H_2O) and the probiotic group did not show an increase in this parameter. The same occurred for mucin-2 (MUC2), the hydrolyzed protein of chia and hydrolyzed protein of chia + probiotic groups showed an increase in MUC2 expression, but only in relation to the group injected with H_2O, and the probiotic group did not change this parameter. When we evaluated the expression of intestinal functionality proteins, we observed that the hydrolyzed protein of chia group had a higher AP expression in relation to the control groups (NI and H_2O) and the hydrolyzed protein of chia + probiotic group had a higher AP expression in relation to the control group injected with H_2O, the probiotic group presented AP expression similar to the group injected with H_2O, and lower than the other groups. In relation to the SI expression, there was no difference between any of the experimental groups (Figure 1).

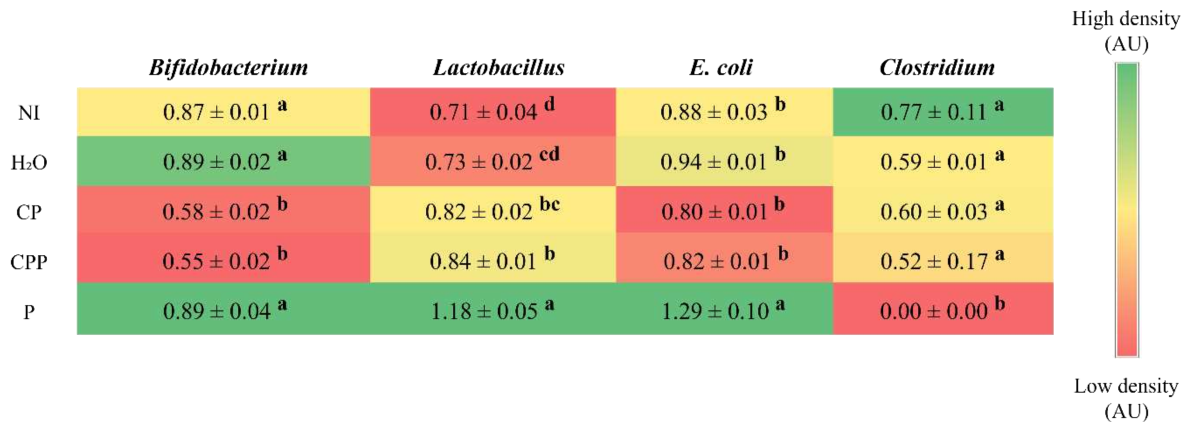


	NF-κβ1	TNF-α	OCLN	MUC2	AP	SI
NI	15.48 ± 0.54 a	4.09 ± 0.09 a	1.06 ± 0.00 b	13.27 ± 0.58 a	1.53 ± 0.01 bc	3.25 ± 0.02 a
H_2O	15.26 ± 0.81 a	3.54 ± 0.10 c	1.06 ± 0.00 b	10.69 ± 0.97 b	1.49 ± 0.01 cd	3.25 ± 0.01 a
CP	16.79 ± 1.07 a	3.56 ± 0.10 c	1.08 ± 0.00 a	12.89 ± 0.66 a	1.58 ± 0.02 a	3.30 ± 0.03 a
CPP	14.67 ± 0.56 a	3.64 ± 0.08 bc	1.08 ± 0.00 a	12.90 ± 0.45 a	1.57 ± 0.01 ab	3.31 ± 0.02 a
P	12.33 ± 0.61 b	3.88 ± 0.07 ab	1.06 ± 0.00 b	9.25 ± 0.33 b	1.46 ± 0.01 d	3.25 ± 0.02 a

Figure 1. Effect of intra-amniotic administration of chia protein and/or probiotic on intestinal (duodenum) gene expression. NI: non-injected; H_2O: 18 MΩ H_2O; CP: hydrolyzed protein of chia; CPP: hydrolyzed protein of chia + *Lacticaseibacillus* paracasei; P: probiotic (*Lacticaseibacillus* paracasei); NF-κβ1: nuclear factor kappa beta; TNF-α: tumor necrosis factor-alpha; OCLN: occludin; MUC2: mucin 2; *Ap*: aminopeptidase; *Si*: sucrose isomaltase; AU: arbitrary unit. Values are means ± SEM, n = 5/group. Per gene (in the same column), red depicts lower gene expression levels, and green depicts higher gene expression levels. a-d Per gene (in the same column), treatment group means not indicated by the same letter are significantly different ($p < 0.05$) according to one way ANOVA followed by post hoc Duncan test.

3.3. Effect of chia protein and/or probiotic on bacterial population in cecum contents

The intra-amniotic administration of hydrolyzed protein of chia and hydrolyzed protein of chia + probiotic reduced the abundance of *Bifidobacterium* compared to both control groups (NI and H_2O) and P group. The group probiotic group showed similar abundance of *Bifidobacterium* to the NI and H_2O groups. The hydrolyzed protein of chia group showed similar abundance of *Lactobacillus* compared to H_2O injection control group and higher than the NI control group. The abundance of *Lactobacillus* in the hydrolyzed protein of chia + probiotic group was similar to the CP and higher than both control groups (NI and H_2O), and the probiotic group showed a higher abundance of *Lactobacillus* compared to all other groups. Furthermore, the probiotic group showed a higher abundance of *E. coli* compared to all other groups, and all other groups presented similar abundance of *E. coli*. The abundance of *Clostridium* was similar among the NI, H_2O, hydrolyzed protein of chia and hydrolyzed protein of chia + probiotic groups and reduced in the probiotic group (Figure 2).



	Bifidobacterium	*Lactobacillus*	*E. coli*	*Clostridium*
NI	0.87 ± 0.01^{a}	0.71 ± 0.04^{d}	0.88 ± 0.03^{b}	0.77 ± 0.11^{a}
H_2O	0.89 ± 0.02^{a}	0.73 ± 0.02^{cd}	0.94 ± 0.01^{b}	0.59 ± 0.01^{a}
CP	0.58 ± 0.02^{b}	0.82 ± 0.02^{bc}	0.80 ± 0.01^{b}	0.60 ± 0.03^{a}
CPP	0.55 ± 0.02^{b}	0.84 ± 0.01^{b}	0.82 ± 0.01^{b}	0.52 ± 0.17^{a}
P	0.89 ± 0.04^{a}	1.18 ± 0.05^{a}	1.29 ± 0.10^{a}	0.00 ± 0.00^{b}

Figure 2. Effect of intra-amniotic administration of chia protein and/or probiotic on genera- and species-level bacterial population from cecal content. NI: non-injected; H_2O: 18 MΩ H_2O; CP: hydrolyzed protein of chia; CPP: hydrolyzed protein of chia + *Lacticaseibacillus* paracasei; P: probiotic (*Lacticaseibacillus* paracasei); AU: arbitrary unit. Values are means ± SEM, n = 5/group. Per bacterial category (in the same column), red depicts lower gene expression levels, and green depicts higher gene expression levels. a-d Per bacterial category (in the same column), treatment group means not indicated by the same letter are significantly different ($p < 0.05$) according to one way ANOVA followed by post hoc Duncan test.

3.4. Effect of chia protein and/or probiotic on duodenal morphological parameters

The villi height and villus surface area were increased in all treatment groups (CP, CPP and P). The hydrolyzed protein of chia (CP) group showed the highest villi height and villus surface area than all other groups. The hydrolyzed protein of chia + probiotic (CPP) group showed higher villi height and villus surface area than both of control groups (NI and H_2O), and the probiotic (P) group presented higher villi height than both control groups (NI and H_2O) and CPP group, the villus surface area was higher than both control groups (NI and H_2O) and similar to CPP and CP groups (Figure 3 and 4).

The depth of the crypts was increased in all treatment groups (CP, CPP and P) compared to both of control groups (NI and H_2O) and was similar among the experimental groups (Figure 3).

The treatment groups (CP, CPP and P) had a higher number of Paneth cells compared to the control groups (NI and H_2O), and the probiotic group had a higher number of Paneth cells compared CP group. The diameter of Paneth was similar among all the groups, except for the comparison between CP and P groups, the administration of probiotic increased the diameter of Paneth cells compared to group injected with hydrolyzed protein of chia (Figure 3).

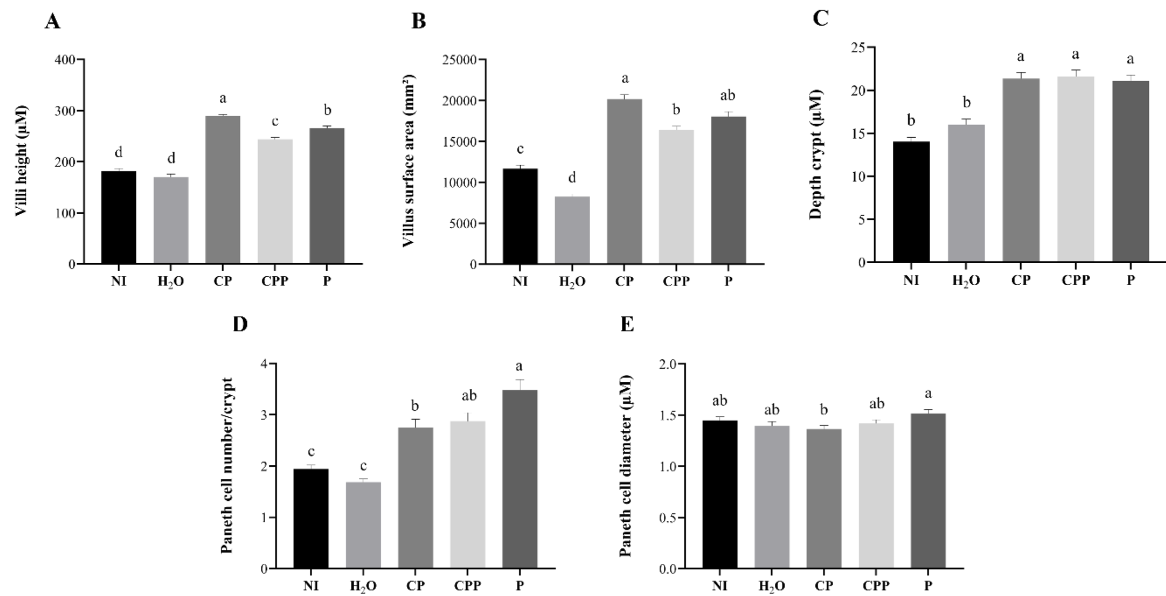


Figure 3. Effect of intra-amniotic administration of chia protein and/or probiotic on duodenal morphological parameters. A) Villi height (µM); B) Villus surface area (mm²); C) Crypt depth (µM); D) Paneth cell number/crypt; E) Paneth cell diameter (µM); NI: non-injected; H_2O: 18 MΩ H_2O; CP: hydrolyzed protein of chia; CPP: hydrolyzed protein of chia + *Lacticaseibacillus* paracasei; P: probiotic (*Lacticaseibacillus* paracasei); Values are means ± SEM, n = 3 animals/group, 4 sections, 10 measurements. Treatment group means for specific variables followed by the same letter are not significantly different ($p > 0.05$) by Kruskal–Wallis and a post-hoc Dunn's test. a-d Treatment group means not indicated by the same letter are significantly different ($p < 0.05$) by Kruskal–Wallis and a post-hoc Dunn's test.

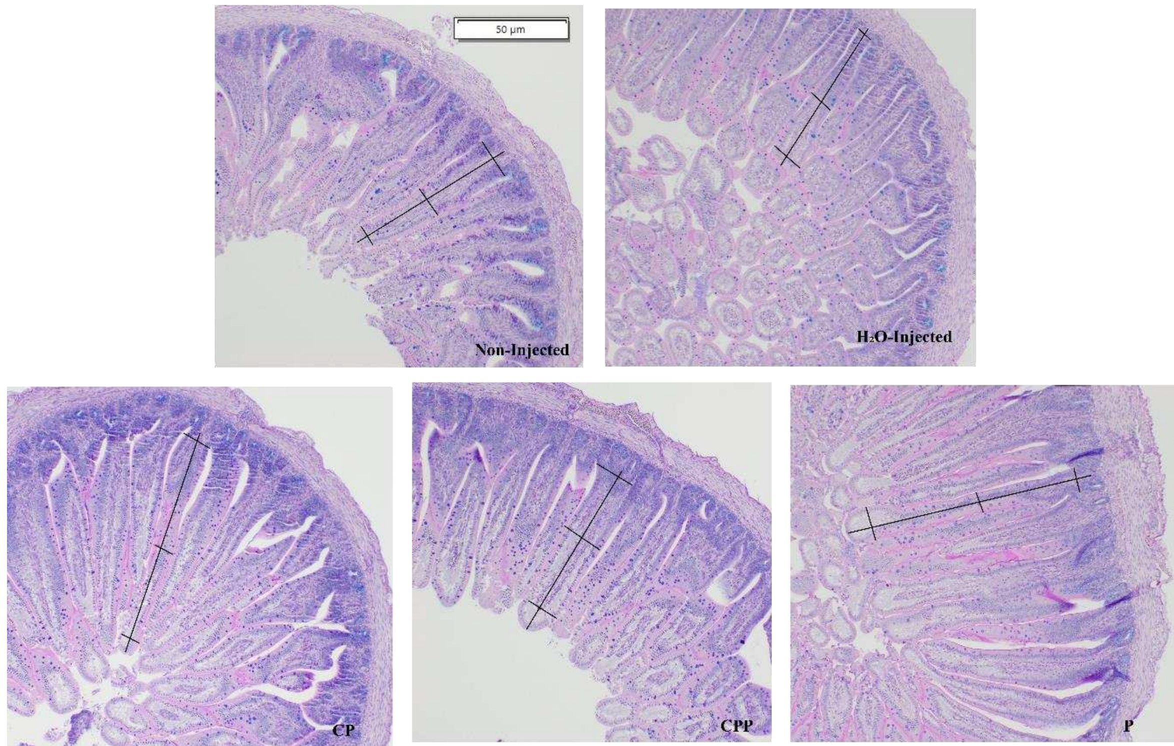


Figure 4. Representation of duodenal cross section morphology, for each treatment group. Villi surface area, depicted in black was obtained by the equation: Villus surface area=2 VW/2 X VL. Where VW = villus width average of three measurements, and VL = villus length. NI: non-injected; H_2O: 18 MΩ H_2O; CP: hydrolyzed protein of chia; CPP: hydrolyzed protein of chia + *Lacticaseibacillus paracasei*; P: probiotic (*Lacticaseibacillus paracasei*).

In the villi, the goblet cell number were not different among the treatment groups (CP, CPP and P), these groups showed number of goblet cells similar to NI group, and lower than H_2O. However, the intra-amniotic administration of hydrolyzed protein of chia, CPP and P increased the diameter of the goblet cells, the CPP and P groups were similar and presented a higher diameter of the goblet cells compared both of control groups (NI and H_2O). CPP were similar to CP, and P was higher than CP. Similar results were founded regarding the diameter of the goblet cells of the crypts. Regarding the goblet cell numbers in the crypt, the groups H_2O, CP, CPP and P showed lower number of goblet cells than the NI group, and the group CPP was lower than CP and P (Table 3).

In the villi, the treatment groups (CP, CPP and P) reduced acidic and neutral goblet cells and increased the mixed goblet cell number. In the crypt, the treatment groups (CP, CPP and P) reduced neutral goblet cells, to the acidic goblet cells, CP and P were similar to both of controls, and CPP were similar to H_2O group, and to the mixed goblet cells, the P group was similar to both of controls and the CP and CPP groups were similar to H_2O group (Table 2).

Table 2. Effect of chia protein and/or probiotic on goblet cells.

	NI	H_2O	CP	CPP	P
Villi Goblet Cell Number	24.68 ± 0.74^{b}	38.38 ± 0.91^{a}	26.40 ± 0.72^{b}	27.46 ± 0.71^{b}	25.79 ± 0.83^{b}
Villi Goblet Cell Diameter (µM)	2.45 ± 0.06^{c}	2.20 ± 0.05^{d}	2.60 ± 0.06^{bc}	2.94 ± 0.10^{ab}	3.13 ± 0.08^{a}
Villi Goblet Cell Type Number					
Acidic	15.28 ± 0.71^{b}	26.71 ± 1.12^{a}	10.29 ± 0.59^{c}	8.49 ± 0.43^{c}	8.82 ± 0.52^{c}
Neutral	0.79 ± 0.13^{a}	0.10 ± 0.04^{b}	0.28 ± 0.09^{b}	0.43 ± 0.12^{b}	0.47 ± 0.12^{b}
Mixed	8.68 ± 0.57^{c}	11.57 ± 0.66^{a}	15.83 ± 0.66^{a}	18.54 ± 0.65^{a}	16.51 ± 0.73^{a}
Crypt Goblet Cell Number	12.67 ± 0.55^{a}	10.95 ± 0.62^{b}	10.03 ± 0.36^{b}	8.04 ± 0.38^{c}	10.18 ± 0.40^{b}
Crypt Goblet Cell Diameter (µM)	2.92 ± 0.05^{d}	3.13 ± 0.05^{cd}	3.20 ± 0.06^{bc}	3.40 ± 0.06^{ab}	3.62 ± 0.07^{a}
Crypt Goblet Cell Type Number					
Acidic	8.53 ± 0.43^{a}	7.88 ± 0.51^{ab}	7.63 ± 0.30^{a}	5.98 ± 0.30^{b}	7.14 ± 0.33^{ab}
Neutral	0.41 ± 0.06^{a}	0.50 ± 0.07^{a}	0.09 ± 0.03^{b}	0.08 ± 0.03^{b}	0.04 ± 0.02^{b}
Mixed	3.73 ± 0.27^{a}	2.58 ± 0.21^{bc}	2.33 ± 0.15^{bc}	1.98 ± 0.17^{c}	3.00 ± 0.17^{ab}

NI: non-injected; H_2O: 18 MΩ H_2O; CP: hydrolyzed protein of chia; CPP: hydrolyzed protein of chia + *Lacticaseibacillus* paracasei; P: probiotic (*Lacticaseibacillus* paracasei), Values are means ± SED, n = 3 animals/group, 4 sections, 10 measurements. a-d Treatment group means not indicated by the same letter are significantly different ($p < 0.05$) by Kruskal–Wallis and a post-hoc Dunn's test.

3.5. Correlation analysis

The Spearman correlation analysis was used to assess the relationship between the intestinal parameters investigated in this study, and significant correlations were observed. Positive correlations were observed between OCLN and MUC2, between AP and villi surface area. Further, negative correlations were observed between *E. coli* and OCLN, MUC2, *Ap*, villi surface area (Figure 5).

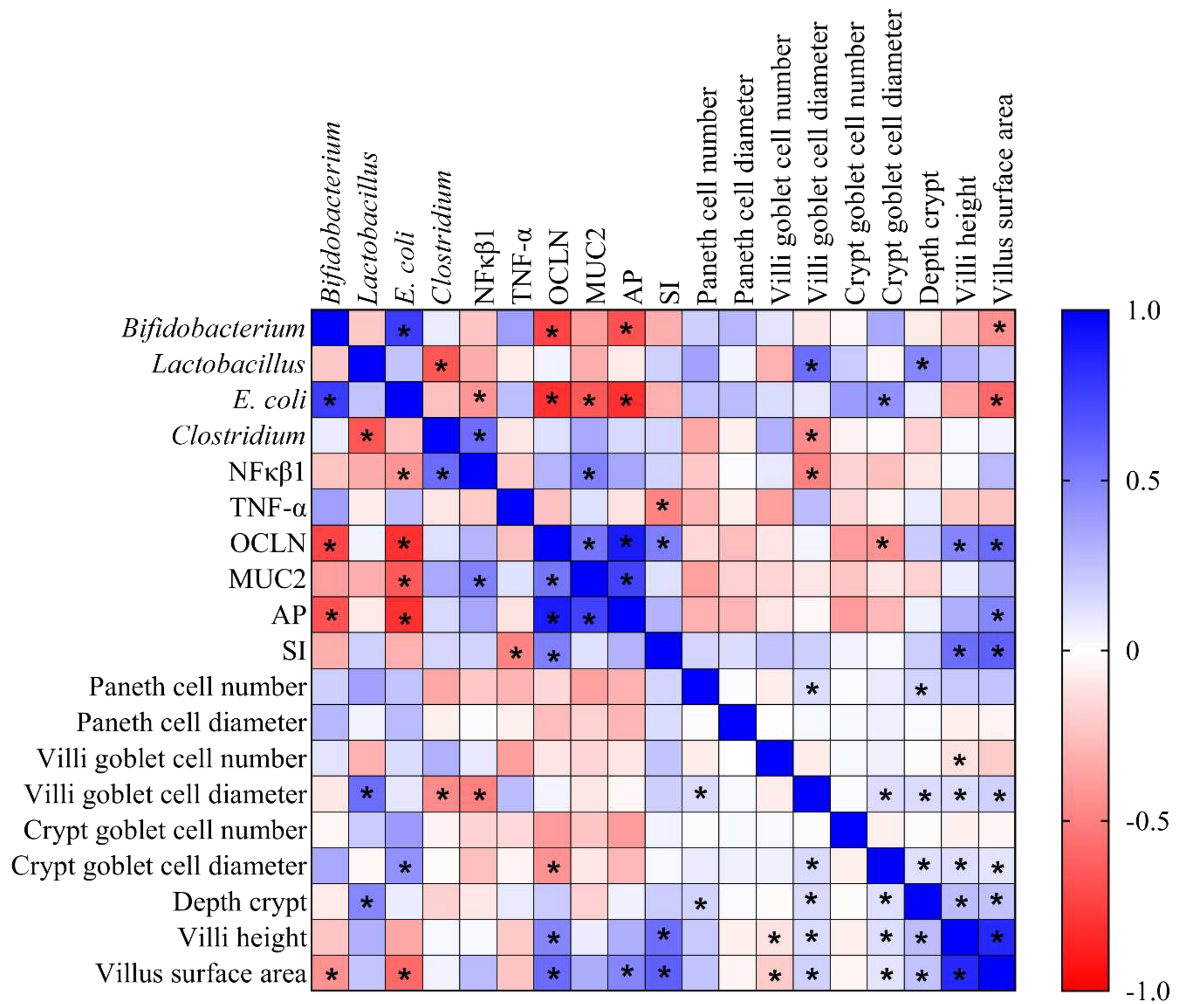


Figure 5. Heatmap of Spearman correlation analysis. NF-κβ1: nuclear factor kappa beta; TNF-α: tumor necrosis factor-alpha; OCLN: occludin; MUC2: mucin 2; *Ap*: aminopeptidase; *Si*: sucrose isomaltase. * Indicates statistically significant difference ($p < 0.05$).

4. Discussion

Chia is a seed with rich nutritional composition and a good source of bioactive peptides [11,12]. However, the potential effects of chia seed hydrolyzed proteins in combination with the probiotic *Lacticaseibacillus paracasei* on intestinal bacterial populations, intestinal barrier, inflammatory response, and BBM functionality *in vivo* have not been investigated. Our results demonstrated that the intra-amniotic administration of the probiotic downregulated NF-κβ1 gene expression, increased the cecal *Lactobacillus* and *E. coli* populations, decreased *Clostridium*, and increase the diameter of goblet cells (Figures 1 and 2 and table 2). Furthermore, hydrolyzed protein of chia downregulated TNF-α expression, the gene expression

of OCLN, MUC2 and AP were improved in the presence of hydrolyzed protein, alone or with the probiotic. Although the hydrolyzed protein of chia has decreased the number of goblet cells, the diameter was increased. Villi height, villi surface area, crypt depth and number of Paneth cells were increased in all treatment groups (CP, CPP and P) comparing to control groups (NI and H_2O).

In the present study, the probiotic administration downregulated NF-κβ1 gene expression comparing to both of control groups (NI and H_2O) and to other treatment groups (CP and CPP). Moreover, TNF-α expression decreased in the groups administered with the hydrolyzed protein of chia (Figure 1). NF-κβ1 is responsible for induce cytokine gene expression to control inflammatory and immune responses [42]. NF-κβ is translocated to the cytosol into the nucleus to initiate the pathway to drive the expression of target genes as TNF-α, IL1β, IL6 and IL10 [43]. The hydrolyzed protein of chia also demonstrated effects in reducing the secretion of TNF-α in an *in vitro* study and in the adipose tissue in an *in vivo* study [14,44]. These observations suggest that probiotic has an anti-inflammatory effect, as was previously reported [20,45], further and similarly, the hydrolyzed protein of chia might have similar effects, as demonstrated in the current study. Probiotic bacteria play a role in the regulation of immune response by stimulating immune cells through the modulation of intestinal microbiota and downregulating the inflammation [21,22]. Some of these bacteria might be useful to mitigate intestinal inflammation, specifically, *Lactobacillus* and *Bifidobacteria* that demonstrate antimicrobial effects by affecting both local and systemic immune response [20,45]. Peptides with hydrophobic amino acids have more ionizable groups that block free radicals and then, increase the antioxidant activity [46]. This activity was also observed in peptides with less than 20 amino acid residues per molecule, since small peptides have a better ability to cross the intestinal barrier and exert their biological effects [47]. Further, peptides with hydrophobicity $\leq$ 20 kcal mol−1 are more effective for penetrating the cell membrane and exercise effects on the molecule [48]. Most of the peptides found in our hydrolyzed protein of chia showed these characteristics, which may explain the benefits against inflammation [12,13]. The main storage protein fractions found in chia seed, are albumin, globulin, glutelin, and prolamin [12,13].

Intestinal inflammation is associated with an impaired barrier function, leading to increased intestinal permeability and bacterial translocation [43]. Here, both groups with hydrolyzed protein of chia (CP and CPP) showed increased gene expression of OCLN and MUC2 compared to H_2O injected control group. TNF-α is a pro-inflammatory cytokine that

plays a key role in the inflammatory cascade that causes increased intestinal epithelial permeability and then chronic intestinal inflammation [26,49,50]. Thus, bioactive peptides present at hydrolyzed protein of chia can reduce the disruption of the intestinal barrier, by reducing inflammatory factors such as TNF-α [51], as found in the current study.

The mucus layer acts as the first barrier on the surface of the gastrointestinal tract, it is an innate host defense mechanism [52]. Mucus is essential for regulating the homeostasis of the intestinal microbiota and preventing disease, by protecting the gastrointestinal barrier from pathogenic microorganisms, toxins or other irritants [53–55]. MUC2 is the main mucin produced specifically in goblet cells [52]. Under intestinal inflammatory conditions, with a reduction in goblet cells, the expression of MUC2 is reduced, the mucus loses its barrier function and exposes the mucous membrane to inflammatory substances, as pathogenic microorganisms, toxins, lipopolysaccharides [52,55]. We observed that hydrolyzed protein of chia alone or when combined with probiotic increased the gene expression of MUC2, probably due to the increased goblet cells diameter, improving the protection of the intestinal barrier. As the tight junction is interdependent with the mucus barrier, loss of one can reduce the other, and this interdependence may result from the regulation of signals that regulate both mucus and the tight junction [52]. Based on the correlation analysis (Figure 5), the gene expression of OCLN was positively correlated with the gene expression of MUC2. Then, with the increase in OCLN and MUC2 the intestinal barrier can protect the host against to the permeability of pathogenic components.

Aminopeptidase (AP) is an exopeptidase that cleaves amino acids from the N-terminus of peptides, located on the enterocyte's BBM [28]. The up regulation of the BBM and functional genes expressions reflects the intestinal development and digestive capabilities. Thus, it also affects the potential increased absorption of nutrients [28,56]. In the current study, AP gene expression was up regulated, probably due to the increase in villi height and villi surface area, which is accordance with our correlation analysis. This increase in villi surface area leads to more enterocytes and consequently higher functionality and absorption capacity [56]. This improvement in absorption capacity is important for the absorption of peptides that hold anti-inflammatory role. In addition, the CP, CPP and P administration keep the gene expression of sucrose isomaltase (*Si*), relative to the non-injected and H_2O injected groups (Figure 1).

The intra-amniotic administration of hydrolyzed protein of chia, hydrolyzed protein of chia + probiotic or just the probiotic (*L. paracasei*) were able to increase villi height, villi surface area and crypt depth, but the increases were higher when these compounds were

administered separately (Figure 3). The intestinal villi play an essential role in the digestion and absorption of nutrients, as the villi increase the internal surface area, and then the digestive and absorptive capacities of the BBM [8,28,56]. The crypts are comprised of continuously proliferating stem cells, which are responsible for the formation of the enterocytes, which have a key role due to their nutrients' absorptive ability [57]. Deeper crypts lead to an increase in the secretion of digestive enzymes [57]. Thus, the surface area of the villi and the crypts' depth are indictors of intestinal developmental and functional status, and then, an increase of these morphometric parameters by CP, CPP or P can improve the digestive and absorptive capabilities of the BBM [28], as demonstrated by the correlation analysis (Figure 5).

Paneth cells are found in the crypts, and they are secretory cells that produce antimicrobial peptides, proteins and other important components in host defense and immunity [58]. Dysfunction of Paneth cells can disrupt these functions, leading to an imbalance in the gastrointestinal tract. Decreased Paneth cells, followed by leakage of bacteria, are often seen in various diseases such as infectious diseases [58]. Here we found that the treatment groups (CP, CPP and P) could increase the number of Paneth cell, but not their diameter, therefore, we suggest that the intra-amniotic administration of hydrolyzed protein of chia, hydrolyzed protein of chia + probiotic or just the probiotic (*L. paracasei*) improved the Paneth cell development without affecting the size of the cells. This indicates that the antimicrobial peptides secreted by Paneth cells were not produced since there were no stimuli such as inflammation or pathogenic bacteria. Similar results were demonstrated post intra-amniotic administration of black corn soluble extract, as a source of phenolics [34].

Goblet cell produces mucin, the most important substance in the mucus layer, which forms a gel barrier against pathogenic bacteria [54]. Our results showed that the investigated probiotic increased the diameter of goblet cells, and the intra-amniotic administration of hydrolyzed protein of chia + probiotic decreased the number of goblet cells, but the diameter was increased. It is possible that the observed reduction in inflammatory biomarker (TNF-α), may lead to increased goblet cells diameter and therefore, increased MUC2 gene expression. Regarding the type of goblet cells, the treatment groups (CP, CPP and P) reduced the number of acidic goblet cells in the villi. An acidic pH in the intestine facilitates the growth of beneficial bacteria over detrimental bacteria [59]. Therefore, the reduction of acidic goblet cells might be associated with the increase in the *E. coli* population verified in the probiotic group [60].

The microbial analysis revealed that the probiotic *L. paracasei* increased the abundance of *Lactobacillus* and *E. coli* and reduced *Clostridium* populations in cecal content in comparison

to all the other groups (Figure 2). Furthermore, the intra-amniotic administration of hydrolyzed protein of chia and hydrolyzed protein of chia + probiotic (*L. paracasei*) reduced the abundance of *Bifidobacterium*, but increased *Lactobacillus*. *Lactobacillus* are considered probiotics due to their immunomodulatory and anti-inflammatory actions, competition with pathogens and by stimulating the release of antimicrobial substances, especially mucin which activates the MUC2 gene and prevents pathogens from adhering to the epithelial barrier [45]. In contrast, the *E. coli* genus may impair the epithelial barrier by disrupting tight junction proteins [53] as indicated by the negative correlation between *E. coli* and OCLN (Figure 5). The intra-amniotic administration of the probiotic (*L. paracasei*) didn't improve the intestinal barrier, as we found in the group administered with the hydrolyzed protein of chia, and this might be related of the increase in *E. coli*. Mucus is an important barrier against potentially pathogenic bacteria [52,55]. In the probiotic group, the *E. coli* population was increased and MUC2 gene expression remained, but when the hydrolyzed protein of chia was added to this probiotic, MUC2 expression was up regulated, and *E. coli* abundance was similar ($p>0.05$) to the control groups. Then, probably, the presence of the hydrolyzed protein of chia can increase MUC2 and prevent the increase in *E. coli*.

It is important to highlight that in this study, we investigated the administration of only one strain of probiotic, but the literature highlighted the importance of administering different strains of probiotics, to obtain greater benefits [20]. The *Lacticaseibacillus paracasei* used in this study is an innovative culture to be tested on plant products without fermentation with a promising probiotic effect.

The administration of hydrolyzed protein of chia, and the administration of the probiotic (*L. paracasei*) were able to improve the intestinal health, and we found more pronounced effects when they were administered separated. When the probiotic is administered with the protein, it can protect the probiotic during digestion, and it can arrive more intact or in different quantity in the intestine. Maybe that is why the difference in effects between the administration of the compounds alone or together. The probiotic had effects on inflammation and microbiota composition, the hydrolyzed protein of chia had effects on inflammation, barrier function and functionality, and both had an impact on intestinal morphology. Thus, the administration of both can bring benefits to intestinal health (Figure 6). The current study is the first to investigate the intestinal effects of hydrolyzed protein of chia and the probiotic *Lacticaseibacillus paracasei in vivo*; thus, future studies aimed to assess the effects in a long-term feeding trial should be

conducted, to see better these effects since the hydrolyzed protein of chia and the probiotic *L. paracasei* presented good results to improve intestinal health.

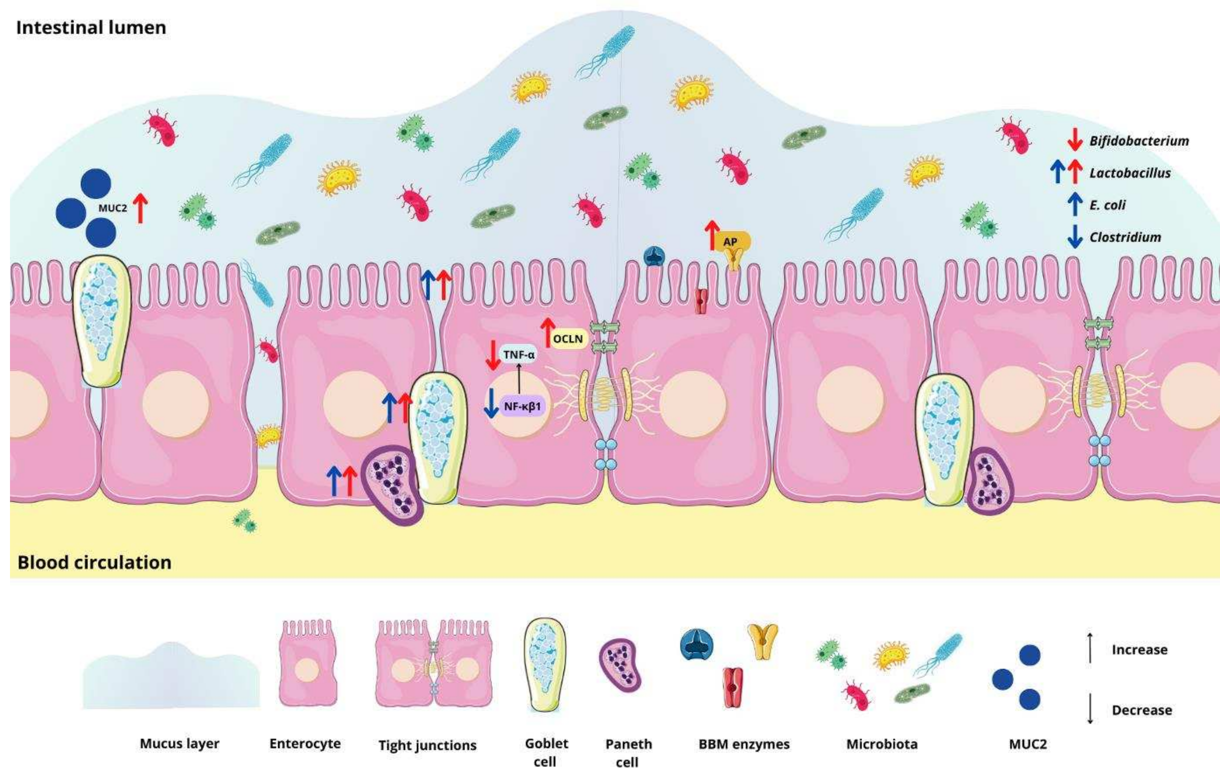


Figure 6. Graphical representation of the results. The intra-amniotic administration of hydrolyzed protein of chia reduced TNF-α and *Bifidobacterium* population and increased OCLN, MUC2, *Ap*, *Lactobacillus* population, villi height, villi surface area and crypt depth, Paneth cell number and the diameter of goblet cells. The probiotic administration reduced NF-κβ1 and *Clostridium* population, increased *Lactobacillus* and *E. coli*, villi height, villi surface area and crypt depth, Paneth cell number and the diameter of goblet cells. NF-κβ1: nuclear factor kappa beta; TNF-α: tumor necrosis factor-alpha; OCLN: occludin; MUC2: mucin 2; *Ap*: aminopeptidase; red arrow: effects of hydrolyzed protein of chia; blue arrow: effects of L. paracasei.

5. Conclusions

The intra-amniotic administration of hydrolyzed protein of chia decreased TNF-α, increased *Lactobacillus* population, OCLN, MUC2 and *Ap*, increased villi height, villi surface area and crypt depth, Paneth cell number and goblet cells diameter. The probiotic (*L. paracasei*) administration promoted NF-κβ1 reduction, increased *Lactobacillus* and reduced *Clostridium*, and increased villi height, villi surface area and crypt depth, Paneth cell number and goblet cells

diameter. Hydrolyzed protein of chia and the probiotic (*L. paracasei*) modulate some aspects of the intestinal health, and we found more pronounced effects when they were administered separated. These finding suggest that intra-amniotic administration of hydrolyzed protein of chia or probiotic (*L. paracasei*) improved intestinal health. Therefore, the current study indicates that additional long-term *in vivo* feeding trials are now warranted to further investigate the observed effects of dietary hydrolyzed protein of chia and the experimental probiotic.

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9.6 Paper 6: Chia Phenolic Extract Appear to Improve Small Intestinal Functionality, Morphology, Bacterial Populations, and Inflammation Biomarkers *In vivo* (*Gallus gallus*)

Article

Chia Phenolic Extract Appear to Improve Small Intestinal Functionality, Morphology, Bacterial Populations, and Inflammation Biomarkers In Vivo (*Gallus gallus*)

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Abstract: Phenolic compounds can act as a substrate for colonic resident microbiota. Once the metabolites are absorbed and distributed throughout the body, they can have diverse effects on the gut. The objective of this study was to evaluate the effects of the intra-amniotic administration of a chia phenolic extract on intestinal inflammation, intestinal barrier, brush border membrane functionality, intestinal microbiota, and morphology in vivo (*Gallus gallus* model). Cornish-cross fertile broiler eggs, at 17 days of embryonic incubation, were separated into groups as follows: non-injected (NI; this group did not receive an injection); 18 MΩ H_2O (H_2O; injected with ultrapure water), and 10 mg/mL (1%) chia phenolic extract (CPE; injected with phenolic extract diluted in ultrapure water). Immediately after hatch (21 days), chickens were euthanized and their small intestine, cecum, and cecum content were collected and analyzed. The chia phenolic extract reduced the tumor necrosis factor-alpha (TNF-α) and increased the sucrose isomaltase (SI) gene expression, reduced the *Bifidobacterium* and *E. coli* populations, reduced the Paneth cell diameter, increased depth crypt, and maintained villus height compared to the non-injected control group. Chia phenolic extract may be a promising beneficial compound for improving intestinal health, demonstrating positive changes in intestinal inflammation, functionality, microbiota, and morphology.

Keywords: *Salvia hispanica* L.; phenolic compounds; intra-amniotic administration; intestinal health; BBM

1. Introduction

Phenolic compounds, originating from plants' secondary metabolism, possess significant functions in plant reproduction and growth while safeguarding against unfavorable environmental factors, pathogens, and herbivores. Moreover, these compounds contribute to several sensory attributes of plants, including color, flavor, and astringency. Their consumption has exhibited preventive effects against conditions like obesity, cardiovascular disorders, diabetes and neurodegenerative diseases [1–4].

Not every phenolic compound is absorbed in the small intestine; the majority of them make their way to the colon, where they act as a substrate for the resident microbiota. This interaction yields various effects, primarily by modulating the intestinal microbiota and promoting its homeostasis. Thus, phenolic compounds can be considered prebiotic substrates [5–9]. Compared to other compounds such as proteins, lipids and carbohydrates, phytochemicals are not required for physiological functions, however, they have the capability to induce biological effects [5,10,11]. Prebiotic effects can occur by increasing the growth and establishment of probiotic bacteria and, by reducing the number of pathogenic bacteria, such as Escherichia coli, Helicobacter pylori and *Clostridium* perfringens. These impacts on the gut microbiota and beneficial properties for health occur through multiple means, including antioxidant, anti-inflammatory, bactericidal, immune, and mucus-producing functions [10,12–14]. Due to its bioavailability, the effects of phenolics are related to the food matrix, depending on whether they are consumed in the whole food or as isolated compounds [5,15–17]. However, the effects of isolating phenolic extracts on gut health, nutrient digestibility, absorption of some vitamins and minerals, and gut microbiota may differ depending on their dosage [1,18]. Therefore, phenolic compounds and other bioactive compounds from food sources need to have their effects and safety validated in *in vivo* studies.

Chia seed is rich in phenolic compounds, which are some of the main components with nutritional and functional activities of the seed, with high antioxidant capacity [19–22]. According to some studies, the main phenolic compounds identified in chia were: rosmarinic, caffeic, salvianolic, gallic, protocatechuic, ferulic acid, daidzin, glycitin, genistin, glycitein and genistein acids [19,20,23,24]. A hydrolyzed phenolic extract of chia, *in vitro*, decreased the activity of gluconeogenic and glycolysis enzymes in cells HepG2. These results suggest that phenolics from chia may reduce metabolic disorders related to obesity linked to impaired gluconeogenesis pathway [23]. Phenolic compounds may favor a diverse bacterial profile of the resident microbiota and promote colon health. The mechanisms of action by which phenolic compounds exert these effects are varied, but include actions such as regulating the expression of genes associated with inflammatory processes and preserving an adequate intestinal barrier [6,25,26].

Currently, the intra-amniotic administration model is widely used as an *in vivo* method to assess the effects of plant-derived bioactive compounds on gut health, including effects on intestinal brush border membrane (BBM) functionality and intestinal morphology. The *Gallus gallus* model contains a complex and dynamic intestinal microbiota, strongly influenced by host

genetics, environment, and diet [27]. The intra amniotic administration of black corn anthocyanin-rich extract promoted an improvement in cecal microbiome while maintaining intestinal morphology and functionality [28]. The intra amniotic administration of a hydrolyzed chia protein was evaluated and demonstrated that chia protein decreased tumor necrosis factor-alpha (TNF-α), increased the *Lactobacillus* population and improved intestinal morphology, barrier, and functionality [29] and the intra amniotic administration of a soluble extract from chia improved the intestinal morphology and microbiota [24]. Therefore, the objective of this study was to evaluate the effects of the intra-amniotic administration of a chia phenolic extract on intestinal inflammation, intestinal barrier, the functionality of brush border membrane, intestinal microbiota, and morphology *in vivo* (*Gallus gallus* model).

2. Materials and Methods

2.1. Sample material

2.1.1. Chia phenolic extract

In this study, chia seeds (*Salvia hispanica* L.) cultivated in Brazil (specifically, in the state of Rio Grande do Sul) were utilized. The seeds were carefully packaged, transported in cardboard boxes, and subsequently stored in hermetically sealed plastic bags, shielded from light, and kept frozen at a temperature of -18°C ± 1°C until analysis. To obtain the chia flour, the seeds were ground, using a knife mill, in three separate replicates (Marconi Equipment, Brazil).

For the extraction of chia phenolics, 20g of chia flour were mixed with 100 mL of methanol: water (80:20, v/v), the mixture was placed in an ultrasonic water bath for 60 min at room temperature and then taken to centrifugation at 1792g for 30 min. The supernatant was collected and taken to a rotatory evaporator at 50°C, thus extracting the total phenolics. In sequence, acid hydrolysis by dissolution was performed, the ratio of 1 mL of sample to 1 mL of HCl (2M) was taken to a water bath at 80°C for 60 min. Then, the extracted hydrolyzed phenolics was lyophilized, resulting in a dried extract.

2.1.2. Determination of total phenolics

The total of phenolic compounds was determined in the dried extract, in three replicates, by the Folin-Ciocalteau reagent [30]. The absorbance was measured in a spectrophotometer (Thermo Scientific, Evolution 606, USA) at 765 nm. Total polyphenols were expressed as milligrams of gallic acid equivalents per gram of sample (mg of GAE/g of sample).

A previous study conducted by our research group characterized the phenolic profile of a chia extract, obtained from the same seed and using the same extraction procedure used in the present study [23]. The main phenolic compounds founded in the extract were rosmarinic acid, danshensu glycoside, ferulic acid and caffeic acid (Figure 1).

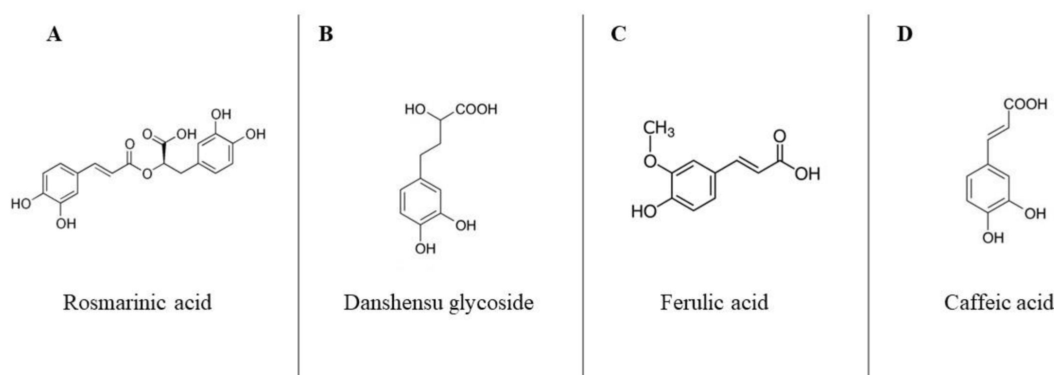


Figure 1: Chemical formulas of the major components of the chia phenolic extract. (A) Rosmarinic acid; (B) Danshensu glycoside; (C) Ferulic acid and (D) Caffeic acid.

2.1.3. Antioxidant Capacity

The antioxidant capacity was determined, in three replicates, by the radical scavenging activity assay using DPPH (1,1-diphenyl-2-picrylhydrazyl). Briefly, in a test tube, protected from light, lyophilized chia phenolic extract (100µL) was added to an ethanolic DPPH solution and stirred by vortex. After incubation (30 min), the absorbance was measured (517nm) [31]. The antiradical activity was expressed in µmol of trolox equivalent per gram of sample (µmol of trolox equivalent/g of sample).

2.2. Intra-amniotic administration

Cornish-cross fertile broiler eggs (n = 27) were acquired from Moyer's chicks, a commercial hatchery located in Quakertown, PA, USA. The eggs were incubated under optimal conditions at the Cornell University Animal Science poultry farm incubator, with a temperature

of 37 ± 2ºC and humidity of 89.6 ± 2%. The animal protocols followed in this study were approved by the Cornell University Institutional Animal Care and Use Committee, under the protocol code 2020-0077.

The chia phenolic extract was diluted in 18MΩH_2O to determine the concentration. The dilution was intended to keep the osmolarity value (Osm) below 320 Osm, ensuring that the injection of the solution would not lead to dehydration in the chicken embryos. At 17 days of embryonic incubation, a candling process was performed to discard eggs that were cracked, infertile, contaminated, or contained early dead embryos. Eggs containing viable embryos were weighed and randomly divided into three groups, ensuring a similar weight distribution (n = 9/group). All eggs underwent disinfection with 70% ethanol. Subsequently, each group received an injection of the specified solution (1 mL per egg) using a 19mm gauge needle. The injection was administered vertically into the amniotic fluid, which was identified through candling. The three groups were assigned as follows: non-injected (NI); 18MΩH_2O (H_2O) and 10mg/mL (1%) chia phenolic extract (CPE). Once all the eggs had been injected, the injection sites were sealed using cellophane tape, and the eggs were arranged in hatching baskets in a way that ensured equal representation of each treatment at every incubator location. Immediately after hatch (21 days) and from each treatment group, the chickens were weighed and then euthanized by CO2 exposure. Subsequently, the small intestine (duodenum), cecum, and cecum content were collected from each chicken.

2.3. Extraction of the total RNA from the duodenum samples

The RNA was extracted from 30mg of the duodenum (n = 5 animals/group) according to the manufacture's protocol (RNeasy Mini Kit, Qiagen Inc., Valencia, CA, USA). All steps were carried out under RNase-free conditions. The total RNA was eluted in 50 µL of RNase-free water. The RNA was quantified by absorbance at 260/280 and the integrity of the 18S ribosomal RNAs was confirmed using 1.5% agarose gel electrophoresis, followed by ethidium bromide staining. Subsequently, the samples were frozen at (−80ºC) until analysis [28].

2.4. Real-time polymerase chain reaction (RT-PCR) and prime design

To obtain the cDNA, a total of 20 µL reverse transcriptase (RT) reaction was carried out in a BioRad C1000 touch thermocycler using the Improm-II Reverse Transcriptase Kit (Catalog #A1250; Promega, Madison, WI, USA). The concentration of the resulting cDNA was determined by measuring the absorbance at 260 nm and 280 nm, applying an extinction coefficient of 33 (for single-stranded DNA).

For gene expression analysis of the duodenum, real-time polymerase chain reaction (RT-PCR) was conducted. The primers used in the real-time qPCR were designed based on gene sequences sourced from the Genbank database, utilizing the Real-Time Primer Design Tool software (IDT DNA, Coralvilla, IA, USA) [29]. The sequences and descriptions of the primers used can be found in Table 1. To assess primer specificity, a BLAST search against the genomic National Center for Biotechnology Information (NCBI) database was performed. The primer for *Gallus gallus* 18S rRNA was designed as a reference gene.

Table 1. Sequence of primers used in this study.

Gene	Oligonucleotides (5'- 3')	
	Forward Primer (5′-3′)	**Reverse Primer (5′-3′)**
BBM functionality		
Ap	CGTCAGCCAGTTTGACTATGTA	CTCTCAAAGAAGCTGAGGATGG
Si	CCAGCAATGCCAGCATATTG	CGGTTTCTCCTTACCACTTCTT
18S rRNA	GCAAGACGAACTAAAGCGAAAG	TCGGAACTACGACGGTATCT
Inflammation		
TNF-α	GACAGCCTATGCCAACAAGTA	TTACAGGAAGGGCAACTCATC
NF-κβ1	CACAGCTGGAGGGAAGTAAAT	TTGAGTAAGGAAGTGAGGTTGAG
Intestinal Barrier		
MUC2	CCTGCTGCAAGGAAGTAGAA	GGAAGATCAGAGTGGTGCATAG
OCLN	GTCTGTGGGTTCCTCATCGT	GTTCTTCACCCACTCCTCCA

BBM: brush border membrane; *Ap*: aminopeptidase; *Si*: sucrose isomaltase; 18S rRNA: reference gene; TNF-α: tumor necrosis factor-alpha; NF-κβ1: nuclear fator-kappa beta; MUC2: mucin 2; OCLN: occludin.

2.5. Real-time qPCR design

The procedures were conducted following previously described methods [24,32]. In summary, cDNA was utilized in 10 mL reactions, each containing 2 × BioRad SSO Advanced Universal SYBR Green Supermix (Hercules, CA, USA). To eliminate DNA contamination in the PCR mix, a "no template" control with nuclease-free water was included. For each reaction (in duplicates), 8 μL of the master mix and 2 μL of cDNA were pipetted into a 96-well plate. A standard curve with seven points in duplicates was assessed. The amplification of double-stranded DNA was carried out using the Bio-Rad CFX96 Touch (Hercules, CA, USA) under

the following PCR conditions: initial denaturation at 95 ºC for 30 s, followed by 40 cycles of denaturation at 95 ºC for 15 s, annealing at various temperatures according to Integrated DNA Technologies (IDT) for 30 s, and elongation at 60 ºC for 30 s. The gene expression data were obtained as Cp values using the "second derivative maximum" method, automatically computed by the Bio-Rad CFX Maestro 1.1 software (Version 4.1.2433.1219, Hercules, CA, USA). The real-time qPCR analysis included a standard curve, and a curve with four points was prepared through a 1:10 dilution (in duplicates). The software generated a graph of Cp values versus log 10 concentrations, and the efficiencies were calculated as 10 (1/slope). The specificity of the amplified real-time RT-PCR products was verified through melting curve analysis (60–95 ºC) after 40 cycles, resulting in distinct specific products with their respective melting temperatures.

2.6. Collection of microbial samples and intestinal contents DNA extraction

The cecum (n = 5 animals/group) was sterilely removed. The cecum contents were placed into a sterile 15 mL tube with 9 mL of sterile PBS and homogenized by vortexing with glass beads (4 mm diameter) for 3 min. Debris was separated by centrifugation at 1000 g for 5 minutes, and the resulting supernatant was collected and subjected to another centrifugation step at 4000 g for 10 minutes. The pellet obtained was washed twice with PBS and stored at -20 ºC until DNA extraction. For DNA purification, the pellet was resuspended in 50 mM ethylenediaminetetraacetic acid (EDTA) and treated with lysozyme (Sigma Aldrich CO., St. Louis, MO, USA; final concentration of 10 mg/mL) for 45 min at 37 ºC. The bacterial genomic DNA was subsequently isolated using the Wizard Genomic DNA purification kit (Promega Corp., Madison, WI, USA) [28].

2.7. Primer's design and PCR amplification of bacterial 16S rDNA

Primers for *Bifidobacterium*, *Lactobacillus*, Escherichia coli and *Clostridium* were used, as established in previous studies [28,32]. Additionally, universal primers were designed to target the conserved region in the bacterial 16S rRNA and were utilized as internal standards. The PCR products were separated by 2% agarose gel, stained with ethidium bromide, and quantified by Quantity One 1-D analysis software (Bio-Ra, Hercules, CA, USA). The relative abundance of each examined bacterium was assessed. All products were normalized to the content of the universal 16S rRNA primer product, allowing for the determination of proportions of each examined bacterial product.

2.8. Intestinal morphology

Intestinal morphology assessments were conducted following previously established method [24,29,32]. Duodenum samples were fixed in fresh 4% (v/v) buffered formaldehyde, dehydrated, cleared, and embedded in paraffin. Multiple 5 µm-thick sections were cut and placed on glass slides. The sections were then deparaffinized in xylene and rehydrated using a series of graded alcohol solutions. Subsequently, the slides were stained with Alcian Blue/Periodic acid-Schiff and examined under a light microscope. The following morphometric measurements were assessed: villus height (µM), villus surface area (µM), crypt depth (µM), Paneth cell number and diameter (µM), goblet cell number and goblet cell diameter (µM) in both villi and crypts, as well as the characterization of goblet cell types (acidic, neutral, and mixed). Each biological sample (n = 3/treatment group) was subjected to examination in four segments, and within each segment, ten randomly chosen villi and crypts were analyzed, resulting in 40 replicates per biological sample. The analysis was performed using a light microscope (CellSens Standard software, Olympus, Waltham, MA, USA).

For the Alcian Blue and Periodic acid-Schiff stain, the segments were only counted for the type of goblet cells (acid, neutral or mixed) in both the villi and crypts. The count of goblet cells was performed on 10 villi and crypts per sample, and the means were calculated for statistical analysis. The villus surface area was determined using the following equation.

Villus surface area=2 VW/2 x VL

Where VW = villus width average of three measurements, and VL = villus length.

2.9. Statistical Analysis

The experimental treatments were arranged in a completely randomized design, and the results were presented as means ± standard error deviation (SED). Statistical significance was determined at a p-value < 0.05. The normality of the data distribution was assessed using the Shapiro-Wilk normality test. For normally distributed data, differences between experimental groups were analyzed using one-way Analysis of Variance (ANOVA), followed by a post-hoc Duncan test. In cases where the data did not follow a normal distribution, the Kruskal-Wallis test was applied, followed by a post-hoc Dunn's test. The correlation between intestinal parameters evaluated in our study was analyzed by Spearman’s correlation coefficient.

The statistical analyzes were carried out utilizing IBM SPSS Statistics®, version 25, while graphing was accomplished with GraphPad Prism® version 9.0 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Chia phenolic extract characterization

The concentration of total phenolic compounds in chia phenolic extract was 405.17 mg of GAE/g of sample, and the antiradical activity was 3.06 µmol of trolox equivalent/g of sample (Table 2).

Table 2. Characterization of chia phenolic extract

Variable	Mean ± SD
Total phenolic compounds (mg of GAE/g of sample)	405.70 ± 17.58
Antiradical activity (µmol of trolox equivalent/g of sample)	3.06 ± 0.05

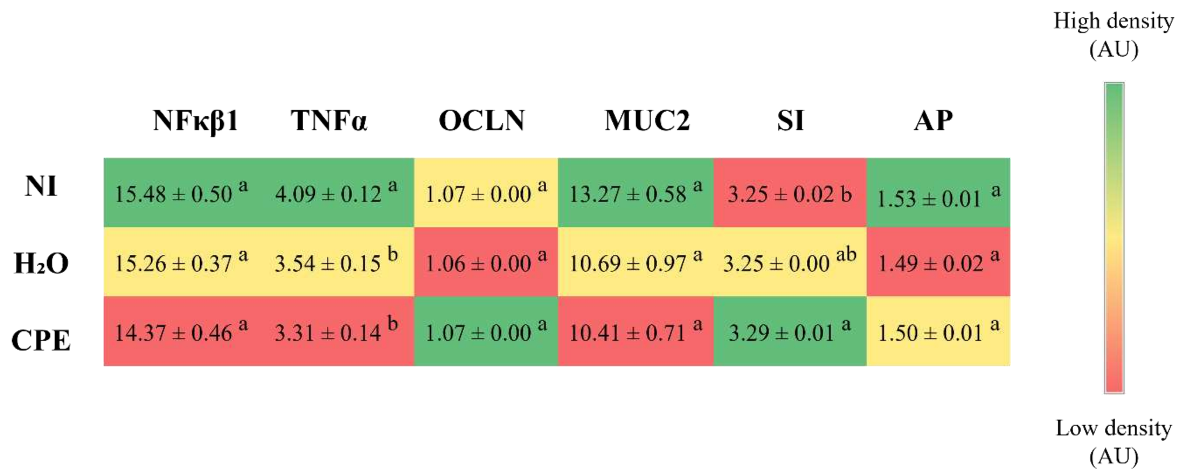
GAE: gallic acid equivalent; SD: standard deviation; total phenolic compounds and antiradical activity were analyzed in three replicates, by spectrophotometry.

3.2. Body weight

Comparing all the groups, the body weight was similar, according to one way ANOVA followed by post hoc Duncan test. NI (35.00 ± 0.82), H_2O (35.13 ± 1.09) and CPE (36.22 ± 0.88). Values are means in grams ± SED, n = 7–9/group (according to hatching).

3.3. Effect of Chia Phenolic Extract on Duodenal Gene Expression

The intra-amniotic administration of chia phenolic extract did not change nuclear factor-kappa beta (NF-κβ1) gene expression but reduced the tumor necrosis factor-alpha (TNF-α) gene expression compared to the non-injected control group. Regarding to the intestinal barrier genes, occluding (OCLN) and mucin 2 (MUC2), chia phenolic extract maintained the expression of these genes. For the intestinal functionality genes, the chia phenolic extract increased the sucrose isomaltase (SI) gene expression compared to the non-injected control group and maintained the aminopeptidase (AP) gene expression (Figure 2).

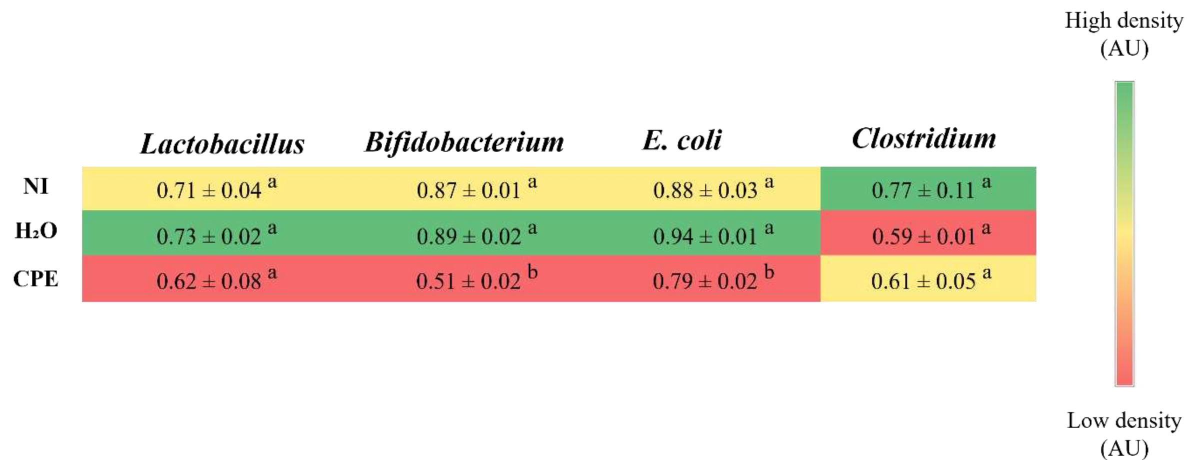


	NFκβ1	TNFα	OCLN	MUC2	SI	AP
NI	15.48 ± 0.50 [a]	4.09 ± 0.12 [a]	1.07 ± 0.00 [a]	13.27 ± 0.58 [a]	3.25 ± 0.02 b	1.53 ± 0.01 [a]
H_2O	15.26 ± 0.37 [a]	3.54 ± 0.15 [b]	1.06 ± 0.00 [a]	10.69 ± 0.97 [a]	3.25 ± 0.00 [ab]	1.49 ± 0.02 [a]
CPE	14.37 ± 0.46 [a]	3.31 ± 0.14 [b]	1.07 ± 0.00 [a]	10.41 ± 0.71 [a]	3.29 ± 0.01 [a]	1.50 ± 0.01 [a]

Figure 2. Effect of intra-amniotic administration of chia phenolic extract on intestinal gene expression. NI: non-injected; H_2O: 18MΩH_2O (water) injected; CPE: Chia Phenolic Extract; NF-κβ1: nuclear factor-kappa beta; TNF-α: tumor necrosis factor-alpha; OCLN: occluding; MUC2: mucin 2; *Si*: sucrose isomaltase; *Ap*: aminopeptidase; AU: arbitrary unit. Values are presented as means ± SED, n = 5/group. For each gene, within the same column, the color red indicates lower gene expression levels, whereas green indicates higher gene expression levels. a - b Per gene, within the same column, treatment groups with different letters indicate significant differences ($p < 0.05$) based on one way ANOVA followed by post hoc Duncan test (for data with normal distribution) or according to Kruskal-Wallis and a post hoc Dunn's test (for data without normal distribution).

3.4. Effect of Chia Phenolic Extract on the Bacterial Population on Cecum Content

The intra-amniotic administration of chia phenolic extract maintained the *Lactobacillus* population, reduced *Bifidobacterium* and *E. coli* populations compared to both of control groups (non-injected and H_2O) and maintained *Clostridium* populations (Figure 3).



	Lactobacillus	*Bifidobacterium*	*E. coli*	*Clostridium*
NI	0.71 ± 0.04 [a]	0.87 ± 0.01 [a]	0.88 ± 0.03 [a]	0.77 ± 0.11 [a]
H_2O	0.73 ± 0.02 [a]	0.89 ± 0.02 [a]	0.94 ± 0.01 [a]	0.59 ± 0.01 [a]
CPE	0.62 ± 0.08 [a]	0.51 ± 0.02 [b]	0.79 ± 0.02 [b]	0.61 ± 0.05 [a]

Figure 3. Effect of intra-amniotic administration of chia phenolic extract on the bacterial population on cecum content. NI: non-injected; H_2O: 18MΩH_2O (water) injected; CPE: Chia Phenolic Extract; AU: arbitrary unit. Values are presented as means ± SED, n = 5/group. For each bacteria, within the same column, the color red indicates lower gene expression levels, whereas green indicates higher gene expression levels. a - b For each bacteria, within the same column, treatment groups with different letters indicate significant differences ($p < 0.05$) based on one way ANOVA followed by post hoc Duncan test (for data with normal distribution) or according to Kruskal-Wallis and a post hoc Dunn's test (for data without normal distribution).

3.5. Effect of Chia Phenolic Extract on Morphological Parameters in Duodenum

The intra-amniotic administration of chia phenolic extract increased the Paneth cell number compared to H_2O control group and maintained compared to non-injected control group. Paneth cell diameter was maintained compared to H_2O control group and reduced compared to non-injected. The chia phenolic extract increased the depth crypt and maintained the villus height compared to both of control groups (non-injected and H_2O) but reduced the villus surface area compared to non-injected control group (Figure 4).

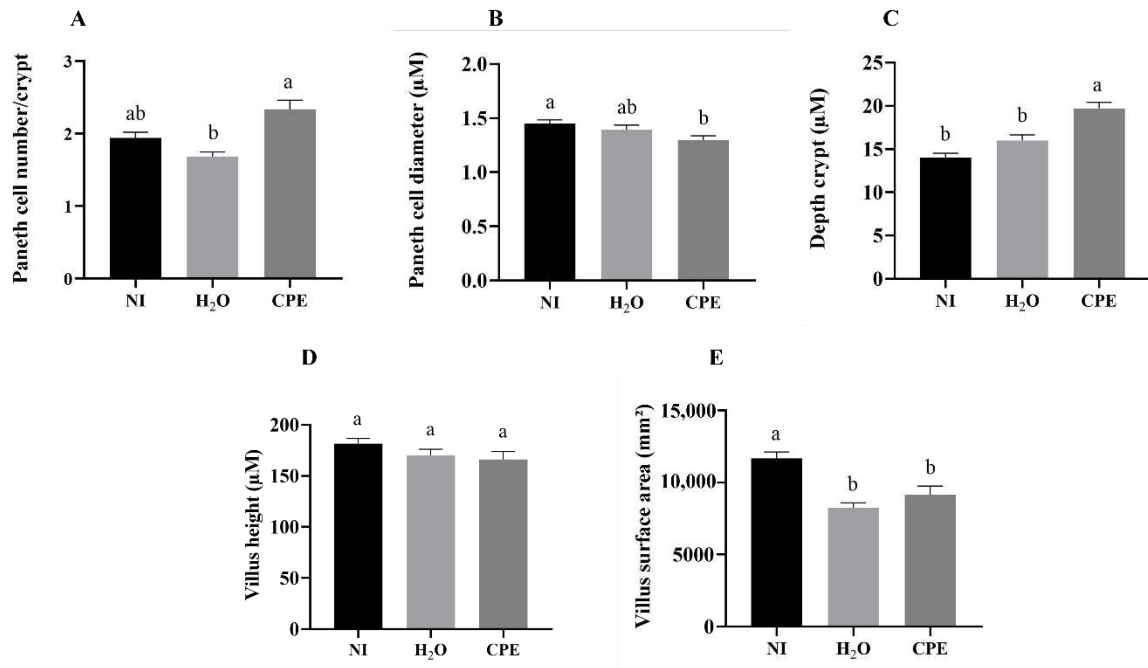


Figure 4. Effect of intra-amniotic administration of chia phenolic extract on morphological parameters in duodenum. NI: non-injected; H_2O: $18M\Omega H_2O$ (water) injected; CPE: Chia Phenolic Extract; AU: arbitrary unit. (A) Paneth cell number/crypt; (B) Paneth cell diameter (µM); (C) Depth crypt (µM); (D) Villus height (µM); (E) Villus surface area (mm2). Values are presented as means ± SED, n = 3 animals/group, 4 sections, 10 measurements. a - b Treatment groups with different letters indicate significant differences ($p < 0.05$) based on Kruskal-Wallis and a post hoc Dunn's test.

The intra-amniotic administration of chia phenolic extract did not change the villus and crypt goblet cell diameter but reduced the goblet cell number in the crypt. For the villi goblet cells, the chia phenolic administration reduced the acidic and neutral types and increased the mixed goblet cells compared to the non-injected control group and for the crypt goblet cells, chia phenolic administration reduced all the types of goblet cells (acidic, neutral, and mixed).

Table 3. Effect of chia phenolic extract on goblet cells.

	NI	**H_2O**	**CPE**
Villi goblet cell diameter (µM)	2.46 ± 0.06^{a}	2.20 ± 0.05^{a}	2.64 ± 0.08^{a}
Crypt goblet cell diameter (µM)	2.92 ± 0.05^{b}	3.13 ± 0.05^{a}	2.96 ± 0.06^{ab}
Villi goblet cell number	24.68 ± 0.74^{b}	38.38 ± 0.91^{a}	23.78 ± 0.60^{b}
Acidic	15.28 ± 0.71^{b}	26.71 ± 1.12^{a}	11.73 ± 0.65^{c}

Neutral	0.79 ± 0.13^{a}	0.10 ± 0.04^{c}	0.50 ± 0.11^{b}
Mixed	8.68 ± 0.57^{b}	11.57 ± 0.66^{a}	11.55 ± 0.46^{a}
Crypt goblet cell number	12.67 ± 0.55^{a}	10.95 ± 0.62^{b}	9.33 ± 0.35^{b}
Acidic	8.53 ± 0.43^{a}	7.88 ± 0.51^{ab}	6.99 ± 0.28^{b}
Neutral	0.41 ± 0.06^{a}	0.50 ± 0.07^{a}	0.10 ± 0.03^{b}
Mixed	3.73 ± 0.27^{a}	2.58 ± 0.21^{b}	2.25 ± 0.16^{b}

Values are presented as means ± SED, n = 3 animals/group, 4 sections, 10 measurements. NI: non-injected; H_2O: 18MΩH_2O (water) injected; CPE: Chia Phenolic Extract. a - c Treatment groups with different letters indicate significant differences ($p < 0.05$) based on Kruskal-Wallis and a post hoc Dunn's test.

3.6. Correlation analysis

The correlation between parameters of intestinal health evaluated in this study was evaluated using the Spearman's correlation analysis. Positive correlations were observed between SI and depth crypt, *Bifidobacterium* and villus surface area, *E. coli* and crypt goblet cell number, and villus surface area and crypt goblet cell number, and negative correlation was observed between villus surface area and depth crypt (Figure 5).

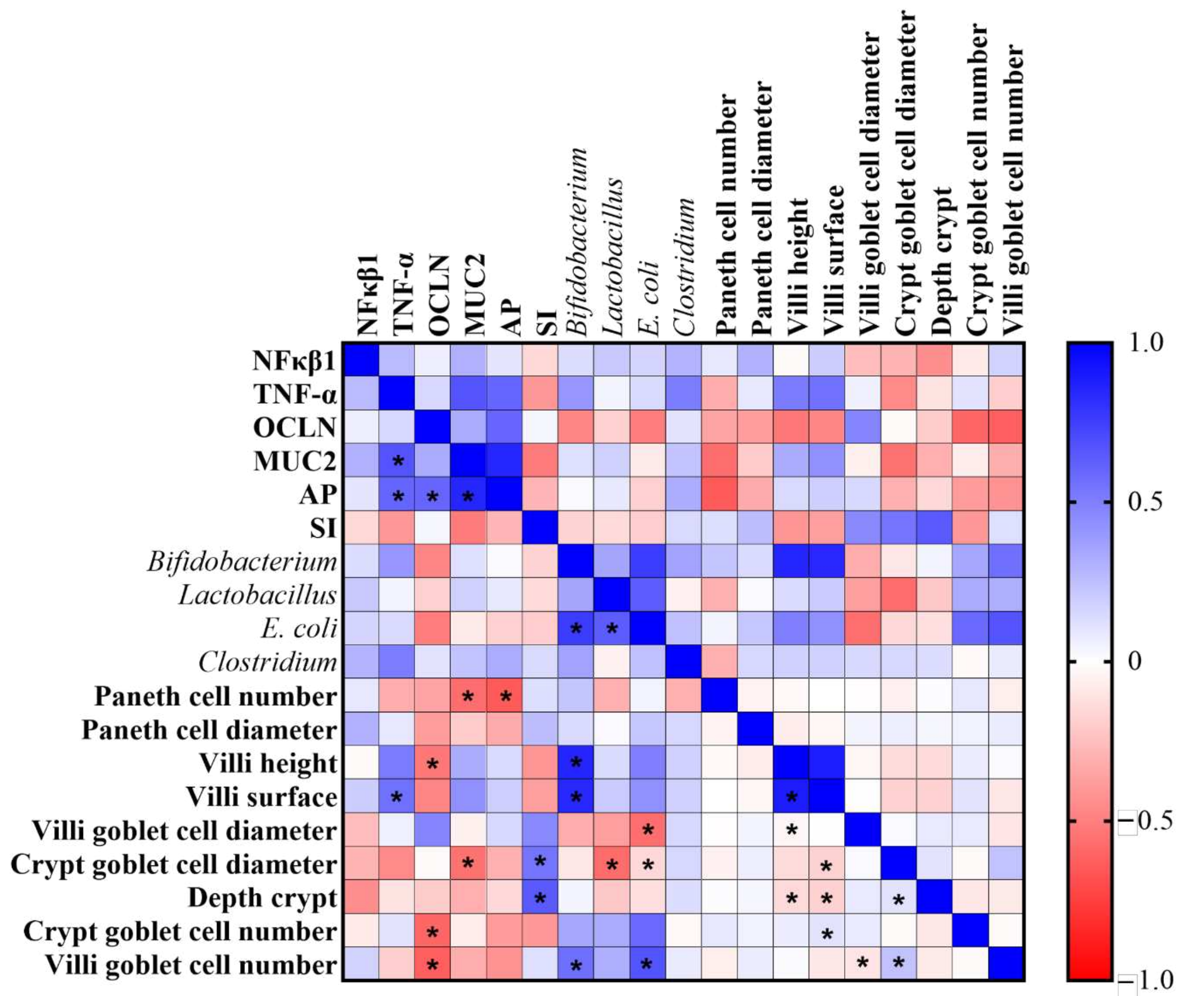


Figure 5. Heatmap demonstrating Spearman's correlation analysis. NF-κβ1: nuclear factor-kappa beta; TNF-α: tumor necrosis factor-alpha; OCLN: occludin; MUC2: mucin 2; *Ap*: aminopeptidase; *Si*: sucrose isomaltase. * Indicates a statistically significant difference ($p < 0.05$).

4. Discussion

Phenolic compounds can reach the colon serving as a substrate for the resident microbiota, after they are absorbed and circulate in the body they can exert a wide range of effects in the intestine, such as anti-inflammatory effects [1,33–35]. In this present study, we aimed to assess the impact of the intra-amniotic administration of a phenolic extract from chia seeds. We investigated its effects on the inflammatory response in the intestine, the intestinal barrier, the functionality of the brush border membrane, bacterial populations, and the overall morphology *in vivo*. Our results demonstrate that the phenolic compounds of chia, reduced the

TNF-α and increased the SI gene expression, reduced the *E. coli* population, reduced the Paneth cell diameter, and increase the Paneth cell number. Further, the chia phenolic extract increased the depth crypt and maintained the villus height in *Gallus gallus* model. These effects might occur based on the concentration of total phenolics and antioxidant capacity (Table 2) found in the extract.

In the present study, the administration of a chia phenolic extract downregulated the TNF-α gene expression compared to the non-injection control group (Figure 2). TNF-α is an activator of the production of different cytokines promoting a pro-inflammatory state. In general, long-term elevated TNF-α levels are associated with conditions such as inflammatory bowel disease, and lowering levels of this cytokine is generally considered to have a beneficial anti-inflammatory effect [36–38]. A previous study characterized the chia phenolic extract and found that the main phenolic compounds in the extract were rosmarinic acid, danshensu glycoside, ferulic acid and caffeic acid [23]. The study by Lu *et al*. (2022) [39] showed that a treatment with rosmarinic acid inhibited the increase of TNF-α, IL-6 and IL-8 in the liver, which indicated that rosmarinic acid could suppress the inflammatory response by inhibiting inflammatory cytokines. Furthermore, the administration of an extract rich in rosmarinic acid decreased TNF-α in mice fed a high fat diet [40]. Danshensu demonstrated to be an efficient antioxidant and also an effective anti-inflammatory impact [41,42] and ferulic acid increased antioxidant capacity, improved intestinal barrier integrity, mucosal immune response and increased stability of ileal microflora [43]. Moreover, it is proposed that the digestion process of chia products could lead to an elevation in antioxidant activity and favor the anti-inflammatory effects [44]. Thus, the phenolic composition of the extract, with anti-inflammatory action, might justify the reduction in TNF-α gene expression.

The expression of the SI gene was found to be increased in the group that received the chia phenolic extract injection (Figure 2). Sucrase isomaltase (SI) is a disaccharidase found in the brush border membrane of the small intestine, responsible for breaking down disaccharides or oligosaccharides into monosaccharides for absorption. This enzyme plays a crucial role in the digestion of starchy foods and foods containing sugars.[45]. A previous study by Enes et al., (2020) demonstrated that, in vitro, a chia phenolic extract downregulated mRNA of enzymes involved on gluconeogenesis and glycolysis (phosphoenolpyruvate carboxykinase and glucose 6-phosphatase, and phosphofructokinase and pyruvate kinase, respectively) [23]. Simsek et al. (2017) [46] demonstrated that caffeic acid, which is found in the phenolic extract of chia [23], induced upregulation of jejunal SI mRNA expression, and suggest this type of regulatory effects

by dietary compounds, such as phenolics, may be some of the mechanisms of action for pharmacological properties. Caffeic acid from propolis, improved glucose uptake and decreased glucose 6-phosphatase expression in insulin resistant *in vitro*, also improved hyperglycemia, glucose tolerance, and hyperlipidemia, and reduced TNF-α expression *in vivo* [47]. Moreover, other chia phenolics may also have provided this effect, such as rosmarinic acid, which has been associated with glucose improvement related to AMPK phosphorilation [48]. The up regulation of SI mRNA expression can be a mechanism for glucose metabolism regulation by phenolic compounds, but more studies are needed to better elucidate the pathway.

Despite the reduction in *Bifidobacterium*, the chia phenolic extract reduced *E. coli* and maintained *Clostridium* and *Lactobacillus* populations compared to non-injected and H_2O groups (Figure 2). Increased *E. coli* has been found in pathologies such as celiac disease, non-alcoholic fatty liver disease, inflammatory bowel disease and gastrointestinal cancer [15]. The ability of polyphenols to reduce *E. coli* has been previously reported [49–51] and some examples are the polyphenols of red wine [52], gallic acid [53] and grape pomace [54]. An extract of black corn, rich in anthocyanin, demonstrated, *in ovo*, a result similar to ours, reduced the *E. coli* and maintained *Lactobacillus* population [28]. Variations in *Lactobacillus* population, in our *in ovo* studies, seem to be more sensitive to the presence of fiber-rich soluble extracts [24,32]. The precise mechanisms through which polyphenols regulate the microbiota are not fully understood, but in general, they possess the ability to intervene both directly and indirectly. The inhibition of certain bacteria is related to the antimicrobial capacity of these compounds. Some phenolic can inhibit the growth of bacteria that are harmful to health, reducing cell adhesion between them and the intestinal epithelium and negatively regulating genes that encode bacteria's membrane proteins. Polyphenols can also act indirectly, influencing the growth of some bacteria which, in turn, modulate the development of others [15,55].

The chia phenolic extract intra-amniotic administration increased the number of Paneth cells and reduced the diameter of Paneth cells (Figure 4A and Figure 4B). In this way, occurred an improvement in Paneth cell development without altering the cell size. The antimicrobial peptides secreted by Paneth cells were not produced, as there were no triggering factors like inflammation or pathogenic bacteria, which is in accordance with our results about the reducing in TNF-α and *E. coli*. Similar results were verified after the intra-amniotic administration of a hydrolyzed protein of chia seed [29]. The intra amniotic administration of chia phenolic extract increased the depth crypt (Figure 4C). In the intestine, the increase in the height of villi and the depth of crypts directly affects the ability to absorb nutrients, as it increases the absorptive area

[56], which is in accordance with the positive correlation we found between SI and depth crypt. In our study, the chia phenolic extract administered could increase the depth of the crypts and maintained the villi height (Figure 4D). The chia phenolic extract reduced the villus surface area compared to the non-injected group (Figure 4E). This effect was also observed with the intra-amniotic administration of grape pomace [57]. The findings were attributed to the ability of phenolic compounds to interact with proteins and form indigestible complexes. As proteins are crucial for cell division and proliferation, the interaction between polyphenols and proteins might decrease protein digestibility, which could explain the decrease in duodenal enterocyte proliferation and, consequently, the reduction in villus surface area [57].

The goblet cell is a type of secretory enterocyte, from which intestinal MUC2 and mucus originate. MUC2 tends to show a positive correlation with the number and function of goblet cells, making it a specific marker indicative of goblet cell secretory activity. [58]. Our results showed that the intra-amniotic administration of a chia phenolic extract was no able to increase the number of goblet cells in the crypt (Table 3). This justifies the fact that there was no increase in MUC2 gene expression, and it is correlated with the reduction in *E. coli* (Figure 5). The same correlation (positive correlation between *E. coli* and crypt goblet cell number) was observed by Verediano *et al*., 2022 [28], that evaluated the intra amniotic administration of a black corn anthocyanin-rich extract.

Chia is rich in dietary fiber, linolenic acid, protein and bioactive compounds, such as, phenolics, which are some of the main components with nutritional and functional activities of the seed [19–21]. Chia has a nutritional composition that suggests promising effects on intestinal health. Therefore, our research group has been evaluating the effects of chia and its fractions on intestinal health. Studies evaluating chia flour demonstrated that the intake of chia increased the production of short chain fatty acids and improved the intestinal morphology in male *Wistar* rats [59], in ovariectomized female *Wistar* rats [60] and in male *Wistar* rats with metabolic disorders caused by the consumption of a high fat high fructose diet [61]. Also, the chia flour intake reduced the intestinal pH [60,61] and increased microbiota richness [59,60]. When we evaluated the chia fractions, the intra amniotic administration of a hydrolyzed chia protein downregulated TNF-α expression, increased the gene expression of OCLN, MUC2, and AP and improved the morphology [29]. The intra amniotic administration of a soluble extract from chia, rich in fiber, improved the intestinal morphology, increased the relative abundance of *Bifidobacterium* and *Lactobacillus*, and up regulated the expression of proteins related to mineral metabolism [24]. In this way, in the present study we aimed to evaluate the effects of a

methanolic extract of chia, rich in phenolic compounds, on the intestinal health, in *Gallus gallus* model. The consumption of a chia flour, the intra amniotic administration of a soluble extract from chia seed and a hydrolyzed protein of chia demonstrated more effects on the intestinal morphology than the intra amniotic administration of an isolated phenolic extract. In this sense, it is important to consider the relevance of nutrient synergy, as we can see with the intake of chia flour, so all compounds (dietary fibers, proteins, and bioactive compounds) are ingested from the same food matrix. Furthermore, the administration of nutrients required for physiological functions (such as fiber and protein) along with phenolic compounds can have a greater impact on intestinal morphology, especially in a naive organism with a developing gastrointestinal tract. The presence of high concentrations of phenolic compounds in food does not always guarantee a high bioaccessibility of these phenolics. The beneficial effects of phenolics are also influenced by other factors such as stability, the composition of the microbiota, and the activity of digestive enzymes [15,62]. Biotransformations mediated by the intestinal microbiota play a role in determining the bioavailability of phenolics, which is further influenced by processes of absorption and metabolism [15,63]. Therefore, in a complex diet with the ingestion of several types of food, it is important to consider how phenolics are ingested in the diet, if they are present in food in which they will relate to other bioactive compounds and nutrients, or if in an isolated form. Maybe, a different dosage of phenolic compounds might be needed to exert more effects, and/or a most appropriate form of administration (in food, isolated, microencapsulated). In this way, more *in vivo* studies, for a longer time and at different dosages and different forms of administration are needed to improve knowledge about the effects of phenolics compounds from chia. The introduction of the use of extracts in medical practice is restricted, due to the lack of possibility of standardization and control of the constancy of the extract composition, but research in this area is important to elucidate questions about which components of certain foods are responsible for their beneficial effects and to guide future efforts towards enabling the use of these compounds as nutraceuticals.

5. Conclusions

The chia phenolic extract intra-amniotic administration improved markers related to inflammation, intestinal functionality, bacterial population and morphology by reducing TNF-α, increasing the SI gene expression, reducing *E. coli* population, and increasing depth crypt in *Gallus gallus* model. Chia phenolic extract may be a promising beneficial compound for improving intestinal health, and further studies are needed to better elucidate the most appropriate form of administration (in food, isolated, microencapsulated) and the dosage.

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Conflicts of Interest: "The authors declare no conflict of interest.".

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10 GENERAL CONCLUSION

Chia (*Salvia hispanica* L.) is a seed with potential biological effects as it has a rich nutritional composition, with high concentrations of dietary fiber, bioactive peptides, and phenolic compounds. In the present study, we investigated the functional properties of chia flour, its hydrolyzed protein and phenolic extract on microbiota and gut health in different conditions and *in vivo* models.

The first study conducted with male rats demonstrated that consuming chia flour for 35 days had a beneficial effect on intestinal health parameters. Chia consumption in a standard and in a high-fat diet increased the production of short-chain fatty acids (acetic acid, propionic acid and butyric acid), fecal moisture and improved intestinal morphology, increasing the circular muscle layer when associated with a standard diet and the crypt length in the group that received high-fat diet. Besides this, chia decreased the gene expression of *Sglt1*, *Si*, *Ap* and *Pept1* in all groups. The intervention did not affect the diversity and abundance of intestinal bacteria but promoted an increase in richness in the group fed with standard diet and chia.

In the study conducted with *Wistar* female rats, the consumption of chia by ovariectomized rats positively affected the gut health by increasing the production of short chain fatty acids, decreasing the pH of cecum content, improved the morphology, increasing the longitudinal and circular muscle layer and crypt thickness, intestinal functionality (increased *Si* and *Ap* gene expression) and improved richness and decreased dominance. Chia consumption by female SHAM rats demonstrated that chia consumption increased the production of acetic and butyric acids in a SD and propionic acid in a HFD, decreased the pH of cecal content, improved the brush border membrane functionality and increased the richness and diversity of microbiota, although it was not able to change the relative abundance of microbiota and showed lower IgA concentration in an HFD condition.

In both of studies carried out with rats, we observed that although chia did not change the bacterial population, it was able to increase the richness and production of short-chain fatty acids, thus being able to reduce intestinal pH, and also have a positive impact on intestinal histomorphometry. According to our results, the effects of chia flour consumption on short-chain fatty acids production, the increased richness with no changes in relative abundance of microbiota and reduction in *Sglt1* and *Pept1* gene expression were similar between male and female *Wistar* rats.

We conducted a systematic review of *in vivo* studies that found the intake of bioactive peptides derived from food can potentially decrease inflammation by reducing gene expression of *Tnf-α* and can improve intestinal health. This improvement occurs not only through modulation of inflammation but also through the increase in IgA levels and improvements in intestinal morphology. These changes in the intestine, when considered together, may represent the mechanisms by which bioactive peptides exerts beneficial effects on intestinal health.

Therefore, with the aim of obtaining a complete understanding of the effects of chia and its fractions on intestinal health, we isolated chia peptides and total phenolics and demonstrated their effects on intestinal health in an *in ovo* experiment, as the first step towards the evaluation of these compounds in intestinal health, thus starting from a naive organism.

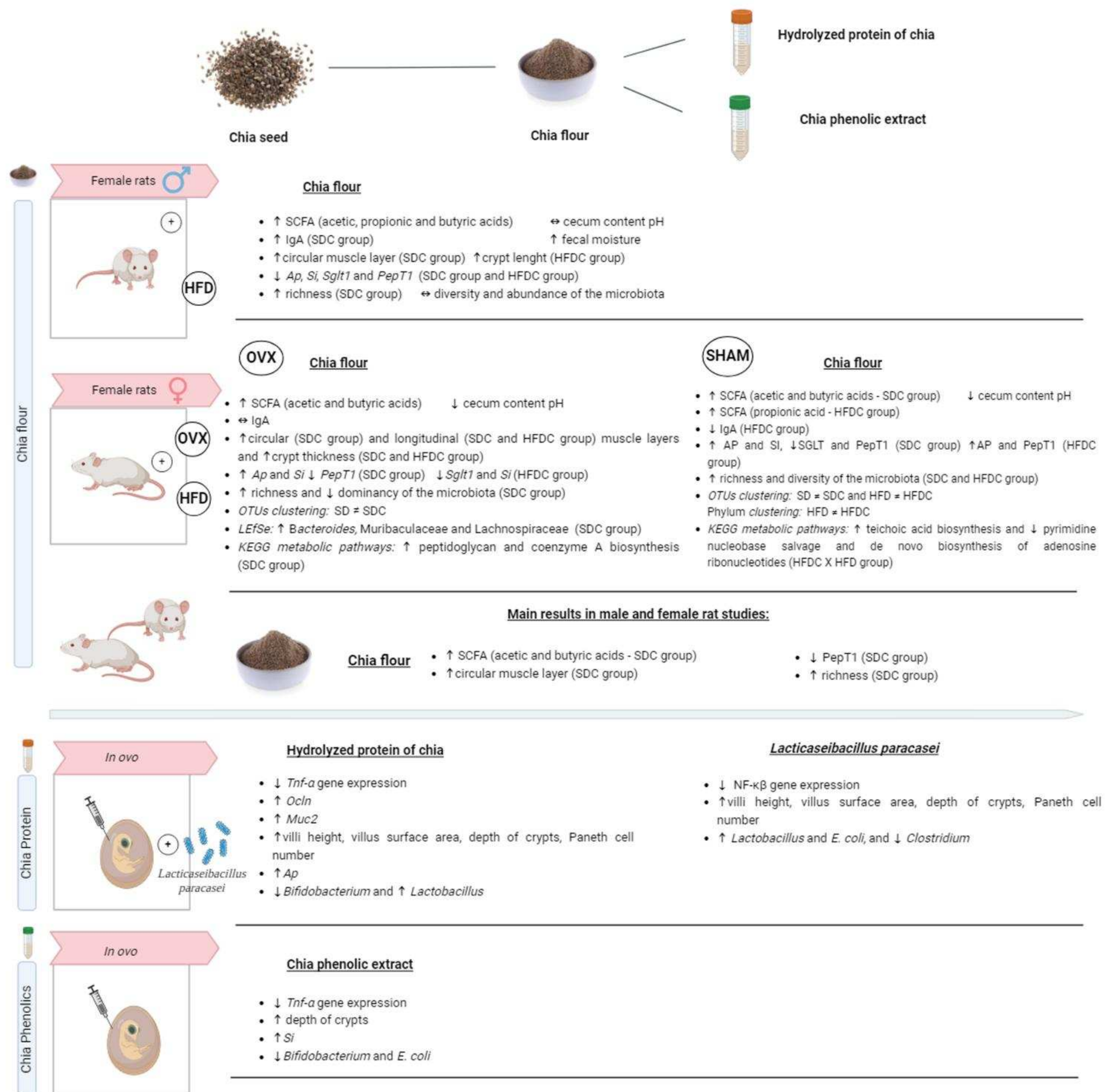
The intra-amniotic administration of hydrolyzed protein of chia decreased the gene expression of *Tnf-α*, increased *Lactobacillus* population, *Ocln*, *Muc2* and *Ap* gene expression, increased villi height, villi surface area and crypt depth, Paneth cell number and goblet cells diameter. The probiotic (*L. paracasei*) administration promoted *Nf-κβ1* reduction, increased *Lactobacillus* and reduced *Clostridium*, and increased villi height, villi surface area and crypt depth, Paneth cell number and goblet cells diameter. Hydrolyzed protein of chia and the probiotic (*L. paracasei*) modulate some aspects of the intestinal health, and it was found more pronounced effects when they were administered separated.

The chia phenolic extract intra-amniotic administration improved markers related to inflammation, intestinal functionality, bacterial population and morphology by reducing *Tnf-α*, increasing the *Si* gene expression, reducing *E. coli* population, and increasing depth crypt in *Gallus gallus* model.

These findings suggest that intra-amniotic administration of hydrolyzed protein of chia or its phenolic extract can improve intestinal health. In these experiments, we also observed a positive impact on morphometric measures of intestine, similar to what was observed in the study with the rats and chia flour. However, here, it was also found that there was a positive impact in inflammatory markers, suggesting that the reduction in intestinal inflammation could be one of the mechanisms by which the chia and its fractions exert beneficial effects on intestinal health.

Thus, our work demonstrated that chia can be considered a food with biological potential to improve intestinal function. These effects were demonstrated in different fractions of the seed: whole grain flour, hydrolyzed protein, and phenolic extract.

Figure 7 – Graphic representation of general conclusion



Functional properties of chia flour, its hydrolyzed protein and phenolic extract on microbiota and gut health in different conditions and *in vivo* models. *Ap*: aminopeptidase; HFD: high-fat diet; HFDC: high-fat diet + chia; IgA: immunoglobulin A; KEGG: Kyoto Encyclopedia of Genes and Genomes; LEfSe: linear discriminant analysis effect size; *Muc2*: mucin 2; *Nf-κβ1*: nuclear factor kappa beta; *Ocln*: occludin; OUT: Operational Taxonomic Units; OVX: ovariectomy; PCR: polymerase chain reaction; *Pept1*: Peptide transporter 1; SDC: standard diet + chia; SFCA: short-chain fatty acids; *Sglt1*: sodium–glucose transport protein; *Si*: isomaltase sucrase; *Tnf-α*: tumor necrosis factor alpha. Source: The author, created with BioRender.com.

11 FINAL CONSIDERATIONS

The present study demonstrated beneficial effects and promising results of chia and its fractions on intestinal health. Considering the importance of the intestinal health in the whole health and the consequences of inflammatory situations, such as a high-fat diet consumption, the chia intake has demonstrated the potential to exert beneficial effects, becoming an option as a functional food to improve the health. Chia has proven to be a seed with important nutritional value, not only its isolated fractions but its whole form consumption (as in flour), showing the importance of nutrient synergy, an important factor to be considered when thinking about nutrition as a way to reduce disease risk and promote health.

Much has already been investigated on chia seeds, but there is still much to be studied. Future efforts, in relation to intestinal health, should be directed towards structuring a robust clinical study, which can fully assess intestinal health. This study can also evaluate the consumption of chia alone or associated with the probiotic used in this study or evaluate the effects on intestinal health of consuming chia in different processes, such as the whole seed, flour, and oil, for example.

In relation to experimental studies, there are pathways that we can consider for future research, such as the intestine-brain axis. The intestinal microbiota, if well-modulated, has the potential to reach various organs of the human body, so it is important to explore these connections. Furthermore, other compounds in addition to dietary fibers have been emerging with prebiotic effects, therefore, given the beneficial results we have had at the intestinal level with chia protein, it would be interesting to advance studies to better understand the mechanisms by which proteins can modulate, not only, the microbiota intestinal, but also, promoting beneficial effects on the intestinal environment. Also, considering the impacts of a high-fat diet, it is important to evaluate the effect of chia consumption, in the form of seeds or oil, on adipose tissue in animals fed a high-fat diet, and how these effects communicate with the rest of the organism, evaluating adipose tissue-brain interactions, for example.

12 OTHER ACTIVITIES

Sandwich Doctorate – 12 months at Cornell University, Ithaca, NY, USA

- Participation in one class as an auditor

Classes – 32 credits completed.

BQI 733, NUT791 (I), TAL 602, NUT 631, NUT 791 (II), NUT634 and MBI661

Estágio em ensino (NUT327)

Seminar Presentation

Project Defense

Qualification

Teaching Assistantship

- NUT 325 - Composição de Alimentos

Participation in Events

- V Congresso Nacional de Alimentos e Nutrição – CONAN
- Nutrition 2023
- Alimentação e impactos na microbiota intestinal
- Como conciliar a mídia e ética profissional?
- Evento comemorativo dos 45 anos do curso de Nutrição e 20 anos do Programa de Pós-Graduação em Ciência da Nutrição da Universidade Federal de Viçosa

Works Presented at Conferences – national and international

- Efeito da farinha de chia associada à dieta hiperlipídica na microbiota intestinal de ratas *Wistar* ovariectomizadas – CONAN (Ouro Preto, MG – Brazil)

- Efeito da chia (*Salvia hispanica* L.) associada à dieta hiperlipídica na microbiota intestinal de ratos *Wistar* – CONAN (Ouro Preto, MG – Brazil)

- Effect of chia (*Salvia hispanica*) on intestinal health of female *Wistar* rats fed with a high-fat diet - International Congress on Bioactive Compounds (Campinas, SP – Brazil)

- Effects of Intra-Amniotic Administration of the Hydrolyzed Protein of Chia and *Lacticaseibacillus paracasei* on Intestinal Parameters *In vivo* (*Gallus gallus*) - Nutrition 2023 (Boston, MA – United States of America)

Book Chapter

- Book: Imunologia aplicada à Nutrição – Chapter: Nutrientes e Compostos Bioativos no Fortalecimento do Sistema Imune contra à Inflamação e a Osteoporose

Seminar Committee

- Efeito de bebidas probiótica (*Lactobacillus rhamnosus*) e simbiótica com sorgo germinado extrusado ([*Sorghum bicolor* (L.) *Moench*]) na resposta glicêmica, saciedade e saúde intestinal de adultos - Doctorade

- Ação moduladora da farinha e óleo de chia na microbiota intestinal de ratos *Wistar* com alterações metabólicas - Masters

- Efeito do consumo de jabuticaba (*Myrciaria cauliflora*) em pó na saúde intestinal de camundongos alimentados com dieta hiperlipídica rica em frutose - Doctorade

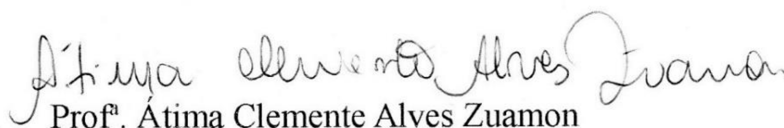
APPENDIX A - Protocol of approval by the Ethics Committee on Animal Use at Universidade Federal de Viçosa (male rats study)

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 97/2014, intitulado *"Chia Salvia hispanica L.: ocorrência e concentração de nutrientes e compostos bioativos, qualidade proteica e biodisponibilidade de ferro"*, coordenado pela professora Helena Maria Pinheiro Sant'Ana 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 13/03/2015, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 97/2014, named " Chia Salvia hispanica L .: occurrence and concentration of nutrients and bioactive compounds, protein quality and bioavailability of iron", is in agreement with the a ctual 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 March 13, 2015 valid for 12 months.



Profª. Átima Clemente Alves Zuamon
Presidente
Comissão de Ética no Uso de Animais - CEUA/UFV

APPENDIX B - Protocol of approval by the Ethics Committee on Animal Use at Universidade Federal de Viçosa (female rats study)

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 20/2017, intitulado **"Efeito da ingestão de chia *Salvia hispanica L.* na biodisponibilidade e na expressão gênica de proteínas envolvidas no metabolismo de cálcio em ratos wistar alimentados com dieta hiperlipídica"**, coordenado pela professora Hércia Stampini Duarte Martno 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 11/07/2017, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 20/2017, named **"Effect of ingestion of chia Salvia hispanica L. on the bioavailability and gene expression of proteins involved in calcium metabolism in wistar rats fed a hyperlipidic diet"**, is in agreement with the a actual Brazilian legislation (Lei Nº 11.794, 2008), Normative Resolutions edited by CONCEA/MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific Purposes and Teaching) and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI therefore being approved by the Committee on July 11, 2017 valid for 12 months.

Profª. Átima Clemente Alves Zuanon

Presidente

Comissão de Ética no Uso de Animais – CEUA/UFV