

BÁRBARA PEREIRA DA SILVA

**EFFECT OF CHIA (*Salvia hispanica* L.) ON THE BIOAVAILABILITY OF
MINERALS, LIPID PROFILE, INFLAMMATION, OXIDATIVE STRESS, AND
INTESTINAL HEALTH**

Thesis submitted to the Federal University of
Viçosa, as part of the requirements of Program in
Science of Nutrition for obtaining the title of
Doctor Scientiae.

Adviser: Hércia Stampini Duarte Martino

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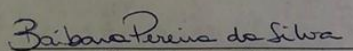
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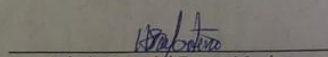
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ABSTRACT

SILVA, Bárbara Pereira da, D.Sc., Universidade Federal de Viçosa, December, 2019. **Effect of chia (*Salvia hispanica* L.) on the bioavailability of minerals, lipid profile, inflammation, oxidative stress, and intestinal health.** Adviser: Hércia Stampini Duarte Martino. Co-advisers: Neuza Maria Brunoro Costa, Maria Eliza de Castro Moreira, and Helena Maria Pinheiro Sant'Ana.

Chia is a pseudocereal that consumed worldwide due to its protective, functional and antioxidant effects, attributed to the presence of lipids, dietary fiber, antioxidant compounds, vitamins and minerals. Among the minerals, calcium, iron and zinc can be highlighted. But although present in good concentration, the bioavailability of these nutrients is unknown. Also, the effect of chia consumption on inflammatory markers and oxidative stress is not known in animals fed a high-fat diet. Furthermore, the effects of intra-amniotic administration of prebiotics extracted from chia on the morphology and functionality intestinal, on intestinal microbiota and on iron and zinc status are not known. In this sense, the objective of the study was to evaluate the effect of chia (*Salvia hispanica* L.) flour consumption on the bioavailability of calcium, iron and zinc, lipid profile, inflammation, oxidative stress, intestinal functionality and morphology, and intestinal microbiota. Chia flour cultivated in Brazil (RS state) was used. To obtain the flour, the seeds were ground. In the first biological essay (**Manuscript 1**), the effect of chia consumption on calcium bioavailability, inflammation, oxidative stress was conducted during 7 weeks. Thirty-two 21-day-old male *Wistar* rats were given either the AIN-93G diet or the high-fat diet. The study consisted of 4 experimental groups: AIN-93G + calcium carbonate, AIN-93G + chia, high-fat diet + calcium carbonate and high-fat diet + chia. The effects of chia intake on calcium bioavailability were measured using the calcium balance technique. In addition, the effect of chia flour on biochemical markers, inflammation and oxidative stress was evaluated. Chia presented low calcium bioavailability, regardless of the type of diet consumed. However, chia consumption reduced inflammatory processes, improved lipid profile and had no effect on oxidative stress. In the second biological essay (**Manuscript 2**), the effect of chia consumption on oxidative stress and inflammation in ovariectomized adult rats was evaluated. Eighty 21-day-old female *Wistar* rats were used. The animals received AIN93-G diet (n = 40) or high fat diet (n = 40) for 7 weeks. At week seven, 40 rats underwent ovariectomy (OVX) and 40 rats underwent surgery, but without organ removal (SHAM). The animals remained in these groups for 3 weeks to recover from surgery. Thus, after 10 weeks, the animals were relocated to the following eight experimental groups, where they remained receiving the experimental diets

for 8 weeks: 1) control diet (SHAM), 2) high fat diet (SHAM), 3) control diet + chia (SHAM), 4) high fat diet + chia (SHAM), 5) control diet (OVX), 6) high fat diet (OVX), 7) control diet + chia (OVX), 8) high fat diet + chia (OVX). After 18 weeks, the animals were euthanized. Gene expression of protein related to inflammation and oxidative stress were measured. It was evaluated chia consumption on biochemical markers. Intake of chia improved lipid profile and increased hepatic and cecal indexes. In addition, chia consumption associated with the standard diet improved antioxidant activity, increased SOD and PPAR- α gene expression, and catalase activity, while reducing NF κ B expression. Chia consumption associated with high fat diet in ovariectomized rats reduced IL-1 β levels and TNF- α expression, increased SOD expression and concentration, and catalase activity. In the third study (**Manuscript 3**), we evaluated the effect of intra-amniotic administration of prebiotics extracted from chia on gene expression of proteins related to iron and zinc metabolism, as well as effect of prebiotic on intestinal functionality, intestinal morphology and bacterial population in a broiler model (*Gallus gallus*). The concentration of dietary fiber and phenolic compounds in chia flour was evaluated. Seven experimental groups were used (Group 1: non-injected; Group 2: 18M Ω H₂O; Group 3: 40mg/mL Inulin; Group 4: 0.5% chia; Group 5: 1% chia; Group 6: 2.5% chia; Group 7: 5% chia). The gene expression of iron and zinc-related proteins was performed by RT-qPCR as well as the expression of brush border membrane proteins and the bacterial composition was evaluated by PCR. Measurements of villi and crypts as well as the number and diameters of goblet cell were evaluated by histological techniques. Intra-amniotic administration of soluble chia extract improved intestinal morphology and protein expression related to Zn metabolism. In addition, chia soluble extract improved gene expression of proteins related to iron metabolism. The consumption of chia demonstrated improve intestinal health and contribute to the absorption of minerals. Thus, in general, our results demonstrated that chia was capable to improve the lipid profile, inflammatory process, oxidative stress, intestinal morphology, intestinal functionality and intestinal microbiota, *in vivo*. In addition, the consumption of this food had low bioavailability of minerals, such as, calcium.

Keywords: Bone metabolism. Calcium. Inflammatory process. Intestinal microbiota. Iron. Zinc.

RESUMO

SILVA, Bárbara Pereira da, D.Sc., Universidade Federal de Viçosa, dezembro de 2019. **Efeito da chia (*Salvia hispanica* L.) na biodisponibilidade de minerais, no perfil de lipídio, na inflamação, no estresse oxidativo e na saúde intestinal.** Orientadora: Prof^a. Hércia Stampini Duarte Martino. Coorientadoras: Neuza Maria Brunoro Costa, Maria Eliza de Castro Moreira e Helena Maria Pinheiro Sant'Ana.

A chia é um pseudocereal que vem sendo consumido pela população mundial devido aos seus efeitos protetores, funcionais e antioxidantes, atribuídos à presença de lipídios, fibra alimentar, compostos antioxidantes, vitaminas e minerais. Dentre os minerais, pode-se destacar o cálcio, o ferro e o zinco. Mas, embora possua boa concentração, a biodisponibilidade desses nutrientes não é conhecida. Também, não se sabe o efeito do consumo de chia sobre os marcadores inflamatórios e de estresse oxidativo em animais alimentados com dieta hiperlipídica. Além disso, não são conhecidos os efeitos da administração intra-aminotica de prebióticos extraídos da chia na morfologia e funcionalidade intestinal, na microbiota intestinal e na absorção de ferro e zinco. Nesse sentido, o objetivo do estudo foi avaliar o efeito do consumo de farinha chia (*Salvia hispanica* L.) na biodisponibilidade de cálcio, ferro e zinco, no perfil de lipídio, na inflamação, no estresse oxidativo, na funcionalidade e morfologia intestinal e na microbiota intestinal. Foi utilizada farinha de chia cultivada no Brasil (RS). Para obtenção da farinha, as sementes foram moídas. No primeiro ensaio biológico (**Manuscrito 1**), avaliou-se o efeito do consumo de farinha de chia sobre a inflamação, estresse oxidativo e biodisponibilidade de cálcio por sete semanas. Foram utilizados 32 ratos *Wistar* machos, com 21 dias de vida, que receberam dieta AIN-93G ou dieta hiperlipídica. O estudo foi constituído de 4 grupos experimentais, sendo eles: AIN-93G + carbonato de cálcio, AIN-93G + chia, dieta hiperlipídica + carbonato de cálcio e dieta hiperlipídica + chia. Foram mensurados os efeitos da ingestão de chia na biodisponibilidade de cálcio, por meio da técnica de balanço de cálcio. Além disso, foi avaliado o efeito da farinha de chia nos marcadores bioquímicos, na inflamação e no estresse oxidativo. A chia apresentou baixa biodisponibilidade de cálcio, independentemente do tipo de dieta consumida. No entanto, o consumo de chia reduziu o processo inflamatório, melhorou o perfil de lipídio e não teve efeito sobre o estresse oxidativo. No segundo ensaio biológico (**Manuscrito 2**), foi avaliado o efeito do consumo de chia no estresse oxidativo e na inflamação em ratas adultas ovariectomizadas. Foram utilizadas 80 ratas *Wistar*, com 21 dias de vida, que receberam dieta AIN93-G (n=40) ou dieta hiperlipídica (n = 40) por 7 semanas. Na sétima semana, 40 ratas foram submetidas a ovariectomia (OVX) e 40 ratas foram

submetidas a cirurgia, porém sem a retirada do órgão (SHAM). Os animais permaneceram nesses grupos por 3 semanas, para recuperação da cirurgia. Assim, após 10 semanas, os animais foram realocados nos seguintes oito grupos experimentais, onde permaneceram recebendo as dietas experimentais por 8 semanas: 1) dieta controle (SHAM), 2) dieta hiperlipídica (SHAM), 3) dieta controle + chia (SHAM), 4) dieta hiperlipídica + chia (SHAM), 5) controle (OVX), 6) dieta hiperlipídica (OVX), 7) dieta controle + chia (OVX), 8) dieta hiperlipídica + chia (OVX). Após 18 semanas, os animais foram eutanasiados. Foi avaliada a expressão gênica de proteínas relacionadas a inflamação e ao estresse oxidativo e o efeito da chia nos marcadores bioquímicos. A ingestão de chia melhorou o perfil de lipídios e aumentou os índices hepático e cecal. Além disso, o consumo de chia associado à dieta padrão melhorou a atividade antioxidante, aumentou a expressão gênica de SOD e PPAR- α e a atividade da catalase, ao mesmo tempo em que reduziu a expressão de NF κ B. A ingestão de chia associada à dieta rica em gordura em ratas ovariectomizadas reduziu os níveis de IL-1 β e a expressão de TNF- α , aumentou a expressão e a concentração de SOD e a atividade da catalase. No terceiro estudo (**Manuscrito 3**), avaliou-se o efeito da administração intra-amniótica de prebióticos extraídos da chia sobre a expressão gênica de proteínas relacionadas ao metabolismo de ferro e zinco, assim como o efeito do prebiótico na funcionalidade e morfologia intestinal e na população bacteriana em modelo de aves (*Gallus gallus*). Foi avaliada a concentração de fibra alimentar e de compostos fenólicos na farinha de chia. Foram utilizados sete grupos experimentais (Grupo 1: não-injetado; Grupo 2: 18M Ω H₂O; Grupo 3: 40mg/mL Inulina; Grupo 4: 0,5% de chia; Grupo 5: 1% de chia; Grupo 6: 2,5% de chia; Grupo 7: 5% de chia). A expressão gênica das proteínas relacionadas ao ferro e ao zinco foi realizada por RT-qPCR, assim como a expressão das proteínas da membrana da borda em escova e a composição bacteriana foi avaliada por PCR. Foi avaliada as medidas de vilosidades e criptas assim como o número de diâmetros das células caliciformes por meio de técnicas histológicas. A administração intra-amniótica de extrato solúvel de chia melhorou a morfologia intestinal e a expressão gênica de proteínas relacionadas ao metabolismo de Zn. Além disso, a administração de extrato aumentou a expressão gênica de proteínas relacionadas ao metabolismo de ferro. Assim, o consumo de chia demonstrou melhorar a saúde intestinal e contribuir para a absorção de minerais. De modo geral, nossos resultados demonstraram que o consumo de chia foi capaz de melhorar o perfil de lipídio, o processo inflamatório, o estresse oxidativo, assim como a morfologia, funcionalidade e microbiota

intestinal, *in vivo*. Além disso, o consumo desse alimento apresentou baixa biodisponibilidade de minerais, como o cálcio.

Palavras-chave: Cálcio. Ferro. Metabolismo ósseo. Microbiota intestinal. Processo inflamatório. Zinco.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABS	Maximum absorbance
AIN-93G	Rodent Diet AIN-93G
ALT	Alanine aminotransferase
AST	Alanine aminoaspartase
ATP	Adenosine triphosphate
ATPase	Adenosinetriphosphatase
ANOVA	Analysis of variance
BBM	Brush border membrane
cDNA	DNA complementary
Cm	Centimeters
CVD	Cardiovascular disease
DcytB	Duodenal cytochrome b
DMT-1	Divalent metal transporter
DNAse	Deoxyribonuclease
EDTA	Acid etilen diamino tetraacetic acid
FAS	Fatty acid synthase
FER	Food efficiency ratio
Fe ⁺²	Ferrous iron
Fe ⁺³	Ferric iron
G	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HCl	Hydrochloric acid
HDL	High density lipoprotein cholesterol
HFD+C	High fat diet + chia
HFD+CC	High fat diet + calcium carbonate
HF	High fat diet
HFC	High fat diet + chia
H ₂ O	Water
H ₂ O ₂	Hydrogen peroxide
H ₃ PO ₄	Phosphoric acid
IκB	IκB kinase

IKK	I κ B kinase
IL-1 β	Interleukin 1 beta
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-12	Interleukin 12
Kcal	Kilocalorie
Kg	Kilogram
LDL	Low density lipoprotein cholesterol
MDA	Malondialdehyde
MTT	1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan
mM	Milimolar
Min	Minutes
Mg	Milligram
M Ω	Megaohm
mRNA	Messenger RNA
μ M	Micromollar
μ L	Microliter
NF- κ B	Nuclear factor kappa B
NO	Nitric oxide
OPG	Osteoprotegerin
OVX	Ovariectomized
PCR	Polymerase chain reaction
PPAR- α	Peroxisome proliferator-activated receptor α
PPAR- γ	Peroxisome proliferator-activated receptor γ
Ppm	Part per million
RS	Rio Grande do Sul
PTH	Parathyroid hormone
PTN	Protein
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
ROS	Reactive oxygen species
RHS	Reactive hydrogen species
RT-qPCR	Polymerase chain reaction in real time

SD	Standard deviation
SD+C	Standard diet + chia
SD+CC	Standard diet + calcium carbonate
SHAM	Non-ovariectomized
SOD	Superoxide dismutase
ST	Standard diet
STC	Standard diet + chia
TBARS	Thiobarbituric acid reactive substances
TC	Total cholesterol
TGL	Triacylglycerides
TNF- α	Tumor necrosis factor alpha
V	Volume
VLDL	Very low density lipoprotein cholesterol
w-3	Omega 3
w-6	Omega 6
ZIP	Zinc transporter ZIP
ZnT	Zinc transporter ZnT

SYMBOL LIST

® - Registered Mark

α - Alpha

β - Beta

γ - Gamma

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1. GENERAL INTRODUCTION

The *Salvia* genus, native to southern of Mexico and northern of Guatemala, comprises approximately 900 seed species of the *Lamiaceae* family (IXTAINA, NOLASCO, TOMÁS, 2008; LU, YEAP FOO, 2002). Of these species, 61 are cultivated in Brazil. The largest cultivation of seeds of this genus occurs in mountainous regions of temperate to subtropical weather (CAPITANI et al., 2012; HARLEY, 2012; GIULIETTI, HARLEY, 2005). Among the species of the *Salvia* genus, chia (*Salvia hispanica* L.) is an herbaceous plant with high nutritional and functional value (IXTAINA, NOLASCO, TOMÁS, 2008).

The energetic value of chia corresponds to 486 kcal/100g (DA SILVA et al., 2017; USDA, 2011). The concentration of lipids in the seed varies between 30 and 38%, of which approximately 70% correspond to α -linolenic acid (w-3) and 19% to linoleic acid (w-6) (FERREIRA, 2013). Chia is also a source of dietary fiber (approximately 30%), mainly of insoluble type, which correspond to approximately 95% of the total fiber concentration (FERREIRA, 2013; VÁZQUEZ-OVANDO et al., 2009; MONROY-TORRES et al., 2008). In addition, the seed has a high protein concentration (18-25%), carotenoids, minerals, vitamins and bioactive compounds, such as phenolics compounds (rosmarinic acid, myricetin, quercetin, kaempferol and caffeic acid) (DA SILVA et al., 2017; OLIVEIRA-ALVES, 2017).

It is known that chia presents hypoglycemic (VUKSAN et al., 2017), anti-inflammatory (HAMEDİ et al., 2016), antioxidant (SCAPIN et al., 2016), antihypertensive (TOSCANO et al., 2014) and cardioprotective actions (ULLAH et al., 2016). These protective, functional and antioxidant effects are attributed to the presence of polyunsaturated lipids, dietary fiber, antioxidant compounds, vitamins and minerals (DA SILVA et al., 2017; CVETKOVİKJ et al., 2013; CAPITANI et al., 2012; MOHD ALI et al., 2012; AYERZA, COATES, 2011).

Among the minerals, chia contains calcium (480 mg/100 g), zinc (3.7 mg/100 g) and iron (9.39 mg/100 g) (DA SILVA et al., 2017). So, a serving portion of chia (40 g/day) provides about 192 mg of calcium, 1.5 mg of zinc and 3.76 mg of iron, which corresponds to 19%, 19%, and 34% of the RDA for an adult man. But, although present in high concentrations, one should take into account the bioavailability of these nutrients for use in metabolic processes and for storage in the human body (ARGYRI et al., 2011; JAFARI, MCCLEMENTS, 2017). It is known that some components present in the food can affect the bioavailability of calcium, iron and zinc, such as phytate, tannin and oxalate, as well as phytate/calcium, zinc/iron and phytate/zinc ratio. However, chia has a low concentration of

tannin and phytate, which could benefit the bioavailability of the minerals (DA SILVA et al., 2017).

Calcium is the most abundant divalent mineral in the human body, concentrating 99% on bones and teeth (ELIAZ, METOKI, 2017). It is an essential mineral for bone maintenance, besides being one of the main intracellular messengers and cofactor of extracellular enzymes and proteins. The percentage of absorption usually varies inversely with consumption, but the absolute amount absorbed increases with consumption (SHARP, 2017). In the current food scenario that the population experiences, with high consumption of sugars and fats, the increase in cases of cardiovascular diseases (CVD) are evident (COCHAIN, ZERNECKE, 2017; LOUZADA et al., 2015). However, although the association between low calcium intake and the development of CVD has been verified (SOARES et al., 2017), it has not been proven that the increase in the levels of inflammatory markers resulting from the ingestion of a high fat diet may be associated with a reduction of calcium absorption.

Iron deficiency is the most common nutritional deficiency in the world (KASSEBAUM et al., 2014). Iron is an essential component of hemoglobin in red blood cells and of myoglobin in muscles, which contain around 60% of total body iron (LOPEZ et al. 2016). It is necessary for the functioning of cellular mechanisms, including enzymatic processes, DNA synthesis, and mitochondrial energy generation (STEINBICKER, MUCKENTHALER, 2013). Dietary iron is available in two forms: haem and non-haem iron. Iron is found in haemoglobin in the haem form, which is present in animal food sources, such as meat, poultry, and seafood. Non-haem iron is present in the plant foods, such as tea, cacao, cereals, dried fruit and seeds, mainly as Fe^{+3} or ferric iron (MCDERMID, LÖNNERDAL, 2012). Haem iron is estimated to contribute 10–15% of total iron intake in meat-eating populations, and is generally better absorbed (HURRELL, EGLI, 2010).

Zinc deficiency is one of the most prevalent nutritional deficiencies in the world (BLACK et al., 2013; WESSELL, BROWN, 2012). Among the factors that contribute to zinc deficiency, it is possible to associate the low intake of the mineral with its low bioavailability in food (MARET, SANDSTEAD, 2006). The deficiency is related to alteration in the process of cell division and differentiation and alteration in protein synthesis and metabolism of fatty acids. Besides this, affects growth and development, as well as increases the risk of infection by infectious diseases (WINTERGERST, MAGGINI, HORNIG, 2007; FISCHER-WALKER et al., 2011).

The use of minerals-rich foods may be a good strategy to contribute to intestinal morphology and physiology, since studies have shown the influence of the nutritional state of minerals, such as iron and zinc, on the intestinal microbiota (REED et al., 2015; ZACKULAR et al., 2016; MAYNERIS-PERXACHS et al., 2016). On the other hand, changes in bacterial concentration are also capable of cause changes in the intestinal microbiota, increasing or reducing certain predominant phyla, which in turn interfere with the absorption and bioavailability of minerals (REED et al., 2015). Thus, both minerals deficiency alters the intestinal bacterial composition and the altered bacterial composition may lead to reduced mineral absorption. In addition, studies show that, in the face of a mineral deficiency situation, there is an increase in inflammation and intestinal permeability, impairing the individual's health (WAPNIR, 2000).

A previous study conducted by our research group found that chia has high bioavailability of iron (DA SILVA et al., 2016). However, there are no *in vivo* studies that evaluated the calcium bioavailability of chia and whether the ingestion of a high fat diet may interfere with the calcium bioavailability, as well as how the bioactive compounds of chia can modulate the inflammation and the oxidative stress. Also, the anti-inflammatory activity of chia in adult female *Wistar* rats submitted to ovariectomy has not been previously reported. In addition, there are no studies evaluating the bioavailability of iron and zinc in soluble extracts from chia as well as the influence of the administration of this extracts on the intestinal microbiota and on intestinal and functional morphology.

2. OBJECTIVES

2.1. General Objective

To investigate chia (*Salvia hispanica* L.) consumption effects on calcium, zinc and iron bioavailability, lipid profile, inflammation, oxidative stress, intestinal morphology, intestinal functionality and intestinal microbiota.

2.2. Specific Objectives

More specifically, the study aimed to evaluate:

- The phenolic compounds of chia flour;
- The effect of chia consumption on the feed efficiency ratio and weight gain of experimental animals;

- Calcium bioavailability of chia flour in male *Wistar* rats fed standard diet and high fat diet;
- The effect of chia on lipid profile;
- The effect of chia flour on biochemical markers and oxidative stress in male and female *Wistar* rats fed standard diet or high fat diet;
- The effect of chia flour on the gene expression and on the quantification of proteins related to oxidative stress and the inflammatory process in male and female *Wistar* rats fed standard diet or high fat diet;
- The prebiotics extracted from chia in terms of total dietary fiber, soluble dietary fiber, insoluble dietary fiber, phytic acid, iron and zinc;
- The effect of prebiotic administration extracted from chia on biomarkers of zinc and iron status, *in ovo* (*Gallus gallus*);
- The effect of prebiotic administration extracted from chia on the microbiota composition, *in ovo* (*Gallus gallus*);
- The effect of prebiotic administration extracted from chia on the intestinal histology and intestinal functionality, *in ovo* (*Gallus gallus*);

3. HYPOTHESIS

The diets containing chia flour will have high bioavailability of calcium, and the consumption of the high fat diet without chia flour will promote a reduction in calcium absorption in *Wistar* rats. Also, chia, as a source of bioactive compounds, can improve lipid homeostasis, and modulate inflammation and oxidative stress, improving metabolic changes caused by the high fat diet consumption. In addition, the administration of prebiotics extracted from chia may be able to promote beneficial changes in intestinal histology and functionality as well as can improve the intestinal microbiota. Besides this, the administration of this prebiotic can increase the bioavailability of iron and zinc *in ovo* (*Gallus gallus*).

4. LITERATURE REVIEW

4.1. Chia: food with functional properties and its effect on inflammation

Functional foods have been increasing the interest of consumers and food industries because of the diversity of bioactive compounds and the broad antioxidant and anti-inflammatory properties present in their food matrix (TRIGUEROS et al., 2013). Chia is a pseudocereal, oval shaped, with a length between 1 and 2 mm, a diameter between 0.8 and 1.3

mm and a width between 0.8 and 1.4 mm, and has a smooth and shiny shell with different colorations: black, brown, gray, black spotted and white (MUÑOZ et al., 2013). It can be consumed whole or ground, added to yogurts, salads, soups and fruits (VUKSAN et al., 2007). In addition, this food has been used in the elaboration of complex preparations such as breads, cakes, cereal bars, biscuits, jellies, beverages, sausages, among other foods (JUSTO et al., 2007; BORNEO, AGUIRRE, LEÓN, 2010).

Although the nutritional composition of chia seeds varies according to cultivation conditions, in general this food has high nutritional value and a large amount of bioactive compounds that are promising for human health (DA SILVA et al., 2017). The high concentration of dietary fiber in the food matrix helps in the volume of the faecal content as well as it promotes greater satiety and consequently reduces the food intake (BURTON-FREEMAN et al., 2017). In addition, the fermentation of soluble dietary fiber produces short chain fatty acids that are associated with many benefits in the body, such as cholesterol reduction, anticancer effect, increased satiety, immunomodulation, anti-inflammatory action, among other benefits (AL-LAHHAM et al., 2010; ZAPOLSKA-DOWNAR, NARUSZEWICZ, 2009; BAILÓN, 2010). Because it has a high concentration of polyunsaturated fatty acids, chia ingestion helps in the reduction of triacylglycerides and plasma LDL in animal and human models (DA SILVA et al., 2016; GUEVARA-CRUZ et al., 2012).

The demographic, cultural and socioeconomic changes that occurred caused changes in eating patterns. Therefore, in the last decades, there is a process of nutritional transition in Brazil, characterized by the progressive decrease of malnutrition and increase of obesity (SCHMIDT et al., 2011; DE SOUZA, 2017). The increase in energy consumption, mainly processed foods, with a high content of simple sugars and saturated fats, associated with lower energy expenditure attributed to the reduction in physical activity, explain the increasing tendency of overweight, obesity and associated diseases in the Brazilian population (SCHMIDT et al., 2011).

Superoxide anion ($O_2^{\bullet}$) and hydroxyl radical ($^{\bullet}OH$) are important reactive oxygen species (ROS) used as biomarkers in physiological and pathological processes. The ROS generation is closely related to the development of a variety of inflammatory diseases. Although $O_2^{\bullet}$ and $^{\bullet}OH$ in normal cells with low concentration are essential for the life, their overaccumulation also can leads to oxidative stress and oxidative damage, and bringing many pathological conditions, such as inflammation, cellular damage and cancer (CHEN et al.,

2011; NATHAN, CUNNINGHAM-BUSSEL, 2013). On the other hand, the inflammation plays a key role in pathogen invasion, tissue repair, adjustment of stress response, and the pathogenesis of various diseases. Inflammation provide a microenvironment, where can produce more $O_2^{\cdot}$ and $\cdot OH$, and the ROS produced is closely related to the development of a variety of inflammatory diseases (LI et al., 2015).

A study conducted with *Wistar* rats who ingested chia seed for five weeks showed that the group that received chia flour had a increase in PPAR- α protein levels whereas TNF- α and IL-10 were lower in these animals. NF κ B mRNA expression and protein levels were lower in the groups that received chia (DA SILVA et al., 2019a). Thus, chia intake was able to mproved inflammation in young rats. Another study with *Wistar* female rats showed that chia consumption for 18 weeks was able to improve the plasma catalase activity, PPAR- α mRNA expression and reduce the NF κ B mRNA expression, and protein levels of TNF- α and IL-1 β . Besides this, chia intake improved antioxidant activity by increasing SOD expression (DA SILVA et al., 2019b). Therefore, one study assessed the anti-inflammatory property of chia seed oil per se and in combination with other vegetable oils. The authors noted that Indian chia seed oil improved the *in vitro* anti-inflammatory effects due the chemical characteristics and composition of this food (GAZEM et al., 2016).

Therefore, consumption of chia may be a viable alternative in the offering of antioxidant and anti-inflammatory compounds, besides contributing vitamins and minerals, among them calcium, zinc and iron. Also, the deficiency of these minerals is directly related to the development of non-communicable chronic diseases, being important their intake in adequate quantities (COCHAIN, ZERNECKE, 2017; LOUZADA et al., 2015).

4.2. Chia: action on the bioavailability of minerals

4.2.1. Calcium

Calcium is an essential mineral and is involved in several physiological processes, among them the maintenance of cellular functions and the development of bones and teeth (ERFANIAN, RASTI, MANAP, 2017; SOTO, GARCIA, SELGAS, 2016). Among the minerals present in the body, calcium is the most abundant, comprising about 1.5-2.0% of body weight (COXAM, 2007).

The daily recommendation for calcium intake is 1,000 mg / day for individuals aged between 19 and 50 years (IOM, 2010), and the low mineral intake is related to the increased risk of developing osteoporosis (SKIBSTED, 2016). Osteoporosis, caused by deficiency of

calcium intake or absorption, affects 75 million people worldwide (THORPE, EVANS, 2011). Currently, calcium intake has been decreasing in all age groups (GREUPNER, SCHNEIDER, HAHN, 2017). Data from the National Health and Nutrition Examination Survey indicate that 68% of American adults do not meet the daily calcium recommendation (BAILEY et al., 2010). The same is true for women (13%-18%, 13-34 years) and for the elderly (8%-15%) in Europe (CASHMAN, 2002). In Brazil, the consumption of this mineral is also below the recommended level, reaching approximately 48.9% of men and 61.3% of women (BRASIL, 2008).

Adequacy of calcium intake is necessary for bone health, both during growth and development in order to maintain a good bone mass concentration (COXAM, HORCAJADA, 2004). The most effective strategy to avoid calcium deficiency is to increase the intake, by consuming food sources such as milk, fish and viscera and some vegetables (ALBARRACIN et al., 2014; TRAILOKYA et al., 2017).

The average calcium absorption in adults corresponds to about 30%, and most of the absorption of this mineral occurs in the small intestine (ALBARRACIN et al., 2014). Calcium uptake occurs through two mechanisms: ATP-dependent, saturated and regulated vitamin D-dependent transcellular transport, occurring predominantly in the proximal duodenum and jejunum, and by unsaturated paracellular transport, where uptake occurs through the intercellular space (SKIBSTED, 2016), occurring mainly in the distal jejunum and ileum (GUÉGUEN, POINTILLART, 2000).

Calcium is accumulated in the skeleton along with phosphate (in the predominant form of hydroxyapatite), whereas soluble calcium is found in the protein-bound extracellular portion (ZHOU, XUE, YANG, 2013). Unabsorbed calcium is excreted as insoluble salts in the faeces and the excess of absorbed calcium is excreted in the urine (SKIBSTED, 2016).

4.2.1.1. Homeostasis and bioavailability of calcium

Calcium is classified as an essential mineral because it has both structural and functional functions. Intracellular calcium acts as the second intracellular messenger, being the cofactor for several proteins and enzymes that regulate neurotransmission and motility processes, hormonal processes, secretion and cell proliferation. Extracellular calcium is the bone mineral component and acts as a cofactor for adhesion molecules, coagulation factors and other proteins (CIVITELLI, ZIAMBARAS 2011; FLAMMINI et al., 2016).

When the intake of calcium is insufficient, the body tries to maintain the homeostasis of this mineral through the activation of the parathyroid glands. Activated, the glands release the parathyroid hormone (PTH), which is able to mobilize calcium from the bones, releasing it into the bloodstream. Since most of the calcium present in the body is found in the bones, the cellular and blood concentrations are mainly maintained (COXAM, 2007; LIM, YEO, TAN, 2017).

The regulation of plasma levels is achieved through a complex physiological system that includes the interaction of calcitropic hormones such as parathyroid hormone (PTH) and dihydrocolecalciferol (vitamin D₃) and calcitonin, which act on specific receptors in the kidneys, bones and intestines and maintain the balance between the three forms of calcium (linked to proteins, complexed with citrate, phosphate or bicarbonate and free ions) (LAMBERT, HAKIM, LANHAM-NEW, 2017).

The secretion of these hormones is governed partially or totally by the plasma concentrations of ionized calcium, in a negative control system. When the concentration is low, parathyroid hormone secretion occurs, which mobilizes calcium from the bones and increases its renal resorption, as well as promote the activation of vitamin D in calcitriol, which in turn acts not only on renal reabsorption and calcium withdrawal from bones, but also increases the intestinal absorption by the active route. An increase in the plasma concentration of calcium ions stimulates the secretion of calcitonin, which favors the deposit of calcium in the bones and increases the renal excretion (LAMBERT, HAKIM, LANHAM-NEW, 2017).

The maintain of adequate serum calcium concentrations is essential, since the intracellular functions and the maintenance of cellular calcium levels should be well regulated. The normal serum calcium level is about 10 mg/100 mL of blood. The receptor is saturable, stimulated by 1,25 (OH) D₃ (calcitriol), regulated by dietary intake and body requirement. Calcitriol influences active transport, increasing membrane permeability, regulating the migration of calcium through intestinal cells and increasing the level of calbindin, which is a calcium-carrying protein. Thus, active transport is characterized as the main mechanism of calcium absorption when the intake of this component is low (LAMBERT, HAKIM, LANHAM-NEW, 2017).

After absorption by the enterocyte, calcium is released into the bloodstream by an ATP-dependent system, in which phosphorylation occurs that induces conformational modifications in the enzyme calcium ATPase. This mechanism is also dependent on vitamin D and is assumed to increase the number of enzymes, and the number of pumps in the

enterocyte. Several minerals such as barium, strontium, manganese and lead compete with calcium for calbindin and some of them have stronger binding than calcium itself (LAMBERT, HAKIM, LANHAM-NEW, 2017).

Calcium-related bioavailability depends on the body's ability to digest, metabolize and absorb this nutrient. However, other factors may also affect the bioavailability of calcium through different mechanisms, such as during absorption, transport, and excretion (LIM, YEO, TAN, 2017). The bioavailability of calcium, in addition to being influenced by exogenous components that interfere with its absorption and excretion, is also controlled by endogenous factors such as age, physiological conditions and hormonal regulation (PEREIRA et al., 2009). Bioavailability depends on several factors, such as the solubility and permeability of the mineral in the intestine (ERFANIAN, RASTI, MANAP, 2017). In addition, other dietary compounds can complex with the mineral, altering its absorption and consequently its use by the organism, as in the case of the proteins, sodium, caffeine, phosphorus, oxalate, phytate, tannins and phenolic compounds present in the food matrix. Therefore, foods of plant origin that contain high concentration of these compounds can reduce the bioavailability of minerals, among them calcium (VAVRUSOVA, SKIBSTED, 2014; AMALRAJ, PIUS, 2015; SKIBSTED, 2016).

The absorption of calcium from the diet has been studied by several methods. Considering that the bioavailability of this nutrient is altered by several factors (ERFANIAN, RASTI, MANAP, 2017), the understanding of the available methods is a crucial factor in the choice of the technique to be performed for mineral dosage *in vivo*. The calcium balance technique estimates the difference between oral intake and fecal and urinary calcium excretion (SATNARAYANA, 2006; KU et al., 2015). The validity of the results obtained depends on the accuracy of the parameters used to evaluate both ingestion and excretion. In addition, this method provides more satisfactory results in animal models than in humans (WEAVER, HEANEY, 2006). The main disadvantage of this method is that it covers calcium that was not absorbed in the gastrointestinal tract and calcium that was absorbed and re-secreted with gastric content, also known as endogenous fecal calcium loss (HEANEY, 2006; GRIFFIN, ABRAMS, 2005).

Considering the important role of calcium in bone health as well as its relation with chronic noncommunicable diseases, special care is necessary to reach the recommendations of the consumption of this nutrient (PEREIRA et al., 2009).

4.2.1.2. Inflammatory process, oxidative stress, calcium and bone remodeling

Inflammation, resulting from adipocyte hypertrophy and consequent infiltration of macrophages, culminates in the activation of specific signaling pathways, thus leading to the production of inflammatory substances. The transcription factor called nuclear factor kappa B (NF- κ B) plays a central role in regulating many essential processes to the body. The NF- κ B transcription factor family comprises five family members that act on the development of the inflammatory process. Faced an equilibrium situation, NF- κ B remains in the cytoplasm bound to inhibitory proteins. However, in inflammation caused by ingestion of high fat diets, NF- κ B signaling occurs in such a way as to allow translocation of the p65 subunit to the nucleus, which culminates in the production of inflammatory cytokines through the following mechanism: the signal (inflammation) activates the receptors present on the membrane, causing activation of the IKK complex. The activated IKK complex is capable of phosphorylate and ubiquitinate I κ B proteins, leading to their proteasomic degradation. NF- κ B molecules become free to translocate to the nucleus, inducing transcriptional expression of target genes (Figure 1) (CATRYSSE, LOO, 2017; SUN, 2011).

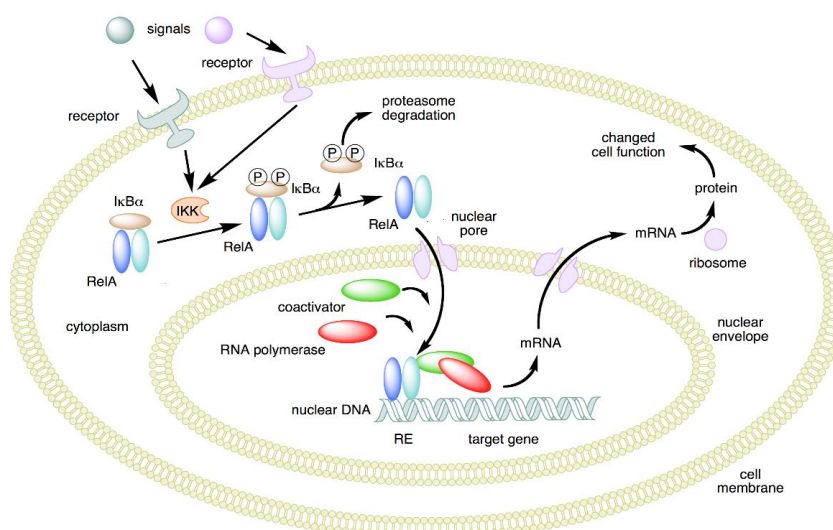


Figure 1. NF- κ B transcription factor signaling pathways. Reference: <https://en.wikipedia.org/wiki/NF-κB>. IKK: protein kinase; I κ B: I κ B kinase; P: phosphorus; RelA: p65; mRNA: Messenger RNA; RE: endoplasmic reticulum; DNA: Deoxyribonucleic acid; RNA: Ribonucleic acid.

PPAR- α (alpha peroxisome proliferator-activated receptor) is a transcription factor that regulates lipid metabolism in the liver. PPAR- α also negatively regulates inflammation.

PPAR- α ligands are able to inhibit the action of proinflammatory cytokines, interfering with NF- κ B in their signaling pathway. In addition to inflammation, PPAR- α plays a role in oxidative stress (DOTSON et al., 2016).

The inflammatory process also acts on bone metabolism through the secretion of cytokines by adipose tissue, such as tumor necrosis factor alpha (TNF- α), interleukin 6 (IL-6), interleukin 1 beta (IL-1 β), interleukin 12 (IL-12) (BASTOS et al., 2009; MORETTINI et al., 2015). High levels of these proinflammatory cytokines stimulate the nuclear κ B factor activator receptor (RANKL)/nuclear κ B factor activator receptor (RANK) that promotes osteoclast differentiation and bone resorption. Osteoprotegerin (OPG) is a protein synthesized by osteoblasts that combines with RANKL, inhibiting RANKL-RANK interaction and osteoclastogenesis, preventing calcium withdrawal from bones (STEEVE et al., 2004; WONG et al., 2016).

Cyclic resorption and reforming basically involves two bone cells, osteoclasts and osteoblasts, coordinated via the nuclear factor activator receptor (RANKL)/nuclear factor activator receptor (RANK)/osteoprotegerin (OPG) ligand pathway. Osteoblasts and osteocytes secrete RANKL that interacts with RANK, a receptor present in the osteoclast precursor cell. This binding leads to the migration, differentiation and fusion of osteoclastic lineage cells that promotes bone resorption, ie, calcium withdrawal from the bones into the bloodstream. Osteoblasts, in turn, produce OPG that binds to RANKL, preventing it from binding to RANK, thereby preventing bone resorption (WONG et al., 2016; RICHARDS et al., 2012).

In addition, the inflammatory process may be intensified by higher lipogenesis that may be caused by lower dietary calcium intake. It is proposed that low dietary calcium elevates parathyroid hormone (PTH) levels and thus increases intracellular calcium in adipocytes that will activate the enzyme fatty acid synthase (FAS), responsible for increase lipogenesis and reduce lipolysis and consequently increase the lipid deposition in the adipocytes, aggravating the inflammatory process (SOARES et al., 2012).

The inflammatory process, associated with higher lipogenesis, may increase oxidized lipid levels. These positively regulate PPAR- γ , a transcription factor that can suppress osteoblast differentiation, preventing bone formation. The oxidized lipids can also block the Wnt- β -catenin signaling pathway, which can stimulate osteoblastogenesis, increasing bone calcium mobilization (WONG et al., 2016). There are no studies showing the regulation of PPAR- α in the face of increased lipogenesis process, which is a factor analyzed in the present work.

The imbalance between the production of reactive oxygen (ROS) and nitrogen (RNS) species as well as their elimination by protective mechanisms leads to oxidative stress (HUSSAIN et al., 2016). This response occurs under certain pathophysiological conditions such as age, inflammation, obesity, cardiovascular and neurodegenerative diseases, leading to alteration of proteins, nucleic acids and lipids (ISLAM, 2017; PANTH et al., 2016; BOCCATONDA et al., 2016). Cellular dysfunctions caused by increased ROS and/or RNS production may lead to impairments in energy metabolism and cellular signaling and lead to genetic mutations. Thus, oxidative stress reduces biological activities, immune function and increases inflammation (DE BARBOSA et al., 2017).

In addition, the nutritional stress produced by eating high-fat diets also generates oxidative stress, leading to the progression of the development of different non-communicable chronic diseases (RANI et al., 2016; RINCÓN-CERVERA et al., 2016). High-fat diets are known to produce redox imbalance and increase oxidative stress due to reduce the calbindin D9k concentration, which leads to an inhibitory effect on intestinal calcium absorption, which occurs due to altered expression of proteins involved in the pathways absorption (transcellular or paracellular) of mineral (XIAO et al., 2010).

Therefore, the use of antioxidant foods, including chia, may be a good therapeutic strategy to prevent oxidative stress caused by high fat diet consumption, which may increase the levels of inflammatory markers, reducing the bioavailability and mineral bone retention (SOARES et al., 2012).

4.2.2. Iron

Anaemia, a major public health problem affecting nearly 40% of the world population, has great implications on quality of life (WHO, 2008; MCLEAN et al., 2009). It can occur during all ages, but it particularly affects young children (≤ 5 years) and women, especially during pregnancy (WHO, 2008; VILLALPANDO et al., 2003). The high prevalence in developing countries (most of Africa, Asia and South America), represents a significant public health problem (WHO, 2008). Anaemia is characterised by reduced red blood cell count and leads to impaired oxygen delivery to tissues (BALARAJAN et al., 2011). Iron deficiency, the leading aetiology of anaemia, is generally assumed to account for more than half of the cases worldwide (HERCBERG, PREZIOSI, GALAN, 2001). In fact, iron deficiency is by itself the most common nutritional disorder, being prevalent even in industrialised nations (MUCKENTHALER et al., 2017; HERCBERG, PREZIOSI, GALAN,

2001). In developed countries, iron deficiency is normally caused by insufficient dietary iron intake or by conditions that cause either iron loss or decrease in iron absorption (JOHNSON-WIMBLEY, GRAHAM, 2011).

Iron is an essential component of haemoglobin in red blood cells and of myoglobin in muscles, which contain around 60% of total body iron content. It is also necessary for the functioning of various cellular mechanisms, including enzymatic processes, DNA synthesis, and mitochondrial energy generation (LOPEZ et al., 2016). In adults, the body contains 3–5 g of iron; 20–25 mg is needed daily for production of red blood cells and cellular metabolism (STEINBICKER, MUCKENTHALER, 2013). Because dietary intake is limited (1–2 mg per day), other sources are needed for iron homeostasis, such as recycling of ageing erythrocytes in macrophages, exchange of iron in iron-containing enzymes, and iron stores (STEINBICKER, MUCKENTHALER, 2013). About 1–2 mg of iron is lost daily as a result of menstrual bleeding, sweating, skin desquamation, and urinary excretion (STEINBICKER, MUCKENTHALER, 2013). Because iron does not have an excretion regulation pathway, dietary intake, intestinal absorption, and iron recycling have to be finely regulated (LOPEZ et al., 2016).

An average diet in developed countries contains 10–15 mg/day of iron, of which only 5–10% is absorbed. Iron in the form of either haem iron or non-haem iron is absorbed mainly in the duodenum and proximal jejunum through enterocytes that are highly specialized for iron absorption and transport (MOLL, DAVIS, 2017). Iron is found in haemoglobin in the haem form, which is present in animal food sources, such as meat, poultry, and seafood. Non-haem iron is present in the plant foods, such as tea, cacao, cereals, dried fruit and seeds, mainly as Fe^{3+} or ferric iron (JOANN, MCDERMID, LÖNNERDAL, 2012). Haem iron, which accounts for 50% of the iron in meat, is more readily absorbed than non-haem iron. Once in the plasma, iron circulates bound to transferrin, which transports most of the iron to the bone for the synthesis of haemoglobin in red cells (MOLL, DAVIS, 2017).

4.2.2.1. Iron metabolism and homeostasis

Iron is present in two predominant oxidation states, Fe^{3+} (ferric) and Fe^{2+} (ferrous) (WALLACE, 2016). Inorganic dietary iron is mainly present in the oxidized form (Fe^{3+}) and must be reduced to the Fe^{2+} form prior to intestinal uptake. This reduction is thought to be mediated by ferrireductases in the intestinal cell apical membrane such as duodenal

cytochrome B (DcytB) (MCKIE et al., 2001). Once reduced, Fe^{2+} is transported across the apical membrane by divalent metal transporter 1 (DMT1) (GUNSHIN et al., 2005).

Iron taken up by enterocytes can be used directly for intrinsic cellular metabolic processes, stored, or exported across the basolateral membrane (DEV, BABITT, 2017). Iron is stored in enterocytes in the form of ferritin. Ferritin storage allows for a more controlled delivery of iron to basolateral iron exporters and the ability to limit systemic iron delivery (VANOAICA et al., 2010). Systemic iron delivery is mediated by the basolateral iron exporter ferroportin (DONOVAN et al., 2000). Although the molecular mechanisms of iron export by ferroportin are still not well understood, it is thought that iron is exported in the Fe^{2+} form, oxidized to Fe^{3+} by hephaestin and ceruloplasmin, and loaded onto transferrin, the main plasma iron carrier (DRAKESMITH, NEMETH, GANZ, 2015).

At the systemic level, the iron hormone hepcidin is a major regulator of body iron balance (GANZ, 2013). Hepcidin controls the iron that entry into circulation from absorptive enterocytes, iron recycling macrophages, and hepatocytes by binding to ferroportin and inducing its internalization and degradation in lysosomes (NEMETH et al., 2004). Stimulating hepcidin expression thereby inhibits iron absorption from the diet and iron release from recycling macrophages and other body stores. In contrast, lowering hepcidin levels promotes iron availability (DEV, BABITT, 2017).

4.2.2.2. Iron bioavailability

Bioavailability is defined as the proportion of the quantity of a nutrient that is ingested and is absorbed by the intestine, then being used by the body (COZZOLINO, 2012). The bioavailability related to iron is the measure of the dietary iron fraction that is able to be absorbed by the gastrointestinal tract and to be stored and incorporated to the heme iron (BRIGIDE et al., 2011). Since the bioavailable iron in foods is an important factor to guarantee the adequate iron intake, many studies have been performed to evaluate the iron bioavailability from different foods and diets (DIAS et al., 2019; DIAS et al., 2018; HOU et al., 2017; PACIFICI et al., 2017; WANG et al., 2019).

The *Gallus gallus* model have been widely used for nutritional research and it is considered an excellent model to evaluate iron bioavailability, since they can respond rapidly to the iron deficiency (DIAS et al., 2019; HOU et al., 2017; PACIFICI et al., 2017; TAKO et al., 2014; WANG et al., 2019). In addition, this model has shown similar results compared to the *in vitro* model using Caco-2 cells. In fact, the combination of this animal model with the

in vitro studies (Caco-2 cells) has shown to be an excellent model to assess dietary iron bioavailability (TAKO, BLAIR, GLAHN, 2011; TAKO, RUTZKE, GLAHN, 2010).

4.2.3. Zinc

Zinc is an essential micronutrient that has action on human health. The mineral is necessary for cell division and differentiation, protein synthesis, growth, nucleic acid metabolism, among others (FISCHER-WALKER et al., 2011; LAMBERTI et al., 2016). Estimates based on the assessment of national food availability indicated that 17% of the global population may have insufficient zinc intake. However, these estimates vary by region, corresponding to 5.7% in Oceania, 7.6% in Europe, 9.6% in the Americas and the Caribbean, 19.6% in Asia and 23.9% in Africa (BLACK et al., 2013).

Among the population groups, infants, children, adolescents, pregnant women and lactating women have higher zinc intake needs and, therefore, are classified as vulnerable groups for depletion of this mineral (KING, COUSINS, 2006). Diagnosis of zinc deficiency is still made based on serum mineral concentration (LAMBERTI et al., 2016). However, new biomarkers have been proposed to more reliably assess zinc nutritional status (REED et al., 2014; REED et al., 2015; KNEZ et al., 2016). Diagnosis is often made when there is a decrease in plasma zinc level below 70-110 µg/dL (GIBSON, 1990).

Symptoms of zinc deficiency are nonspecific and often only become apparent with severe deficiency. Children with zinc deficiency may present, in addition to infectious diseases such as pneumonia, severe diarrhea and growth deficiency (FISCHER-WALKER et al., 2013). Pregnant women may have complications such as hypertension, preeclampsia, intrapartum hemorrhage, infection, premature birth, as well as congenital anomalies (KING, 2000).

4.2.3.1. Intestinal zinc absorption

Zinc is released from food as ions during digestion. These ions can then bind to endogenous transporters to be absorbed into the duodenum and jejunum (TUBEK, 2007). These specific carriers may facilitate the passage of zinc through the cell membrane to the portal circulation. In a nutritional state where zinc intake is high, the mineral can also be absorbed through paracellular transport. About 80% of circulating zinc is bound to albumin, and any condition that changes serum albumin concentration can have a secondary effect on serum zinc levels. Although serum zinc accounts for only 0.1% of total body zinc, circulating

zinc is rapidly released from the bloodstream to meet the needs of cells and organs (WHO, 2004; TUBEK, 2007, BROWN et al., 2004).

Zinc is absorbed into the small intestine by a carrier mediated mechanism. Under normal physiological conditions, absorption transport processes are not saturated. The absorbed zinc fraction is difficult to determine because zinc is secreted from the gut. Zinc administered in aqueous medium is absorbed more efficiently (60-70%) compared to zinc supplied in solid diets (approximately 33%). In addition, the rate of mineral absorption varies with zinc content and diet composition (COUSINS, 1985; ROOHANI et al., 2013), since some factors are capable of interfering with its absorption, such as phytate (ROOHANI et al., 2013).

Zinc absorption in the duodenum and jejunum is concentration dependent and increases with increasing dietary zinc to a maximum rate where it reaches the plateau. (WHO, 2004). In addition, zinc status may influence its absorption, and in the face of mineral deficiency absorption is more effective whereas in the case of adequate nutritional status, the efficiency of zinc absorption becomes reduced (KREBS, 2000).

4.2.3.2. Zinc metabolism and homeostasis

Adults have about 2-3 g of zinc in their body. About 60% of zinc is stored in skeletal muscle, while 30% is stored in bone, 5% in liver and skin and 2-3% in other tissues (KAMBE et al., 2015). Serum zinc represents only 0.1% of body zinc, with approximately 80% being bound to albumin and 20% being bound to α 2-macroglobulin (BARNETT et al., 2013), while cellular zinc is distributed in the cytoplasm, nucleus and membrane in the following proportions: 50%, 30-40% and 10%, respectively (HAASE, RINK, 2014).

Maintaining homeostasis of zinc nutritional status is essential for the survival of animals and individuals. This mechanism occurs due to adjustments in both zinc absorption and endogenous intestinal excretion of the mineral (HAMBIDGE, KREBS, 2001), and the mechanisms between gastrointestinal absorption and endogenous zinc excretion are synergistic. In situations of ingestion of zinc below or much below the recommended, secondary homeostatic mechanisms are able to reduce its urinary excretion and increase the retention of zinc released from tissues such as bones, for example (BROWN et al., 2004).

In general, more than 30 proteins, including ZnT and ZIP transporters act under strict regulations to maintain systemic and cellular zinc homeostasis. However, humoral mediators that indicate the nutritional status of zinc in a cell or tissue have not been identified in mineral

metabolism, unlike iron, as in this case a hormone called hepcidin is able to play a critical role in homeostasis of this mineral (NEMETH et al., 2004).

ZnT-type transporters mobilize cytosol zinc for extracellular space and lumen of intracellular compartments (KAMBE, 2012; HUANG, TEPAAMORNDECH, 2013) while ZIP-type carriers act by replenishing cytosolic zinc from the extracellular space and the lumens of the intracellular compartments (KAMBE, 2012; JEONG, EIDE, 2013). Most ZIP-type carriers are located on the surface of the plasma membrane. Both transporters play various roles in immune cell functions controlling zinc signaling. Thus, any changes in these transporters result in immune dysfunctions (MURGIA et al., 2009; HAASE, RINK, 2014).

4.2.3.3. Zinc bioavailability

Zinc-related bioavailability is a measure of the fraction of food zinc that can be absorbed into the gastrointestinal tract and stored and incorporated into the body. The bioavailability of zinc depends on its solubility, chemical form as well as the presence of inhibiting or facilitating compounds.

Phytic acid is the major dietary factor known to reduce zinc bioavailability (HAMBIDGE, MILLER, KREBS, 2011). Due to the high concentration of phosphate groups, phytate forms salts with minerals, altering their absorption (LOPEZ et al., 2002). Phytate inhibitory effects may be related to the phytate: zinc molar ratio in the diet. Molar ratio greater than 15:1 inhibits zinc absorption (WHO, 2004). Regarding calcium, study showed that this mineral does not seem to interfere with zinc absorption (LÖNNERDAL, 2000). However, interactions between zinc and calcium are complex and not all studies have shown that calcium further increases the impact of phytic acid on zinc absorption (LÖNNERDAL, 2000; HUNT, BEISEIGEL, 2009).

The interaction between zinc and iron is not yet well established in the literature (ROOHANI et al., 2013). A study showed that decreased concentration of Zn could contribute to reduced plasma Fe concentrations (KNEZ et al., 2017). However, it is worth mentioning the limitations of serum zinc dosage, since its concentration can be influenced by a number of factors. A study by Lonnerdal (2000) that analyzed the interaction between zinc and iron, observed a significant reduction in zinc absorption in the fasting state when iron was added. In addition, the authors noted that the interaction seems less pronounced when zinc intake is closer to a physiological level than when it is lower than recommended. However, Szymlek-Gay et al. (2016) observed that iron is not able to alter zinc absorption, concluding that

neither supplemental or fortification Fe nor the amount of Fe habitually consumed altered Fe absorption, Fe utilisation, Zn absorption, Zn status or growth in Fe-sufficient infants. There are some compounds that can increase the body's absorption of zinc, such as proteins (MCDOWELL, 2003). Animal protein consumption improves zinc bioavailability from plant sources, since amino acids released from animal protein keep zinc in solution or due to the protein binding mechanism to phytate, increasing mineral solubility (GIBSON, RABOY, KING, 2018).

4.3. *In ovo* feeding administration

In ovo exogenous nutrients administration was first applied in the 1980s for vaccination against Marek's disease (SHARMA, BURMESTER, 1982). Over the years, further research on *in ovo* nutrients administration was conducted in order to potentially improve poultry production (KADAM et al., 2013). Besides this, some nutrients have been applied for *in ovo* feeding administration, including amino acids, carbohydrates, and vitamins, are used to improve the quality of broiler chickens (TAKO, FERKET, UNI, 2004; OHTA, KIDD, 2001), specifically in the context of hatch weights, feed utilization, growth, and marketing size (KADAM et al., 2013).

Two time points during embryonic development were suggested for the *in ovo* procedure for *Gallus gallus* model. First, is on day 12 of embryonic development, when the chorioallantoic membrane is fully developed and vascularized, and the embryo is surrounded by the amniotic fluid that remains in contact with the embryonic gastrointestinal tract, which allows the transport of substances from the air chamber into the intestine (SOBOLEWSKA et al., 2017). The second point is on day 17-18, since Uni and Ferket (2003) illustrated that *in ovo* feeding must be applied while the embryo consumes the amniotic fluid (17–18 days of the embryonic development), just prior to the embryo's oral consumption of the amniotic fluid, which occurs by Day 19 (UNI, FERKET, 2003). Salahi et al. (2011) provided evidence that the best *in ovo* injection time might be at 453 h of incubation, since the embryos are transferred from the setter to the hatching basket at day 17–18, which should be an appropriate time to administer nutrients practically.

Actually, *in ovo*-feeding administration has been used as an *in vivo* method to assess the prebiotic effects on mineral absorption, gut microbiota population, intestinal development, and short-chain fatty acids (SCFA) content (DA SILVA et al., 2019; PACIFICI et al., 2017;

HOU et al., 2017). Hence, it is important to highlight the potential of the *in ovo* feeding approach, as a technique for the evaluation of prebiotic effects.

4.4. *In ovo* administration x Prebiotics x Intestinal Microbiota

It has been reported that the gut microbiome contains an estimated 3–8 million unique genes, which expands the genetic capacity of humans by 100-fold (CONSORTIUM, 2012). In recent years, it was demonstrated that the gut microbiome community participates in abundant bioactivities, such as: maturation and regulation of the immune system (PARTIDA-RODRIGUEZ et al., 2017); digestion and release of essential nutrients (ZHANG et al., 2017); improvement of intestinal barrier function (MCCABE, BRITTON, PARAMESWARAN, 2015); and the potential inhibition of pathogenic bacteria (MCCABE, BRITTON, PARAMESWARAN, 2015). Besides this, diet is an important factor that affect gut microbiota (SUBRAMANIAN et al., 2014).

The habitual dietary patterns are associated with the composition of individual's gastrointestinal microbiota, and significant changes in macronutrient and dietary fiber intake, specifically prebiotics can rapidly induce changes (WU et al., 2011). The prebiotic effect is defined as “the selective stimulation of growth and/or activities of one or a limited number of microbial genus in the gut microbiota that confer health benefits to the host” (ROBERFROID et al., 2010). It is known that the consumption of dietary fiber promotes extensive metabolic interactions among bacterial species present in the gastrointestinal microbial community, stimulating the growth of different kinds of bacteria (HOLSCHER, 2017). Bacterial fermentation of prebiotics results in the production of acidic fermentation end products, primarily lactic acid and SCFAs (short chain fatty acid), that reduce the colonic pH, which in turn impacts the composition of the microbial communities present in the gastrointestinal tract (HOLSCHER, 2017).

It is known that the intestinal microbial populations play an essential role in human and animal health. Because of this, recent studies suggested that cecal microbial populations are a useful indicator of gut health; this hypothesis was also confirmed in recent *in ovo* prebiotics administration studies (PACIFIC et al., 2017; HOU et al., 2017; CALIK et al., 2016; TAKO et al., 2014; HARTONO et al., 2015). Study showed that *Bifidobacterium* and *Lactobacillus* genera proportions were higher ($p < 0.05$) in intestinal contents of *Gallus gallus* after the intra-amniotic administration and dietary inulin treatment (TAKO et al., 2012). The increase of *Bifidobacterium* and *Lactobacillus* genera proportions were also observed in the

intra-amniotic administration of wheat prebiotics (TAKO et al., 2014), raffinose, stachyose (PACIFIC et al., 2017), and chickpea and lentil extracts (HOU et al., 2017).

The most studies are using *Gallus gallus* as a model for microbiota analyses. This model present a complex and dynamic gut microbiota, strongly influenced by host genetics, environment and diet (YEGANI, KORVER, 2008). Further, there is considerable similarity at the phylum level between the gut microbiota of broilers (*Gallus gallus*) and humans, with *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria* representing the four dominant bacterial phyla in both (BACKHED, 2005; DIAS et al., 2018; HOU et al., 2017; REED et al., 2017).

Thus, prebiotic intake may be related to microbiota modulation and intestinal health, and it is therefore interesting to investigate the action of ingestion of a food with high concentration of soluble fiber (*Gallus gallus*), on the intestinal microbiota.

4.5. *In ovo* administration x Prebiotics x Intestinal Functionality (BBM gene expression)

Intestinal barrier is a morphologic and physiologic structure placed between tissues and intestinal lumen which is known as external environment and it ensures continuing of events such as absorption and secretion between them (MATUR, ERASLAN, 2012). Thus, the small intestine is considered as the most important part of the gastrointestinal tract, because the majority of the enzymatic digestion and absorption occurs in this tissue. The small intestine is composed of four layers which are: mucosa, submucosa, muscularis and serosa (MIRZA, 2018). The mucosa is composed of fingerlike form called villi. The villi is able to increase the surface area exposed to the absorption, and an increase of the length of villi leads to a higher efficiency of the digestion process and absorption and also favours protection against many kinds of pathogens. Submucosa is a layer of dense tissue that supports the mucosa and muscularis layer is composed of smooth muscle fibres, keeping the mucosal surface and underlying glands in a constant state of gentle agitation to expel contents of glandular crypts and enhance contact between epithelium and the contents of the lumen. The serosa consists of a thin layer of loose connective tissue covered by mesothelium. The villi, present in mucosa layer, are formed by many columnar epithelial cells called enterocytes, and all the enterocytes contain a large number of microvilli which form the brush border membrane (MIRZA, 2018). In this membrane, there are functional genes which are used as biomarkers of BBM (brush border membrane) digestive and absorptive capabilities and overall tissue functionality (REED et al., 2015). Diet is a factor that can affect positively

or negatively the intestinal function. The consumption of prebiotics can improve the intestinal health (PACIFICI et al., 2017; HOU et al., 2017; SOBOLEWSKA et al., 2017; SLAVIN, 2013) increasing the gene expression of some proteins present in BBM, such as sucrase-isomaltase (SI), aminopeptidase (AP), sodium glucose cotransporter-1 (SGLT1), and peptide transporter 1 (PepT 1).

Sucrase-isomaltase (SI) is one of the major disaccharidases of the small intestine that hydrolyze disaccharides or oligosaccharides to monosaccharides for absorption (DIAZ-SOTOMAYOR et al., 2013). Aminopeptidase (AP) is an exopeptidase that cleaves amino acids from the N-terminus of peptides and resides on the brush border membrane (HOU, TAKO, 2018). Already SGLT1 is highly expressed on the brush border membrane of villus enterocytes in the proximal part of the small intestine and is responsible for dietary glucose absorption (OGUMA et al., 2015). The peptide transporter (PepT1) is expressed most abundantly in the duodenum, and its function is moving peptides from the lumen of the small intestine into the enterocyte (CHEN et al., 2002). Pacific et al. (2017) performed a study focusing on the administration of prebiotics (raffinose and stachyose) in different concentrations (50 mg/mL and 100 mg/mL) on brush border membrane functional genes in *Gallus gallus* model. The authors observed that prebiotics intra-amniotic administration, regardless of the concentrations used, increased gene expression of AP, SI, SGLT1 compared with non-inject and water inject groups. In another study, Hou et al. (2017) aimed to investigate how the prebiotics extracted from chickpea and lentil could affect the calcium status, intestinal functionality, and health-promoting bacterial populations in *Gallus gallus* model. The results showed that the lentil associate with calcium *in ovo* administration (50 mg/mL lentil + 4 mmol/L CaCl₂) was able to increase the SI gene expression. In another study, the authors evaluated the action of wheat bran prebiotics on mineral metabolism gene expression, intestinal functionality, and bacterial populations *in vivo* (*Gallus gallus*), using the intra-amniotic administration. They found that wheat bran prebiotics increase the expression of SGLT1 gene expression compared to the other experimental groups (cellulose wheat bran and arabinose). However, gene expression of SGLT1 in wheat bran group did not differ in relation to non-inject group. The AP gene expression was higher in wheat bran group compared to arabinose and water inject groups. Besides this, gene expression of SI did not differ among all groups (WANG et al., 2019).

Dias et al. (2019) assessed the effects of different varieties of common beans, biofortified or not, on BBM proteins functionality, iron metabolism and intestinal bacterial

populations. The authors showed that SI, SGLT1 and AP gene expression did not differ among groups fed bean (carioca bean, black bean and white bean, biofortified or not) compared to the inulin group (positive control). Thus, in general, the results that related prebiotic intra-amniotic administration with BBM functionality indicate that prebiotics can potentially enhance the absorptive and digestive capacity of the body in experimental *in vivo* models.

4.6. *In ovo* administration x Prebiotics x Intestinal Morphology

The morphological parameters such as length of villi, depth of crypt, villi/crypt proportion, and surface area of villi are used to investigate the effects of prebiotics on intestinal morphology and cell proliferation (MATUR, ERASLAN, 2012). The small intestine is specialized in the digestion and absorption process of nutrients and constitutes the barrier between the host's external and internal environment (SOBOLEWSKA et al., 2017). The intestine consists of microvilli, which act in the process of nutrient's metabolism (SOBOLEWSKA et al., 2017), as the villi increase the internal surface area, as well as the digestive and absorptive capacities of the brush border membrane (BBM) (YAMAUCHI, INCHAROEN, YAMAUCHI, 2010). Thus, an increase of any of these morphometric parameters is expected to improve the digestive and absorptive capabilities of the intestine. The histomorphological parameters of the small intestine, such as the villi measures and the crypt depth, are some of the indicators of the health and functional status of the intestine (SOBOLEWSKA et al., 2017). Both shorter villi and deeper crypts lead to a decrease in the absorption of nutrients (FAN et al., 2014), because shorter villi reduce the total surface area of the intestinal absorption which results in poorer absorption of nutrients, and deeper crypt contributing to an increased secretion of digestive enzymes (XU et al., 2014). The consumption of prebiotics can improve intestinal morphology. This fact may occur due to increased motility of the digestive tract by the intact form that the dietary fiber is found in the food, besides the formation of gel promoted by the soluble fiber fraction, leading to hyperplasia and/or hypertrophy of intestinal cells (DA SILVA et al., 2016). The cells hyperplasia and/or hypertrophy is responsible for increasing the villus depth, villus height and crypt depth (LIANG et al., 2014). Another explanation is that dietary prebiotics increase butyrate production, which may lead to enterocyte proliferation. This fact was observed in birds that received intra-amniotic administration of carbohydrates. The animals developed

villi with greater surface areas compared to the untreated animals (TAKO, FERKET, UNI, 2004).

Studies have shown that the prebiotics administration was able to increase the villus measures, crypt depth, goblet cell number and goblet cell diameter. Pacific et al. (2017) observed that raffinose and stachyose intra-amniotic administration in *Gallus gallus* model, regardless of the concentrations used, increased villus surface area compared to the control. The same result was observed by Hou et al. (2017). The authors showed that the prebiotics extracted from chickpea and lentil increased villus surface area in the same experimental model. Bogucka et al. (2016) evaluated the effect of prebiotics administration on the development of the intestinal villi and the number of goblet cells in the small intestine on the 1st and the 4th days of life of broilers. They observed that, on day one, the villus height did not differ among experimental groups. However, the villus width, villus surface area and crypt depth was lower in prebiotic group (inulin). On day four, the inulin group showed lower villus width, villus surface area and crypt depth (BOGUCKA et al., 2016). Moreover, a study that evaluated the raffinose administration on the ileum mucosa health showed that villus height and villus height to crypt depth ratio of post-hatching birds increased linearly with higher dosages of this prebiotic. However, raffinose did not affect the crypt depth (BERROCOSO et al., 2017). Mista et al. (2017) evaluated the effect of *in ovo* administered prebiotics on the development of the small intestine of broiler chickens and found that prebiotics did not significantly affect the villus lengths but increase the crypt depths of animals. However, villus length: crypt depth ratio did not alter between the group that receives or not the prebiotic (MISTA et al., 2017).

The cells hyperplasia and/or hypertrophy can improve the goblet cell number (PIEL et al., 2005). Mucus is a barrier to prevent water loss and to remove inhaled foreign substances such as microbes and inflammatory cells. The major macromolecular components of mucus, the mucin glycoproteins, are secreted by surface epithelial goblet cells (VOYNOW, MENGRO, 2009). The thick mucus gel layer formed by mucins maintains the integrity of the gastrointestinal mucosal surface and acts as a medium for protection, lubrication, and transport between luminal contents and the epithelial lining (ANDRIANIFAHANANA, MONIAUX, BATRA, 2006). Nowadays, it has been studying the action of the consumption of prebiotics to enhance mucosal barrier function directly, potentially via its impact on goblet cell function. Generally, the studies show that the prebiotic consumption can increase the goblet cell diameters and the goblet cell numbers, suggesting an increase in the production of

mucus, hence changing the bacterial composition and function (OUWEHAND et al., 2005; DEPLANCKE, GASKINS, 2001). Pacific et al. (2017) noted that stachyose and raffinose administration in the dose of 50 and 100 mg.mL⁻¹ increased goblet cell diameters. The same result was observed by Hou et al. (2017) in chickpea and lentil *in ovo* feeding administration. Although, Bogucka et al. (2016) observed that the administration of inulin and synbiotic with the addition of inulin on the 1st day of life of chicks was able to improve the number of goblet cell in the duodenum and jejunum and caused a decrease on the 4th day after hatching. Bogucka et al. (2017) determined the effect of prebiotic and synbiotic preparations injected *in ovo*, on day 35 of rearing and noted that the no difference was found for goblet cells on duodenal villus area. However, in relation to jejunum and ileum, it was observed that Bi2tos administration, regardless if on prebiotic or synbiotic group, increase the number of goblet cells in the animals. To confirm these beneficial results, Wang et al. (2019) observed that the administration of wheat bran, cellulase wheat bran and arabinose increased the number of goblet cells (per villi), most increased in the presence of arabinose. In addition, the diameter of goblet cells increased in all treatment groups.

5. GENERAL METHODOLOGY

This study was divided into three stages, namely: Evaluation of calcium bioavailability and inflammation in male *Wistar* rats (**Paper 1**); Effects of chia on oxidative stress and inflammation in ovariectomized adult female *Wistar* rats (**Paper 2**); and Evaluation of intra-amniotic administration of soluble extract from chia in the intestinal health, microbiota population and bioavailability of iron and zinc in *Gallus gallus* model (**Paper 3**).

Working Sites

The phases developed in this study were performed in the following laboratories:

- Experimental Nutrition Laboratory (Nutrition and Health Department/UFV): analysis of calcium bioavailability *in vivo*, inflammation and oxidative stress;
- Trace Minerals Laboratory and Nutrition Unit (Cornell University Agriculture and Health Center/USDA/USA): analysis of intestinal microbiota, intestinal morphology, intestinal functionality, and zinc and iron bioavailability *in Gallus gallus* model;
- Soil Analysis Laboratory (Soil Department/UFV): analysis of minerals;
- Metabolism and Fermentation Laboratory (Biochemistry Department/UFV): inflammation and oxidative stress analysis;

- Biochemistry and Molecular Biology of Infectious and Parasitic Agents Laboratory (Biochemistry Department/UFV): RT-qPCR analysis;
- Clinical Analysis Laboratory (Health Division/UFV): biochemical analysis;
- Veterinary Hospital (Department of Veterinary Medicine/UFV): ovariectomy procedure.

Stage 1: Evaluation of calcium bioavailability in male Wistar rats

5.1. Raw material

Chia seeds (*Salvia hispanica* L.) produced in Brazil and grown in Catuípe, Rio Grande do Sul (RS) were used. In the laboratory, the seeds were ground in a knife mill with a particle size of 850 micrometers (MA 090 CFT) (2000 rpm; 30 mesh). Subsequently, the flours were packed in polyethylene bags covered with aluminum foil and stored in a freezer ($-18 \pm 1^\circ \text{C}$) until the time of analysis.

5.2. Biological assay

The positive control group received standard diet with calcium carbonate. The test groups received chia flour, associated with standard diet or high fat diet, depending on which group they belonged. Thus, we used 4 groups, namely:

- 1- Standard diet + calcium carbonate;
- 2- Standard diet + chia;
- 3- High fat diet + calcium carbonate;
- 4- High fat diet + chia;

5.3. Experimental animals

Thirty-two male rats (*Rattus norvegicus*, Wistar, *albinus* variation), weanling, with 21 days of life were systematically divided into 4 groups with 8 animals each, being each animal considered a repeat, so that the mean difference weights between the groups did not exceed 5 grams. The animals were distributed in individual metabolic cages of stainless steel in controlled temperature environment ($21 \pm 1^\circ \text{C}$) and light and dark cycle of 12 hours, automatically controlled.

The animals received distilled water and their respective experimental diets *ad libitum*. The experimental groups received the following diets: standard diet + calcium carbonate; standard diet + chia flour; high fat diet + calcium carbonate and high fat diet + chia flour

(Figure 1). In the last week of the intervention, calcium balance analysis was performed for three non-consecutive days, and the animals' faeces and urine were collected. After 35 days, after 12 hours fasting, the animals were anesthetized with isoflurane (Isoforine®, Cristália) and euthanized by cardiac puncture. The blood was collected in tubes with heparin, 16 x 100 mm (BD Vacutainer®) and centrifuged (Hermle®, Z216MK model, Germany) at 800 g for 10 minutes to separate the plasma. In addition, fragments of left femur were fixed in formaldehyde 10% and kept at room temperature for subsequent analyse of bone resistance. The liver were stored at -80°C until molecular biology analyzes. The study was submitted and approved by the Ethics Committee on Animal Research of the Federal University of Viçosa, Brazil (Potocol 97/2014) (Appendix I).

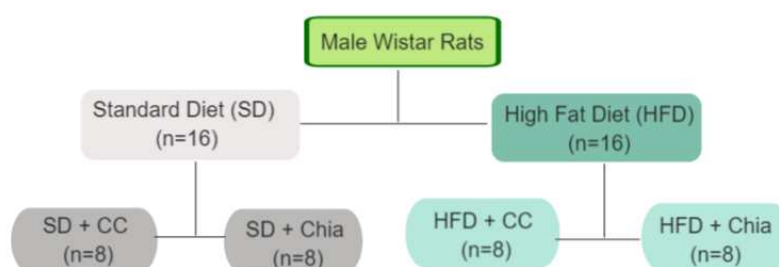


Figure 1. Experimental design of the calcium bioavailabilty study in male *Wistar* rats. SD: standard diet; HFD: high fat diet; CC: calcium carbonate.

5.3.1. Experimental diets and calcium consumption

The composition of the diets were based on the AIN-93G diet (REEVES et al., 1993), in powder for growing animals (20% protein, 30% lipid and 50% carbohydrate) or high fat diet (64% lipids, 16% protein and 20% carbohydrates) (RESEARCH DIETS, 2017), with modifications, depending on which group they were allocated. Albumin was used as a protein source, replacing casein, due to the lower concentration of contaminating calcium. The chia seed was ground at the time of preparation of the diets, to obtain the flour.

The amount of chia flour offered in the diets (SD + C and HFD + C) was determined, based on their composition, in order to provide 50% of the recommendation (2.5 g of calcium per 1 kg of diet) for rodents (BRYK et al., 2016). This quantity was determined based on the average consumption of the world population (BALK, et al., 2017). The other ingredients of these diets were added in sufficient quantity to match the composition of lipids, proteins, carbohydrates, fibers and calories of the corresponding diets (SD + CC and HFD + CC), respectively. Thus, the concentration of fibers and lipids in the experimental diets were higher

than the AIN-93G diet (Table 1). For the calculation of the experimental diet, it was considered the centesimal composition of the chia flour obtained previously by our research group (32.2g/100g lipids, 18.2g/100g protein, 33.4g/100g fiber, 4.6 g/100 g of carbohydrates and 480 mg of calcium/100 g) (DA SILVA et al., 2017). All ingredients were weighed in a semi-analytical balance (Gehaka, BG2000, Brazil), manually mixed, sieved in plastic sieve and homogenized in an industrial mixer (Leme) for 15 minutes. After the pre-preparation, the diets were packed in polyethylene bags, properly labeled and stored in a freezer ($-18^{\circ}\text{C} \pm 1^{\circ}\text{C}$).

Table 1. Nutritional composition of experimental diets used on calcium bioavailability study with male *Wistar* rats.

<i>Ingredients (per kg of diet)</i>	Experimental Diets			
	SD + CC	SD + C	HFD + CC	HFD + C
Calcium carbonate (mg)	5.00	0.00	5.00	0.00
Chia (g)	0.00	416.80	0.00	416.80
Albumin (g)	217.90	117.60	217.90	117.60
Dextrinized starch (g)	132.00	132.00	132.00	115.10
Sucrose (g)	100.00	100.00	100.00	100.00
Soybean oil (mL)	134.20	0.00	134.20	0.00
Lard (g)	0.00	0.00	200.00	200.00
Microcrystalline cellulose (g)	139.20	0.00	139.20	0.00
Calcium-free mineral mix (g)	35.00	35.00	35.00	35.00
Vitamin mix (g)	10.00	10.00	10.00	10.00
L-cystine (g)	3.00	3.00	3.00	3.00
Choline bitartrate (g)	2.50	2.50	2.50	2.50
Corn starch (g)	221.20	183.10	21.20	0.00
<i>Nutritional composition</i>				
Total calories (kcal)	3700.42 ^b	3624.75 ^b	4700.42 ^a	4624.11 ^a
Caloric density (kcal.g ⁻¹)	3.70 ^b	3.62 ^b	4.70 ^a	4.62 ^a
Calcium (g.kg ⁻¹)*	2.41 $\pm$ 0.00 ^a	2.53 $\pm$ 0.02 ^a	2.57 $\pm$ 0.04 ^a	2.58 $\pm$ 0.00 ^a

* Analyzed according to the methodology proposed by Gomes (2011). SD + CC: standard diet + calcium carbonate; SD+C: standard diet + chia; HFD+CC: high fat diet + calcium carbonate; HFD+C: high fat diet + chia. Means with different letters in the same row indicate a significant difference ($p < 0.05$) according to the Newman-Keuls test.

5.4. Determination of calcium content of diets

The calcium content of the experimental diets was determined by atomic absorption spectrophotometry (GOMES, 2011). This determination confirmed the calcium concentrations of the diets.

5.5. Weight gain, food efficiency ratio (FER) and indices

Body weight gain and food consumption were monitored weekly during the experimental period to determine the feed efficiency ratio (FER), which was obtained by the following formula: $FER\% = (\text{Body Weight Gain} / \text{Diet Consumption}) \times 100$ (DA SILVA et al., 2016). The hepatosomatic index and cecal index were calculated as a relation between liver weight or cecum weight and body weight, respectively, multiplied by 100 (KIM et al., 2016).

5.6. Determination of calcium metabolic balance

In the week prior to euthanasia, each rat was housed individually in a metabolic cage. Samples of urine and feces were collected for three non-consecutive days over the 24-hour period using specific collectors (FRANCO, 2009). The mineral intake of each animal was estimated according to its daily dietary intake, and the absorption and retention rates were calculated using the equations proposed by Ku et al. (2015):

$$\text{Calcium Absorption Rate (\%)} = (\text{Ca Ingested} - \text{Ca Fecal}) / \text{Ca Ingested} \times 100;$$

$$\text{Calcium Balance (mg / day)} = \text{Ca Ingested} - (\text{Urinary Ca} + \text{Fecal Ca});$$

$$\text{Calcium retention rate (\%)} = (\text{Ca balance} / \text{Ca Ingested}) \times 100.$$

It was calculated the mean of the diet consumed by the animals in one day for calcium balance calculation. To facilitate the identification of feces from the diet consumed on the day of analysis, indigo carmine (200 mg/100 g diet) was added to mark the faeces with dark blue coloration. The total feces content was quantified and divided by three to obtain the daily mean (FRANCO, 2009).

The feces were weighed, hermetically packed in plastic bags and stored in a freezer at -20°C until analysis. The urine was packed in falcon tubes and also stored in a freezer at -20°C until the time of analysis (LOBO et al., 2007; LOBO et al., 2009). Urinary calcium was analyzed by specific kit (Bioclin®) using the BS-200 Chemistry Analyzer (Bioclin®) while the calcium present in feces and femur were analyzed by atomic absorption spectrophotometry (GOMES, 2011). The size and thickness of the femur were measured by

means of a 200-mm digital pachymeter (resolution, 0.01 mm; Model 530-312; Mitutoyo). Maximum tensile strength was determined using a Universal Mechanical Testing Machine (Instron, Norwood, MA USA). Resistance curves were built on a microcomputer in the Blue Hill software.

5.7. Biochemical analysis

For the determination of biochemical parameters, 0.5 mL of plasma was used. Plasma glucose concentrations, total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), very-low density lipoprotein (VLDL), triacylglycerides (TGL), uric acid, creatinine, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured by colorimetric methods using commercially available kits (Bioclin®, Belo Horizonte, Brazil). Analyses were conducted on a BS-200 Chemistry Analyzer (Bioclin®).

5.8. Oxidative stress levels analysis

To obtain the homogenate, approximately 200 mg of tissue were initially weighed into 2 ml microtubes. Then the contents were macerated with pistil. It was added 1000 µl of phosphate buffer 50 mM with 1 mM EDTA (pH 7.4) and the sample was centrifuged at 12,000 g for 15 minutes at 4°C. The supernatant was carefully removed, and stored in ultra-freezer until analysis.

5.8.1. Malondialdehyde (MDA)

400 µL of homogenate and 400 µL of TBARS solution (15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid and 0.25 M HCl) were aliquoted into 2 mL microtubes. Then, the samples were vigorously vortexed and heated in the water bath at 95°C for 30 minutes. After this procedure, 600 µl of n-Butanol were added, and the mixture was vortexed and centrifuged at 800 g for 10 minutes. The supernatant was carefully removed and pipetted in triplicate on Elisa plate for reading in Multiskan GO spectrophotometer (Thermo Scientific) at 535 nm (KOHN, LIVERSEDGE, 1944; PYLES et al., 1993). The MDA concentration was calculated using the molar absorptivity coefficient $E_0 = 1.56 \times 10^5 \text{ mol L}^{-1} \text{cm}^{-1}$ (BUEGE; AUST, 1978), and the results expressed in nmol of MDA per milligrams of protein (MDA/PTN). Total liver homogenate protein was quantified according to the Bradford method (1976).

5.8.2. Nitric oxide

This analysis was performed in liver tissue and plasma: 50 μL of plasma or liver homogenate was mixed with solutions A (1% sulfanilamide in 2.5% H_3PO_4) and B (0.1% naphthyl 1 ethylene diamide dihydrochloride in 2.5% H_3PO_4) in the ratio (1:1) and the microtiter plate was incubated in the dark for 10 minutes. Absorbance was read on a spectrophotometer (Multiskan Go, Thermo Cientific) at 570 nm and the results were calculated using the standard curve and expressed in $\mu\text{mol NO/mg}$ protein in hepatic tissue and in μM in plasma (GREEN et al., 1982).

5.8.3. Superoxide dismutase enzymatic activity

The quantification of superoxide dismutase (SOD) enzyme was performed on homogenate samples, as described above. To the aliquotted samples in the microtubes (30 μL) were added 249 μL of Tris-HCl buffer 50 mM, pH 8.2, containing 1 mM EDTA; 6 μL MTT (1.25 mM) and 15 μL pyrogallol (10 mM). For the standard, it was added 6 μL of MTT; 15 μL of pyrogallol (10 mM) and 279 μL of buffer and to obtain the blank, 6 μL of MTT and 294 μL of buffer were applied to the wells. The samples, standard and blank were incubated at 37°C for 5 minutes and the reading was performed on a Multiskan GO (Thermo Scientific) spectrophotometer at 570 nm. Results were expressed as units of SOD/mg protein. The calculations were performed in relation to the absorbance value of the standard, considering that it has one U of SOD, that is, 100% of pyrogallol oxidation (MARKLUND, 1995).

5.8.4. Catalase enzymatic activity

The determination of catalase enzyme activity was based on its ability to cleave hydrogen peroxide (H_2O_2) in water and molecular oxygen, as described by Aebi (1984). In a tube, it was added 20 μL of the homogenate supernatant (1:10 diluted in water), 1 ml of phosphate buffer 100 mM (pH 7.2) + H_2O_2 (in each 25 ml buffer, 40 μL of H_2O_2 30%). The absorbance was determined at 0, 30 and 60 seconds at 240 nm in a spectrophotometer (T70 + UV/VIS Spectrometer). The equipment was cleared prior to each analysis with 100 mM phosphate buffer pH 7.2 and the respective sample. Catalase activity was calculated according to Lambert Beer's law. The absorbance used for the calculation was the delta obtained from the absorbance read at times 0 and 60 (final absorbance - initial absorbance).

5.8.5. Determination of the antioxidant capacity of liver and plasma

To obtain the homogenate, about 200 mg of tissue was weighed into 2 mL microtubes and then the contents were macerated with pestle. 500 μ L of 1X buffer (Antioxidant Assay kit, Sigma Aldrich) was added and the sample was centrifuged at 12,000 g for 15 minutes at 4°C. The supernatant was removed, and stored in an ultra freezer (-80°C) until analysis.

For the analysis, 10 μ L of trolox standards were pipetted in the well of the Elisa plate, in triplicate, at increasing concentrations to obtain the standard curve. Aliquots of 10 μ L of homogenate and plasma were added to the remaining wells identified for the samples. 20 μ L of the metmyoglobin reagent and 150 μ L of the ABTS solution were added to all wells. The plate was covered with aluminium foil and incubated at room temperature (22°C) for 5 minutes. After incubation, the absorbance was reading at 405 nm on Elisa reader. For the calculation, it was used the line equation obtained by the construction of the analytical curve and the values were expressed in mM trolox equivalent.

5.9. Extraction of mRNA from liver tissue and cDNA synthesis

The liver was macerated in liquid nitrogen in gral shroud free of RNase and the samples were aliquotted for RNA extraction. Total animal liver RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) using the manufacturer's recommendations (100 mg of tissue were homogenized with 1 mL of TRIzol). After extraction with chloroform (0.2 mL/1 mL TRIzol), the aqueous phase RNA was precipitated with isopropyl alcohol (0.5 mL/100 mg tissue), the tubes centrifuged at 12,000 g for 10 minutes (4°C) and RNA washed with 75% ethanol and centrifuged at 9,500 g for 5 minutes (4°C). The pellet was resuspended in 50 μ L of diethylpyrocarbonate-treated ultra pure water (DEPC water).

After extraction, the RNA samples were treated with DNase (RQ1 RNase Free-DNase Kit; Promega, Madison, WI, USA) using the manufacturer's protocol as follows: 7 μ L of the sample (2 μ g RNA) were transferred to a tube and treated with 1 μ L of DNase buffer and 2 μ L of DNase and left at 37°C for 30 minutes for the action of the DNase enzyme. After that period, 1 μ L of stop solution was added and the samples remained in a water bath at 65°C for 10 minutes. Total isolated RNA was used to synthesize cDNA using reverse transcription M-MLV kit (Invitrogen Corp, Grand Island, NY) according to the manufacturer's protocol (LIVAK, SCHMITTGEN, 2001).

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

mRNA Expression levels of genes in the liver that are involved in inflammation processes was analyzed by RT-qPCR. The SYBR Green PCR master mix from Applied Biosystems (Foster City, CA) was employed, and the analyses were performed on the StepOne™ Real-Time PCR System (Thermo Fisher Scientific) by means of the measurement system involving SYBR-Green Fluorescence and Primer Express software (Applied Biosystems, Foster City, CA). The PCR cycle involved the initial denaturation at 95°C (10 min) and 40 cycles, with 1 min of denaturation (95°C), 1 min. (62°C) and 2 min. (72°C), followed by the analytical curve of dissociation. Sense and antisense primer sequences were ordered (Choma Biotechnologies) to amplify PPAR- α (ID: 2404316), NF κ B (ID: 234230511), Zn-SOD1 (ID: 5615763), TNF- α (ID: 234230513), and IL-10 (ID: 537719). The relative expression levels of mRNA were normalized to the endogenous control: glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All the steps were performed under conditions with RNase free.

5.11. NF- κ B p65 and PPAR- α quantification

To determine the concentrations of PPAR- α and NF- κ B p65 in the liver, hepatic tissue samples were homogenized by means of the NE-PER Nuclear and Cytoplasmic Extraction Kit reagents (Thermo Scientific Fisher, USA). The nuclear fractions of PPAR- α and NF- κ B p65 were assessed by immunoassay using the Rat PPAR (Cat # E-EL-R0725-ra; Elabscience, USA) and Rat NF- κ B p65 (Cat #E-EL-R0674, Elabscience, USA), ELISA kits, respectively. The microplates that were provided in the ELISA kits were respectively precoated with anti-PPAR- α and anti-NF- κ B p65 antibodies. The concentrations of PPAR- α and NF- κ B p65 were calculated by comparison to the corresponding standard curves.

5.12. Statistical analysis

The treatments were conducted in a completely randomized design, with eight replicates. The results were subjected to analysis of variance. To determine “F-value” significance, the t-test and post hoc Newman-Keuls test were carried out to compare means among the diet groups. The main dispersion was expressed as standard deviation. Statistical analyzes were performed in the SPSS software, version 20.0. Data with a P-value < 0.05 were considered statistically significant.

Stage 2: Effects of chia on oxidative stress and inflammation in ovariectomized adult female Wistar rats

5.13. Raw material

Chia seeds (*Salvia hispanica* L.) produced in Brazil and grown in Catuípe, Rio Grande do Sul (RS) were used. In the laboratory, the seeds were ground in a knife mill with a particle size of 850 micrometers (MA 090 CFT) (2000 rpm; 30 mesh). Subsequently, the flours were packed in polyethylene bags covered with aluminum foil and stored in a freezer ($-18 \pm 1^\circ\text{C}$) until the time of analysis.

5.14. Experimental animals

Eighty female rats (*Rattus norvegicus*, Wistar, *albinus* variation), weanling, with 21 days of life were used. The animals were systematically divided into 2 groups with 40 animals each and received, during 7 weeks, standard diet (n = 40) or high-fat diet (n = 40), depending on which group they were allocated. After that period, 40 rats were submitted to ovariectomy and 40 rats were submitted to surgery, but without removal of the organ (SHAM group). The animals remained receiving this diet for another 3 weeks for recovery from surgery.

After 10 weeks, the animals were relocated to the following eight experimental groups (n = 10), where they remained for 8 weeks on the following diets: 1) standard diet (SHAM); 2) high fat diet (SHAM); 3) standard diet + chia (SHAM); 4) high fat diet + chia; 5) standard diet (OVX); 6) high fat diet (OVX); 7) standard diet + chia (OVX); 8) high fat diet + chia (OVX) (Figure 2). All animals received calcium carbonate as a source of calcium. The animals were distributed in individual metabolic cages of stainless steel in controlled temperature environment ($21 \pm 1^\circ\text{C}$) and light and dark cycle of 12 hours, automatically controlled. The animals received deionized water and their respective experimental diets *ad libitum*.

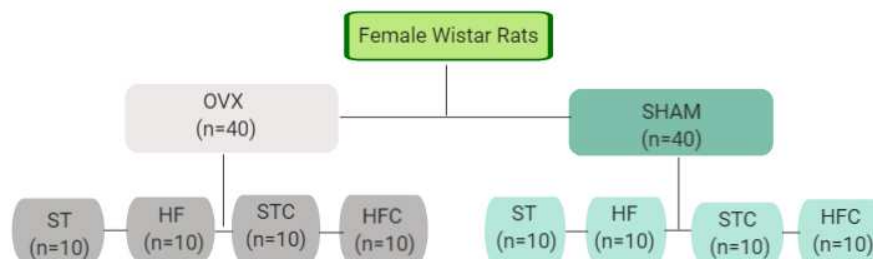


Figure 2. Experimental design of the calcium bioavailability study in female Wistar rats. OVX: ovariectomized; SHAM: non-ovariectomized; ST: standard diet; HF: high fat diet; STC: standard diet + chia; HFC: high fat diet + chia.

After 126 days and 12 hours of fasting, the animals were anesthetized with isoflurane (Isoforine®, Cristália) and euthanized by cardiac puncture. The blood was collected in tubes with heparin, 16 x 100 mm (BD Vacutainer®) and centrifuged (Hermle®, Z216MK model, Germany) at 800 g for 10 minutes to separate the plasma. The liver were stored at -80°C until molecular biology analyzes. The study was submitted and approved by the Ethics Committee on Animal Research of the Federal University of Viçosa, Brazil (Protocol 97/2014).

5.15. Ovariectomy procedure

At 10 weeks, the animals were submitted to ovariectomy (OVX), with abdominal incision and ovarian removal, 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 the anti-inflammatory flunixinameglumine (0.68 mg/kg) and the antibiotic enrofloxacin (10 mg/kg) via subcutaneous. The animals were anesthetized with isoflurane diluted in 100% oxygen by inhalation via a calibrated vaporizer. The isoflurane concentration was adjusted to maintain the appropriate anesthetic plane. After induction and anesthetic stabilization, the animals were placed in dorsal decubitus on a mattress with active heating and the operative field was prepared with iodopovidone 10%. The analgesic morphine was administered subcutaneously at a dose of 5 mg/kg.

After the surgery, the animals remained in a heated chamber in order to maintain body temperature, and then returned to the individual cages. The surgical-anesthetic and post-surgical procedures were performed at the Veterinary Hospital of Veterinary Department (UFV), under the responsibility of the Veterinary Medicine Lukiya Silva Campos Favarato. The onset of osteoporosis induction after OVX was 3 weeks prior to initiation of the intervention, where the rats remained in individual cages and received AIN diet or high fat diet and water *ad libitum*.

5.16. Experimental diets

The composition of the diets were based on the AIN-93G diet (REEVES et al., 1993), in powder for growing animals (16% protein, 34% lipid and 50% carbohydrate) or high-fat diet (64% lipids, 17% protein and 19% carbohydrates) (RESEARCH DIETS, 2017), with modifications, depending on which group they were allocated. Albumin was used as a protein source, replacing casein, due to the lower concentration of contamination. The chia seed was ground at the time of preparation of the diets, to obtain the flour.

The amount of chia flour offered in the diets (STC and HFC) was determined, based on their composition, in order to provide 100% of calcium recommendation (5 g of calcium per 1 kg of diet) for rodents and chia contributed with 20% of calcium. The other ingredients of these diets were added in sufficient quantity to adjust the composition of lipids, proteins, carbohydrates, fibers and calories of the corresponding diets, respectively (Table 2). For the calculation of the experimental diet, it was considered the centesimal composition of the chia flour obtained previously by our research group (30.2 g/100g lipids, 19.7 g/100g protein, 37.0 g/100g fiber, 2.2 g/100 g of carbohydrates and 430 mg of calcium/100 g) (SILVA et al., 2017). All ingredients were weighed in a semi-analytical balance (Gehaka, BG2000, Brazil), manually mixed, sieved in plastic sieve and homogenized in an industrial mixer (Leme) for 15 minutes. After the pre-preparation, the diets were packed in polyethylene bags, properly labeled and stored in a freezer ($-18^{\circ}\text{C} \pm 1^{\circ}\text{C}$).

Table 2. Nutritional composition of experimental diets used on study with female *Wistar* rats.

<i>Ingredients</i>	Early Stage		Experimental Diets			
	10 weeks		8 weeks			
	ST	HF	ST	STC	HF	HFC
Calcium carbonate (mg)	6.25	6.25	12.50	10.00	12.50	10.00
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 without calcium (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	78.45	347.50	319.40	151.00	122.90
Cholesterol	-	1.50	-	-	1.50	1.50
<i>Nutritional composition</i>						
Total calories (Kcal)	3778.00 ^b	4971.80 ^a	3759.80 ^b	3647.40 ^b	4728.80 ^a	4616.48 ^a
Caloric density (Kcal/g)	3.78 ^b	4.97 ^a	3.76 ^b	3.65 ^b	4.73 ^a	4.62 ^a

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 the Duncan test.

5.17. Weight gain, food efficiency ratio (FER) and indices

Body weight gain and food consumption were monitored weekly during the experimental period to determine the feed efficiency ratio (FER), which was obtained by the following formula: $\text{FER}\% = (\text{Body Weight Gain} / \text{Diet Consumption}) \times 100$ (DA SILVA et al., 2016). The hepatosomatic index and cecal index were calculated as a relation between

liver weight or cecum weight and body weight, respectively, multiplied by 100 (KIM et al., 2016). The percentage of adiposity was calculated using the following formula: (visceral + gonadal + retroperitoneal + mesenteric + inguinal adipose tissues) / total body weight x 100 (PEREIRA et al., 2012).

5.18. Biochemical analysis

For the determination of biochemical parameters, 0.5 mL of plasma was used. Plasma glucose concentrations, total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), very-low density lipoprotein (VLDL), triacylglycerides (TGL), urea, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured by colorimetric methods using commercially available kits (Bioclin®, Belo Horizonte, Brazil). Analyses were conducted on a BS-200 Chemistry Analyzer (Bioclin®).

5.19. Oxidative stress levels analysis

To obtain the homogenate, approximately 200 mg of tissue were initially weighed into 2 ml microtubes, then the contents were macerated with pistil. It was added 1000 µl of phosphate buffer 50 mM with 1 mM EDTA (pH 7.4) and the sample was centrifuged at 12,000 g for 15 minutes at 4°C. The supernatant was carefully removed, and stored in ultra-freezer until analysis.

5.19.1. Liver lipid peroxidation (MDA)

The hepatic lipid peroxidation was quantified by the thiobarbituric acid reactive substances (TBARS) method. 400 µL of sample and 400 µL of TBARS solution (15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid and 0.25 M HCl) were aliquoted into 2 mL microtubes. Then, the samples were vigorously vortexed and heated in the water bath at 95°C for 30 minutes. After this procedure, 600 µl of n-Butanol were added, and the mixture was vortexed and centrifuged at 800 g for 10 minutes. The supernatant was carefully removed and pipetted in triplicate on Elisa plate for reading in Multiskan GO spectrophotometer (Thermo Scientific) at 535 nm (KOHN, LIVERSEDGE, 1944; PYLES et al., 1993). The MDA concentration was calculated using the molar absorptivity coefficient $E0 = 1.56 \times 10^5 \text{ mol L}^{-1}\text{cm}^{-1}$ (BUEGE; AUST, 1978), and the results expressed in nmol of MDA per milligrams of protein (MDA/PTN). Total liver homogenate protein was quantified according to the Bradford method (1976).

5.19.2. Superoxide dismutase enzymatic activity (SOD)

The quantification of superoxide dismutase (SOD) enzyme was performed on homogenate samples, as described above. To the aliquotted samples in the microtubes (30 μ L) were added 249 μ L of Tris-HCl buffer 50 mM, pH 8.2, containing 1 mM EDTA; 6 μ L MTT (1.25 mM) and 15 μ L pyrogallol (10 mM). For the standard, it was added 6 μ L of MTT; 15 μ L of pyrogallol (10 mM) and 279 μ L of buffer and to obtain the blank, 6 μ L of MTT and 294 μ L of buffer were applied to the wells. The samples, standard and blank were incubated at 37°C for 5 minutes and the reading was performed on a Multiskan GO (Thermo Scientific) spectrophotometer at 570 nm. Results were expressed as units of SOD/mg protein. The calculations were performed in relation to the absorbance value of the standard, considering that it has one U of SOD, that is, 100% of pyrogallol oxidation (MARKLUND, 1995).

5.19.3. Catalase enzymatic activity

The determination of catalase enzyme activity is based on its ability to cleave hydrogen peroxide (H_2O_2) in water and molecular oxygen, as described by Aebi (1984). In a tube, it was added 20 μ L of the homogenate supernatant (1:10 diluted in water), 1 ml of phosphate buffer 100 mM (pH 7.2) + H_2O_2 (in each 25 ml buffer, 40 μ L of H_2O_2 30%). The absorbance was determined at 0, 30 and 60 seconds at 240 nm in a spectrophotometer (T70 + UV/VIS Spectrometer). The equipment was cleared prior to each analysis with 100 mM phosphate buffer pH 7.2 and the respective sample. Catalase activity was calculated according to Lambert Beer's law. The absorbance used for the calculation was the delta obtained from the absorbances read at times 0 and 60 (final absorbance - initial absorbance).

5.19.4. Determination of the antioxidant capacity of liver

To obtain the homogenate, about 200 mg of tissue was weighed into 2 mL microtubes and then the contents were macerated with pistil. 500 μ L of 1X buffer (Antioxidant Assay kit, Sigma Aldrich) was added and the sample was centrifuged at 12,000 g for 15 minutes at 4°C. The supernatant was removed, and stored in an ultra freezer until analysis.

For the analysis, 10 μ L of trolox standards were pipetted in the center of the well of the Elisa plate, in triplicate, at increasing concentrations to obtain the standard curve. Aliquots of 10 μ L of homogenate were added to the remaining wells identified for the samples. 20 μ L of the metmioglobin reagent and 150 μ L of the ABTS solution were added to all wells. The plate was covered with alumina paper and incubated at room temperature for 5 minutes. After

incubation, the absorbance was reading at 405 nm on Elisa reader. For the calculation, it was used the line equation obtained by the construction of the analytical curve and the values were expressed in mM trolox equivalent.

5.20. Histological analysis

Semi-serial histological sections of fragments of the liver with 3µm thickness were obtained in automatic microtome (Reichert-Jung®, Genossen, Germany) and were stained by hematoxylin and eosin (HE) technique. The slides were examined under an Olympus BX43 light microscope. The samples were semi-quantified in: absence steatosis (0), minimal steatosis (1), mild steatosis (2), moderate steatosis (3) and severe steatosis (4).

5.21. Biomolecular Analysis

5.21.1. Extraction of mRNA from liver tissue and cDNA synthesis

The liver was macerated in liquid nitrogen in gral shroud free of RNase and the samples were aliquotted for RNA extraction. Total animal liver RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) using the manufacturer's recommendations (100 mg of tissue were homogenized with 1 ml of TRIzol). After extraction with chloroform (0.2 mL/1 mL TRIzol), the aqueous phase RNA was precipitated with isopropyl alcohol (0.5 mL/100 mg tissue), the tubes centrifuged at 12,000 g for 10 minutes (4°C) and RNA washed with 75% ethanol and centrifuged at 9,500 g for 5 minutes (4°C). The pellet was resuspended in 50 µl of diethylpyrocarbonate-treated Milli-Q water (DEPC water).

After extraction, the RNA samples were treated with DNase (RQ1 RNase Free-DNase Kit; Promega, Madison, WI, USA) using the manufacturer's protocol as follows: 7 µL of the sample (2 µg RNA) were transferred to a tube and treated with 1 µl of DNase buffer and 2 µl of DNase and left at 37°C for 30 minutes for the action of the DNase enzyme. After that period, 1 µL of stop solution was added and the samples remained in a water bath at 65 °C for 10 minutes. Total isolated RNA was used to synthesize cDNA using reverse transcription M-MLV kit (Invitrogen Corp, Grand Island, NY) according to the manufacturer's protocol (LIVAK, SCHMITTGEN, 2001).

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

mRNA Expression levels of genes in the liver that are involved in inflammation processes was analyzed by RT-qPCR. The SYBR Green PCR master mix from Applied Biosystems (Foster City, CA) was employed, and the analyses were performed on the StepOne™ Real-Time PCR System (Thermo Fisher Scientific) by means of the measurement system involving SYBR-Green Fluorescence and Primer Express software (Applied Biosystems, Foster City, CA). The PCR cycle involved the initial denaturation at 95°C (10 min) and 40 cycles, with 1 min of denaturation (95°C), 1 min. (62°C) and 2 min. (72°C), followed by the analytical curve of dissociation. Sense and antisense primer sequences were ordered (Choma Biotechnologies) to amplify PPAR- α (ID: 2404316), NF κ B (ID: 234230511), Zn-SOD1 (ID: 5615763), TNF- α (ID: 234230513). The relative expression levels of mRNA were normalized to the endogenous control: glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Table 2). All the steps were performed under conditions RNase free.

5.22. IL-1 β and TNF- α quantification

To determine the concentrations of TNF- α and IL-1 β in the liver, hepatic tissue samples were homogenized using a specify extraction kit reagents (Invitrogen). The fractions of TNF- α and IL-1 β were assessed by immunoassay using the Rat TNF- α (Cat#88-7340, Invitrogen) and Rat IL-1 β (Cat#88-7340, Invitrogen), ELISA kits, respectively. The microplates that were provided in the ELISA kits were respectively precoated with anti-TNF- α and anti- IL-1 β antibodies. The concentrations of TNF- α and IL-1 β were calculated by comparison to the corresponding standard curves.

5.23. Statistical analysis

The treatments were conducted in a completely randomized design, with ten repetitions. The results were subjected to analysis of variance at 5% probability. To "F-value" significance, the post hoc Duncan test was carried out to compare means among the groups with ovariectomy or not. Test-t was used to compare the same diet group with ovariectomy or not. The classification results of histological analyzes were assessed by non-parametric Kruskal-Wallis test and Fisher's LSD test for post-hoc comparisons between groups. Parametric tests were expressed as mean and standard deviation and non-parametric tests

were expressed as mean. Statistical analyzes were performed in the SAS software, version 9.0. Data with a p -value ≤ 0.05 were considered statistically significant.

Stage 3: Evaluation of the intra-amniotic administration of soluble extract from chia in the intestinal health, microbiota population and bioavailability of iron and zinc in Gallus gallus model

5.24. Extraction of soluble extract from chia

The extraction of soluble extract was performed according to Tako et al., (2014) and Hou et al., (2017). Briefly, the chia flour samples were dissolved in distilled water (50 g/L) (60°C, 60 min) and then centrifuged at 3000 rpm (4°C) for 25 min to remove particulate matter. The supernatant was collected and dialysed (MWCO 12–14 kDa) exhaustively against distilled water for 48 hours. At last, the dialysate was collected and then lyophilized to yield a fine off-white powder.

5.25. Liquid chromatography–mass spectrometry (LC-MS) analysis for polyphenols determination

Extracts and standards were analyzed by an Agilent 1220 Infinity Liquid Chromatograph (LC; Agilent Technologies, Inc., Santa Clara, CA, USA) coupled to an Advion expressionL® compact mass spectrometer (CMS; Advion Inc., Ithaca, NY, USA). Ten-microliter samples were injected and passed through an XBridge Shield RP18 3.5 μ m 2.1 $\times$ 100 mm column (Waters, Milford, MA, USA) at 0.6 mL/min. The column was temperature-controlled at 40°C. The mobile phase consisted of ultra-pure water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). Polyphenols were eluted using linear gradients of 94.0 to 84.4% A in 1.50 min, 84.4 to 81.5% A in 2.25 min, 81.5 to 77.0% A in 6.25 min, 77.0 to 55.0% in 1.25 min, 55.0 to 46.0% in 2.25 min, 46.0 to 94.0% in 2.25 min and hold at 94.0% A for 2.25 min for a total run time of 18 min. From the column, the flow was directed into a variable wavelength Ultraviolet (UV) detector set at 280 nm. The flow was then directed into the source of an Advion expressionL® CMS, and Electrospray ionization (ESI) mass spectrometry was performed in the negative ionization mode using selected ion monitoring with a scan time of 200 ms. The capillary temperature and voltages were 250°C and 180 volts, respectively. The ESI source voltage and gas temperature were 2.5 kilovolts and 250°C, respectively. The desolvation gas flow was 240 L/h. Advion Mass Express™ software (Advion, Ithaca, USA) was used to control the LC and compact mass

spectrometers (CMS) instrumentation and data acquisition. Individual polyphenols were identified and confirmed by comparison of m/z and LC retention times with authentic standards. The analysis of MS and UV data was performed using Advion Data Express™ software (Advion, Ithaca, USA).

5.26. Phytate, Dietary Fiber, Iron and Zinc analysis in chia seeds and chia extract

Dietary phytic acid (phytate)/total phosphorous was measured as phosphorus released by phytase and alkaline phosphatase, following the kit manufacturer's instructions ($n = 5$) (K-PHYT 12/12. Megazyme International. Bray, Ireland). The determination of total fiber and soluble and insoluble fractions was performed by the enzymatic-gravimetric method according to AOAC (2012), using the enzymatic hydrolysis for a heat-resistant amylase, protease and amyloglucosidase (total dietary fiber assay Kiyonaga, Sigma®, Kawasaki, Japan). For the determination of iron and zinc, chia seed and chia extract (0.5 g) were treated with 3.0 mL of 60:40 HNO_3 and HClO_4 mixture into a Pyrex glass tube and left for overnight to destroy organic matter. The mixture was then heated to 120°C for two hours and 0.25 mL of 40 $\mu\text{g/g}$ Yttrium added as an internal standard to compensate for any drift during the subsequent inductively coupled plasma atomic emission spectrometer (ICP-AES) analysis. The temperature of the heating block was then raised to 145°C for 2 hours. Then, the temperature of the heating block raised to 190°C for ten minutes and turned off. The cooled samples in the tubes were then diluted to 20 mL, vortexed and transferred into auto sample tubes to analyze via ICP-AES. The model of the ICP used was a Thermo iCAP 6500 series (Thermo Jarrell Ash Corp., Franklin, MA, USA) (DIAS et al., 2018).

5.27. Animals and Design

Cornish-cross fertile broiler eggs ($n = 105$) were obtained from a commercial hatchery (Moyer's chicks, Quakertown, PA, USA). The eggs were incubated under optimal conditions at the Cornell University Animal Science poultry farm incubator. All animal protocols were approved by Cornell University Institutional Animal Care and Use committee (ethic approval code: 2007-0129). Prebiotics in powder form were separately diluted in 18 $\text{M}\Omega\text{H}_2\text{O}$ to determine the concentrations necessary to maintain an osmolarity value (Osm) of less than 320 Osm to ensure that the chicken embryos would not be dehydrated upon injection of the solution. At 17 day of embryonic incubation, eggs containing viable embryos were weighed

and divided into 7 groups (n = 15). All treatment groups were assigned eggs of similar weight frequency distribution.

Each group was then injected with the specified solution (1 mL per egg) with a 21-gauge needle into the amniotic fluid, which was identified by candling. The 7 groups were assigned as follows: (1) non-injected; (2) 18 MΩ H₂O; (3) inulin; (4) chia extract 0.5%; (5) chia extract 1%; (6) chia extract 2.5%; (7) chia extract 5%. After all the eggs were injected, the injection holes were sealed with cellophane tape and the eggs placed in hatching baskets such that each treatment was equally represented at each incubator location. Immediately after hatch (21 days) and from each treatment group, chicks were euthanized by CO₂ exposure and their small intestine, blood, pectoral, cecum and liver were collected.

5.28. Iron and zinc content in serum and liver

The liver (0.5 g) and serum (50 µL) were treated with 3.0 mL of 60:40 HNO₃ and HClO₄ mixture into a Pyrex glass tube and left for overnight to destroy organic matter. The mixture was then heated to 120°C for two hours and 0.25 mL of 40 µg/g Yttrium added as an internal standard to compensate for any drift during the subsequent inductively coupled plasma atomic emission spectrometer (ICP-AES) analysis. The temperature of the heating block was then raised to 145°C for 2 hours. Then, the temperature of the heating block raised to 190°C for ten minutes and turned off. The cooled samples in the tubes were then diluted to 20 mL, vortexed and transferred into auto sample tubes to analyze via ICP-AES. The model of the ICP used was a Thermo iCAP 6500 series (Thermo Jarrell Ash Corp., Franklin, MA, USA).

5.29. Isolation of total RNA from chicken duodenum and liver

Total RNA was extracted from 30 mg of the proximal duodenal tissue or liver tissue (n = 10) using Qiagen RNeasy Mini Kit (RNeasy Mini Kit, Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. Briefly, tissues were disrupted and homogenized with a rotor-stator homogenizer in buffer RLT®, containing β-mercaptoethanol. The tissue lysate was centrifuged for 3 minutes at 8,000 g in a micro centrifuge. An aliquot of the supernatant was transferred to another tube, combined with 1 volume of 70% ethanol and mixed immediately. Each sample (700 µL) was applied to an RNeasy mini column, centrifuged for 15 s at 8000 g, and the flow through material was discarded. Next, the RN easy columns were transferred to new 2-mL collection tubes, and 500 µL of buffer RPE® was pipetted onto the RNeasy column followed by centrifugation for 15 s at 8000 g. An additional

500 µL of buffer RPE were pipetted onto the RNeasy column and centrifuged for 2 min at 8000 g. Total RNA was eluted in 50 µL of RNase free water. All steps were carried out under RNase free conditions. RNA was quantified by absorbance at A 260/280. Integrity of the 28S and 18S ribosomal RNAs was verified by 1.5% agarose gel electrophoresis followed by ethidium bromide staining. DNA contamination was removed using TURBO DNase treatment and removal kit from AMBION (Austin, TX, USA).

5.30. Real time polymerase chain reaction (RT-PCR)

To create the cDNA, a 20 µL reverse transcriptase (RT) reaction was completed in a BioRad C1000 touch thermocycler using the Improm-II Reverse Transcriptase Kit (Catalog #A1250; Promega, Madison, WI, USA). The first step consisted of 1 µg of total RNA template, 10 µM of random hexamer primers, and 2 mM of oligo-dT primers. The RT protocol was to anneal primers to RNA at 94°C for 5 min, copy the first strand for 60 min at 42°C (optimum temperature for the enzyme), then heat inactivate at 70°C for 15 min and hold at 4°C until ready to analyze by Nanodrop (Waltham, MA, 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). Genomic DNA contamination was assessed by a real-time RT-PCR assay for the reference genes samples.

5.31. Primer design

The primers used in the real-time PCR was designed based on 11 gene sequences from Genbank database, using Real-Time Primer Design Tool software (IDT DNA, Coralville, IA, USA). The sequences and the description of the primers used in this work are summarized in Table 3. The amplicon length was limited to 90 to 150 bp. The length of the primers was 17–25-mer and the GC content was between 41% and 55%. 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. Results obtained from the qPCR system were used to normalize those obtained from the specific systems as described below.

5.32. Real-time qPCR design

cDNA was used for each 10 µL reaction together with 2×BioRad SSO Advanced Universal SYBR Green Supermix (Cat #1725274, Hercules, CA, USA) which included

buffer, Taq DNA polymerase, dNTPs and SYBR green dye. Specific primers (forward and reverse) (Table 1) and cDNA or water (for no template control) were added to each PCR reaction. The specific primers used can be seen in Table 3. For each gene, the optimal $MgCl_2$ concentration produced the amplification plot with the lowest cycle product (C_p), the highest fluorescence intensity and the steepest amplification slope. Master mix (8 μ L) was pipetted into the 96-well plate and 2 μ L cDNA was added as PCR template. Each run contained 7 standard curve points in duplicate. A no template control of nuclease-free water was included to exclude DNA contamination in the PCR mix. The double stranded DNA was amplified in the Bio-Rad CFX96 Touch (Hercules, CA, USA) using the following PCR conditions: initial denaturing at 95°C for 30 s, 40 cycles of denaturing at 95°C for 15 s, various annealing temperatures according to Integrated DNA Technologies (IDT) for 30 s and elongating at 60°C for 30 s.

The data on the expression levels of the genes were obtained as C_p 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). For each of the 13 genes, the reactions were run in duplicate. All assays were quantified by including a standard curve in the real-time qPCR analysis. The next four points of the standard curve were prepared by a 1:10 dilution. Each point of the standard curve was included in duplicate. A graph of C_p vs. log 10 concentrations was produced by the software and the efficiencies were calculated as $10[1/\text{slope}]$. The specificity of the amplified real-time RT-PCR products were verified by melting curve analysis (60–95°C) after 40 cycles, which should result in a number of different specific products, each with a specific melting temperature.

5.33. Collection of Microbial Samples and Intestinal Contents DNA Isolation

The cecum were sterilely removed and treated as described previously (TAKO et al., 2008; HARTONO et al., 2014). The contents of the cecum were placed into a sterile 50 mL tube containing 9 mL of sterile PBS and homogenized by vortexing with glass beads (3 mm diameter) for 3 min. Debris was removed by centrifugation at 700 g for 1 min, and the supernatant was collected and centrifuged at 12,000 $\times$ g for 5 min. The pellet was washed twice with PBS and stored at -20°C until DNA extraction. For DNA purification, the pellet was re-suspended in 50 mM EDTA and treated with lysozyme (Sigma Aldrich CO., St. Louis, MO, USA; final concentration of 10 mg/mL) for 45 min at 37°C. The bacterial genomic DNA was isolated using a Wizard Genomic DNA purification kit (Promega Corp., Madison, WI, USA).

5.34. Primers design and PCR amplification of bacterial 16S rDNA

Primers for *Lactobacillus*, *Bifidobacterium*, *Clostridium* and *E. coli* were designed according to previously published data (TAKO et al., 2008; ZHU et al., 2002). To evaluate the relative proportion of each examined bacteria, all products were expressed relative to the content of the universal primer product and proportions of each bacterial group are presented. PCR products were separated by electrophoresis on 2% agarose gel, stained with ethidium bromide, and quantified using the Quantity One 1-D analysis software (Bio-Rad, Hercules, CA, USA).

Table 3. DNA sequences of the primers used in *in ovo* study.

Analyte	Forward Primer (5'-3')	Reverse Primer (5'-3')	Base Pair	GI Identifier
DMT1	TTGATTCAGAGCCTCCCATTAG	GCGAGGAGTAGGCTTGTATTT	101	206597489
Ferroportin	CTCAGCAATCACTGGCATCA	ACTGGGCAACTCCAGAAATAAG	98	61098365
Dcytb	CATGTGCATTCTCTTCCAAAGTC	CTCCTTGGTGACCGCATTAT	103	20380692
Hepcidin*	GAGCAAGCCATGTCAAGATTTC	GTCTGGGCCAAGTCTGTTATAG	132	XM_015291382.1
ZnT 1	GGTAACAGAGCTGCCTTAACT	GGTAACAGAGCTGCCTTAACT	105	54109718
SI	CCAGCAATGCCAGCATATTG	CGGTTTCTCCTTACCACTTCTT	95	2246388
AP	CGTCAGCCAGTTTGACTATGTA	CTCTCAAAGAAGCTGAGGATGG	138	45382360
SGLT1	GCATCCTTACTCTGTGGTACTG	TATCCGCACATCACACATCC	106	8346783
LPL*	TGCTCAGATGCCCTACAAAG	TCTCGTCTAGAGTGCCATACA	119	396219
CEL*	ATGCTGCTGACATCGACTAC	TTCTGAAGTGGACGGTTGATAG	97	417165
18S rRNA*	GCAAGACGAACTAAAGCGAAAG	TCGGAAC TACGACGGTATCT	100	7262899

DMT1, Divalent metal transporter 1; Dcytb, Duodenal cytochrome b; Znt 1, Zinc transporter 1; SI, Sucrose isomaltase; AP, Amino peptidase; SGLT1, Sodium-Glucose transport protein 1; LPL, Lipoprotein lipase; CEL, Carboxyl ester lipase; 18S rRNA, 18S Ribosomal subunit. *liver analyses.

5.35. Glycogen analysis

At hatch, the pectorals muscle (20 mg) was dissected and placed on ice before freezing for subsequent glycogen analysis. The pectoral muscle samples were then homogenized in 8% perchloric acid, and glycogen content was determined using modified methods described by Dreiling et al (1987). After homogenization, the samples were centrifuged at 12,000 rpm at 4°C for 15 min. The supernatant was removed, and 1.0 mL of petroleum ether was added to each tube. After mixing, the petroleum ether fraction was removed, and samples from the bottom layer were transferred to a new tube containing 300 µL of color reagent. All samples were read at a wavelength of 450 nm in ELISA reader and the amount of glycogen was calculated according to a standard curve. The amount of glycogen present in pectoral sample was determined by multiplying the weight of the tissue by the amount of glycogen per 1 g of wet tissue.

5.36. Morphological examination

Liver and intestine samples were collected at the conclusion of the study and from each treatment group. Samples were fixed in fresh 4% (v/v) buffered formaldehyde, dehydrated, cleared, and embedded in paraffin. Serial sections were cut at 5 µm and placed on glass slides. Sections were deparaffinized in xylene, rehydrated in a graded alcohol series, stained with hematoxylin/ eosin or Alcian Blue/Periodic acid-Schiff, and examined by light microscopy. In the intestine, morphometric measurements of villus height, villus width, depth of crypts, goblet cell number and goblet cell diameter were performed with a light microscope using EPIX XCAP software (Standard version, Olympus, Waltham, MA, USA).

Villi height was measured using the lamina propria as the base; villi width, depth of the crypt (defined as the depth of the invagination between adjacent villi); number of goblet cell were measured per side of a longitudinal section through the villus; goblet cell size was measured as the diameter of the goblet cells (µm²). Villi surface area was calculated from the villus height and width at half height (UNI et al. 1999). For the Alcian Blue and Periodic acid-Schiff stain, the segments were only counted for the types of goblet cells in the villi epithelium, goblet cells within the crypts and the mucus layer thickness. Goblet cells were enumerated on 10 villi/sample, and the means were utilized for statistical analysis. The liver was stained with hematoxylin-eosin (H&E) for standard microscopy and visualize using the same light microscope. Mean fat cells diameter was determined by random utilizing the EPIX

XCAP software (standard version, Olympus, Waltham, MA, USA) by enumerating 10 fat cells/segment/sample, and the means were utilized for statistical analysis.

5.37. Statistical analysis

All values are expressed as means and standard deviation. Experimental treatments for the *in ovo* assay were arranged in a completely randomized design. The results were analyzed by ANOVA. For significant “p-value”, post hoc Duncan test was used to compare test groups. Statistical analysis was carried out using SPSS version 20.0 software. The level of significance was established at $p < 0.05$.

5.38. Ethical aspects

The research project related to *Wistar* rats (male and female) was submitted and approved (process n° 20/2017) by the Animal Research Ethics Committee of the Federal University of Viçosa (UFV) (Appendix I). The iron and zinc bioavailability assay project was submitted to Cornell University's Animal Research Ethics Committee (ethic approval code: 2007-0129). All experimental procedures with the animals were performed in accordance with ethical principles in animal experimentation.

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RESULTS

PAPER 1: Effects of chia (*Salvia hispanica* L.) on calcium bioavailability and inflammation in *Wistar* rats

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Effects of chia (*Salvia hispanica* L.) on calcium bioavailability and inflammation in *Wistar* rats



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1. Introduction

Chia (*Salvia hispanica* L.), is an herbaceous plant with high nutritional and functional value (Ixtaina, Nolasco, & Tomás, 2008) due to high concentrations of essential fatty acids, dietary fiber, phenolic compounds, and proteins (Ayerza & Coates, 2011; Chicco et al., 2009; Silva et al., 2017; Vuksan et al., 2007). Moreover, the seeds of this plant stand out due to their high concentrations of minerals, including calcium (Silva et al., 2017). Although calcium is present at high concentrations in chia, the bioavailability of this mineral for use in metabolic processes or storage in humans must be evaluated (Jafari & McClements, 2017).

The prevalence of calcium deficiency is around 51% (3.5 billion people) worldwide (Kumssa et al., 2015). Calcium intake is currently decreasing across all age groups, resulting in a higher prevalence of osteoporosis and osteomalacia (Greupner, Schneider & Hahn, 2017). In addition to low calcium intake, the current diets of many populations are characterized by high sugar and fat consumption, which can impair calcium bioavailability. This modification in food consumption is associated with an increased risk of cardiovascular diseases, obesity, and other disorders due to inflammation and oxidative stress (Catrysse & Van Loo, 2017; Cochain & Zernecke, 2017).

Changing in food intake can induce the inflammation, culminates in the activation of specific signaling pathways, thus leading to the production of inflammatory substances. These pathways include NF- κ B signaling and the production of inflammatory cytokines (Catrysse, & Loo, 2017). PPAR- α negatively regulates inflammation. PPAR- α ligands can inhibit the action of proinflammatory cytokines by interfering with NF- κ B activity in their signaling pathway. In addition to inflammation, PPAR- α plays a role in oxidative stress (Dotson et al., 2016). In addition, the inflammatory process also acts on bone metabolism through the secretion of cytokines by adipose tissue, e.g., tumor necrosis factor alpha (TNF- α), and interleukins (Morettini et al., 2015). The high levels of these proinflammatory cytokines can promote the differentiation into osteoclasts and consequently the process of bone resorption (Wong et al., 2016). Studies have shown that ingestion of chia can act beneficially on the pathways of inflammation in animals fed high fat diet or not (Marineli et al., 2015; Rincón-Cervera; Poudyal et al., 2012a; Poudyal et al., 2012b; Poudyal et al., 2013).

Although the association between low calcium intake and the development of cardiovascular disease has been verified (Soares et al., 2017), the association between calcium absorption and inflammatory biomarkers of high-fat intake has not been investigated. In addition, consumption of a high-fat diet contributes to the activation of inflammatory

pathways, promoting increased oxidative stress, deregulating lipid metabolism, and inducing dyslipidemia, which, in turn can decrease calcium absorption (Ventura et al., 2017). The hypothesis of the present study is that chia, as a source of calcium and bioactive compounds, can modulate bone metabolism, inflammation, and oxidative stress. Thus, the objective of the study was to investigate the bioavailability of calcium in chia and the influence of chia consumption on the blood lipid profile, oxidative stress, and inflammation in young *Wistar* rats fed a standard or high-fat diet.

2. Materials and Methods

2.1. Raw materials and preparation of flours

Chia seeds (*Salvia hispanica* L.) grown in the state of Rio Grande do Sul (Brazil) were used for the study. 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 . Subsequently, chia flour was packed in polyethylene aluminum bags and stored in a freezer ($-18^{\circ}\text{C} \pm 1^{\circ}\text{C}$) until analysis.

2.2. Determination of calcium content and chemical composition

Calcium content in chia was quantified according to ref. (Gomes, 2011). The calcium concentration was determined by coupled plasma atomic emission spectrometry (instrument model Optima 3300 DV, Perkin Elmer, Massachusetts, USA) with an inducible plasma argon source. Calibration curves were constructed by means of standard solutions of calcium, according to Pires et al. (2015). The analyses were performed in triplicate.

The analyses of moisture, ash, lipids, carbohydrates, proteins, total dietary fiber, calcium and total phenolic compounds were performed as reported previously (Silva et al., 2017) and the data were used to determine the composition of the experimental diets, because we employed the same chia sample for the standard and high-fat diet group (lipids: 32.2%; carbohydrates: 4.6%; proteins: 18.2%; total dietary fiber: 33.4%; calcium: 0.25%; total phenolic: 0.97%). The chemical composition was determined according to AOAC (2012). The total energy value of chia was estimated considering the conversion factors of $4 \text{ kcal}\cdot\text{g}^{-1}$ for protein or carbohydrate and $9 \text{ kcal}\cdot\text{g}^{-1}$ for lipids.

2.3. Animals and diets

A controlled experimental study was carried out to assess the bioavailability of calcium by measuring calcium balance, retention, and absorption. Thirty-two male rats (*Rattus norvegicus*, Wistar, *albinus* variation), newly weaned, 21 days old, from the Central Animal Facility of the Center for Biological Sciences and Health at Federal University of Viçosa, Minas Gerais, Brazil, were systematically subdivided into 4 groups with 8 animals each, randomized by body weight. The animals were distributed into individual stainless-steel cages in a controlled temperature environment (22°C) and automatically controlled light and dark cycles of 12 hours. The animals received deionized water and their respective experimental diets ad libitum. The experimental diets were based either on the standard AIN-93G diet (Reeves, Nielsen, & Fahey, 1993) or high-fat diet (Research Diets, New Brunswick, NJ) with modifications. The standard diet was composed of 20% protein, 30% fat and 50% carbohydrate. The high fat diet was prepared in the following proportions: 64% fat, 16% protein and 20% carbohydrate. The amount of chia offered in the diets, which was based on composition, provided 50% of the recommended amount (0.0025 kg of calcium per 1 kg of diet) (Bryk et al., 2016). The other ingredients were added in sufficient quantities to provide the planned amounts of lipids, proteins, carbohydrates, fiber, and calories (Table 1).

Table 1. Nutritional composition of experimental diets.

<i>Ingredients (per kg of diet)</i>	Experimental Diets			
	SD+CC	SD+C	HFD+CC	HFD+C
Calcium carbonate (mg)	5.00	0.00	5.00	0.00
Chia (g)	0.00	416.80	0.00	416.80
Albumin (g)	217.90	117.60	217.90	117.60
Dextrinized starch (g)	132.00	132.00	132.00	115.10
Sucrose (g)	100.00	100.00	100.00	100.00
Soybean oil (mL)	134.20	0.00	134.20	0.00
Lard (g)	0.00	0.00	200.00	200.00
Microcrystalline cellulose (g)	139.20	0.00	139.20	0.00
Calcium-free mineral mix (g)	35.00	35.00	35.00	35.00
Vitamin mix (g)	10.00	10.00	10.00	10.00
L-cystine (g)	3.00	3.00	3.00	3.00
Choline bitartrate (g)	2.50	2.50	2.50	2.50
Corn starch (g)	221.20	183.10	21.20	0.00
<i>Nutritional composition</i>				
Total calories (kcal)	3700.42 ^b	3624.75 ^b	4700.42 ^a	4624.11 ^a
Caloric density (kcal.g ⁻¹)	3.70 ^b	3.62 ^b	4.70 ^a	4.62 ^a
Calcium (g.kg ⁻¹)*	2.41±0.00 ^a	2.53±0.02 ^a	2.57±0.04 ^a	2.58±0.00 ^a

* Analyzed according to the methodology proposed by Gomes (2011). SD + CC: standard diet + calcium carbonate; SD+C: standard diet + chia; HFD+CC: high fat diet + calcium carbonate; HFD+C: high fat diet + chia. Means with different letters in the same row indicate a significant difference ($p < 0.05$) according to the Newman-Keuls test.

The experimental groups received one of the following four diets: standard diet + calcium carbonate (SD + CC), standard diet + chia (SD + C), high-fat diet + calcium carbonate (HFD + CC), or high-fat diet + chia (HFD + C). Chia served as a source of calcium in the SD + C and HFD + C groups, whereas calcium carbonate was used as the source of calcium in the SD + CC and HFD + CC groups. On the 35th day, after 12 hours of fasting, the

animals were anesthetized with isoflurane (Isoforine, Cristália®) and blood was collected by cardiac puncture. Blood was centrifuged at 4°C for 600 s (Fanem-204, São Paulo, Brazil) and the plasma was stored at -80°C. Urine, feces, liver tissue, and the right femur were collected and stored at -80°C before analysis. Prior to euthanasia, each animal was individually housed in a metabolic cage. Samples of urine and feces were collected during 3 days for determination of the calcium bioavailability.

Body weight gain and food consumption were monitored weekly during the experimental period to determine the feed efficiency ratio (FER) (Silva et al., 2016). The hepatosomatic index and cecal index were calculated as a relation between liver weight or cecum weight and body weight, respectively, multiplied by 100 (Kim et al., 2016).

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).

2.4. Biochemical analysis

For the determination of biochemical parameters, 0.5 mL of plasma was used. Plasma glucose concentrations, total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), very-low density lipoprotein (VLDL), triacylglycerides (TGL), uric acid, creatinine, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured by colorimetric methods using commercially available kits (Bioclin®, Belo Horizonte, Brazil). Analyses were conducted on a BS-200 Chemistry Analyzer (Bioclin®).

2.5. Calcium bioavailability

For each animal, calcium intake was estimated according to daily food intake, and calcium absorption, balance, and retention were calculated using equations proposed by Ku et al. (2015).

Blood plasma and urine calcium levels were measured by colorimetric methods involving commercially available kits (Bioclin®, Belo Horizonte, Brazil). Analyses were performed on a BS-200 Chemistry Analyzer (Bioclin®). The calcium concentrations in feces and the femurs of experimental animals were determined by atomic absorption spectrophotometry (Gomes, 2011).

The size and thickness of the femur were measured by means of a 200-mm digital pachymeter (resolution, 0.01 mm; Model 530-312; Mitutoyo). Maximum tensile strength was determined using a Universal Mechanical Testing Machine (Instron, Norwood, MA USA). Resistance curves were built on a microcomputer in the Blue Hill software.

2.6. Extraction of mRNA from liver tissue and cDNA synthesis

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

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

mRNA Expression levels of genes in the liver that are involved in inflammation processes was 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 PPAR- α (ID: 2404316), NF κ B (ID: 234230511), Zn-SOD1 (ID: 5615763), TNF- α (ID: 234230513), and IL-10 (ID: 537719). The relative expression levels of mRNA were normalized to the endogenous control: glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 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
GAPDH	AGGTTGTCTCCTGTCACTTC	CTGTTGCTGTAGCCATATTC
PPAR- α	CCTGCCTTCCCTGTGAACT	ATCTGCTTCAAGTGGGGAGA
NF κ B	ACCGAAGCAGGAGCTATCAA	GCGTACACATTCTGGGGAGT
Zn-SOD1	GAGCAGAAGGCAAGCGGTGAA	CCACATTGCCCAGGTCTG
TNF- α	ACGGCATGGATCTCAAAGAC	AGATAGCAAATCGGCTGACG
IL-10	ACTACCATAGCCACAACGCA	TTTCTGTTTCCTACGGCGCT

GAPDH: glyceraldehyde 3-phosphate dehydrogenase; PPAR- α : peroxisome proliferator-activated receptor alpha; NF κ B: factor nuclear kappa B; SOD: superoxide dismutase; TNF- α : tumor Necrosis Factor Alpha; IL-10: interleukin 10.

2.8. NF- κ B p65 and PPAR- α quantification

To determine the concentrations of PPAR- α and NF- κ B p65 in the liver, hepatic tissue samples were homogenized by means of the NE-PER Nuclear and Cytoplasmic Extraction Kit reagents (Thermo Scientific Fisher, USA). The nuclear fractions of PPAR- α and NF- κ B p65 were assessed by immunoassay using the Rat PPAR (Cat # E-EL-R0725-ra; Elabscience, USA) and Rat NF- κ B p65 (Cat #E-EL-R0674, Elabscience, USA), ELISA kits, respectively.. The microplates that were provided in the ELISA kits were respectively precoated with anti-PPAR- α and anti-NF- κ B p65 antibodies. The concentrations of PPAR- α and NF- κ B p65 were calculated by comparison to the corresponding standard curves.

2.9. Lipid peroxidation and oxidative stress levels analysis

2.9.1. Homogenate preparation

To obtain a liver homogenate, 200 mg of liver was mixed with 1000 μ L of phosphate buffer (50 mM) and 1 mM EDTA (pH 7.4). The sample was macerated and centrifuged at 12,000 $\times$ g and 4°C for 10 minutes, and then the supernatant was removed and stored in an ultra-freezer until analysis.

2.9.2. Malondialdehyde (MDA)

MDA was quantified by the thiobarbituric acid reactive substances (TBARS) method (Kohn & Liversedge, 1944; Pyles et al., 1993). MDA concentration was calculated via the

molar absorptivity coefficient (Buege & Aust, 1978), and the results were expressed in nanomoles of MDA per milligram of protein (MDA/PTN). Total protein in the liver homogenate was quantified by the Bradford method (1976).

2.9.3. Nitric oxide

This analysis was performed in liver tissue and plasma: 50 μ L of plasma or homogenate was mixed with solutions A (1% sulfanilamide in 2.5% H_3PO_4) and B (0.1% naphthyl 1 ethylene diamide dihydrochloride in 2.5% H_3PO_4) in the ratio (1:1) and the microtiter plate was incubated in the dark for 10 minutes. Absorbance was read on a spectrophotometer (Multiskan Go, Thermo Cientific) at 570 nm and the results were expressed in ($\mu\text{mol NO}$)/(mg protein) in hepatic tissue and in μM in plasma (Green et al., 1982).

2.9.5. Superoxide dismutase (SOD)

The quantification of SOD was performed in relative units, and one unit was defined as the amount of SOD enzyme that inhibits the pyrogallol oxidation rate by 50%. The analysis was carried out on a spectrophotometer (Multiskan GO, Thermo Scientific) at 570 nm, and the results were expressed as units of SOD activity per milligram of protein (Marklund, 1995).

2.9.4. Catalase (CAT)

Catalase was analyzed according to the methodology proposed by Aebi (1984). At 0, 30, and 60 seconds after the reaction was initiated, the absorbance at 240 nm was determined on a spectrophotometer (T70 + UV/VIS Spectrometer). Enzymatic activity was recorded in micromoles per milliliter of a sample. Catalase activity was calculated according to Lambert Beer's law.

2.9.5. Liver and plasma antioxidant capacity

Aliquots (10 μ L) of the liver homogenate or plasma were added to the wells along with 20 μ L of the reagent metmyoglobin and 150 μ L of the ABTS solution. Then, 10 μ L of increasing concentrations of trolox standard were pipetted into the wells, in triplicate, to construct a standard curve. The microtiter plate was incubated at room temperature, and then the absorbance at 405 nm was read by means of a spectrophotometer (Multiskan GO). The values are expressed in mM Trolox equivalents.

2.10. Statistical analysis

The treatments were conducted in a completely randomized design, with eight replicates. The results were subjected to analysis of variance. To determine "F-value" significance, the post hoc Newman-Keuls test were carried out to compare means among the diet groups. The main dispersion was expressed as standard deviation. Statistical analyzes were performed in the SPSS software, version 20.0. Data with a P-value <0.05 were considered statistically significant.

3. Results

All four diets contained the same amount of calcium ($p > 0.05$), which was approximately 0.0025 kg per kg of diet. At the beginning of the study, until the third week, the total food intake ($p \leq 0.05$) was higher in the rats fed the standard diet (SD+CC and SD+C) than in the rats fed the high-fat diet. By contrast, at the end of the experiment, animals that were fed the standard diet containing chia (SD+C) showed higher ($p \leq 0.05$) food intake than did the other diet groups (Fig. 1). Consumption of phytic acid and total phenolics was higher ($p < 0.05$) in animals fed the standard diet containing chia (SD+C) than in animals feed the high-fat diet with chia (HFD+C; Table 3). In the groups without chia, these parameters were not measured.

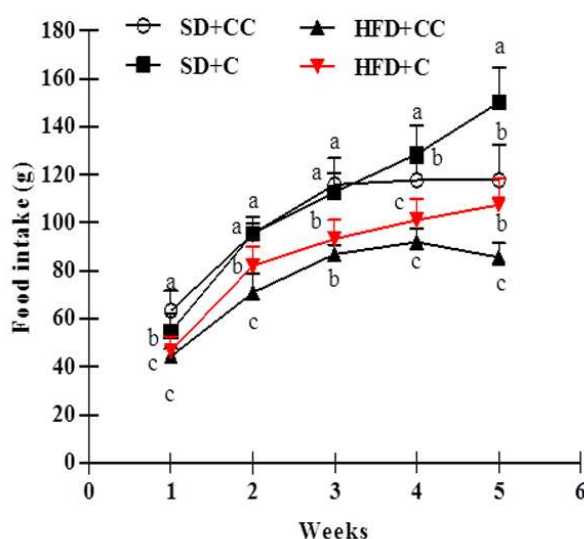


Figure 1. Food intake of the experimental animals. *Means followed by the same letter in the same graphic did not differed by Newman-Keuls test at 5% of probability. SD+CC: standard diet + calcium carbonate; SD+C: standard diet + chia; HFD+CC: high fat diet + calcium carbonate; HFD+C: high fat diet + chia.

Animals that were fed with calcium carbonate showed higher ($p \leq 0.05$) saturated fatty acid intake and lower ($p \leq 0.05$) polyunsaturated fatty acid intake than did the animals that were fed chia (Table 3). In addition, calcium intake was higher ($p \leq 0.05$) in the rats fed a standard diet with chia (SD+C) owing to their higher food intake (Table 3). Animals fed the standard and high-fat diets with calcium carbonate (SD+CC and HFD+CC) showed lower ($p \leq 0.05$) weight gain than did the other groups (Table 3). The food efficiency ratio was higher ($p \leq 0.05$) in the animals fed the high-fat diet (HFD+CC and HFD+C) when compared to the animals fed a standard diet (SD+C and SD+CC; Table 3).

Liver weight and the hepatosomatic index were higher ($p \leq 0.05$) in the groups that were fed the high-fat diet (HFD+CC and HFD+C) than in the groups that were fed the standard diet (SD+CC and SD+C). The cecal weight and cecal index did not differ significantly among the four experimental groups ($p > 0.05$; Table 3).

Table 3. Weight gain, FER, indexes and consumption of phytic acid, phenolic compounds, fatty acids and calcium by the experimental animals (n = 8) for 35 days

Groups	SD+CC	SD+C	HFD+CC	HFD+C
Weight gain (g)	164.40 ± 15.50 ^b	183.20 ± 14.34 ^a	150.76 ± 11.36 ^b	182.14 ± 13.97 ^a
FER	31.82 ± 1.53 ^b	33.88 ± 1.77 ^b	39.67 ± 2.70 ^a	42.31 ± 1.51 ^a
Liver index	5.66 ± 0.38 ^b	5.20 ± 0.23 ^b	6.35 ± 0.33 ^a	6.14 ± 0.36 ^a
Cecum index	2.23 ± 0.30 ^a	2.60 ± 0.47 ^a	2.68 ± 0.54 ^a	2.23 ± 0.34 ^a
Phytic acid (g/kg/day)	-	2.21 ± 0.15 ^a	-	1.73 ± 0.13 ^b
Total phenolic intake (mg GAE/ kg/day)	-	2.20 ± 0.17 ^a	-	1.75 ± 0.13 ^b
Fatty acids intake (g/kg/day)				
Saturated	86.27 ± 5.03 ^a	6.55 ± 0.58 ^c	63.59 ± 2.89 ^b	5.20 ± 0.19 ^c
Polyunsaturated	26.00 ± 1.52 ^c	60.74 ± 2.13 ^a	19.16 ± 0.87 ^d	50.15 ± 2.85 ^b
n-3	0.20 ± 0.01 ^c	14.77 ± 0.98 ^a	0.15 ± 0.01 ^c	11.74 ± 0.65 ^b
n-6	3.05 ± 0.14 ^c	4.12 ± 0.27 ^a	2.27 ± 0.11 ^d	3.28 ± 0.18 ^b
Calcium intake (g/kg/day)	1.19 ± 0.07 ^b	1.32 ± 0.01 ^a	0.97 ± 0.04 ^c	1.11 ± 0.08 ^b

SD + CC: standard diet + calcium carbonate; SD+C: standard diet + chia; HFD+CC: high fat diet + calcium carbonate; HFD+C: high fat diet + chia. FER: weight gain/food intake x 100. Indexes: cecum and liver weight/ body weight x 100. The phytic acid and total phenolic

intake were analyzed by the *t* test. Means followed by the same letter in the same row did not differ significantly according to the Newman–Keuls test at the 5% threshold of probability.

Chia consumption in the rats fed either the standard or high-fat diet led to a lower ($p \leq 0.05$) calcium balance and calcium absorption and retention rates as compared to the rats fed either the standard or high-fat diet with calcium carbonate. In addition, the urinary calcium concentration was lower ($p \leq 0.05$) in the groups that were fed chia (SD+C and HFD+C) and in the group fed the high-fat diet with calcium carbonate (HFD+CC). Nonetheless, fecal calcium content was lower ($p \leq 0.05$) in the animals that were fed calcium carbonate (SD+CC and HFD+CC). The calcium concentration in the femur and blood did not differ among the four groups ($p > 0.05$) (Figure 2).

Although bone calcium content did not differ among the experimental groups, the bone resistance of rats fed chia (SD+C and HFD+C) and HFD+CC rats was lower ($p \leq 0.05$; 31.3-, 51.4-, and 35.8 fold lower, respectively) than that in the rats fed the standard diet with calcium carbonate (SD+CC) (Figure 2).

	SD + CC	SD + C	HFD + CC	HFD + C	
Urinary Ca (mg/day)	a 0.53±0.08	b 0.46±0.03	b 0.47±0.04	b 0.43±0.04	High values Low values
Fecal Ca (mg/day)	b 2.40±0.60	a 22.15±1.17	b 1.84±0.92	a 18.60±2.95	
Ca femur (g.100 ⁻¹)	a 5.61±0.53	a 5.34±0.49	a 5.35±0.34	a 5.20±0.36	
Ca serum (mg/dL)	a 11.73±0.50	a 11.57±0.63	a 11.65±0.78	a 12.16±0.38	
Ca balance (mg/day)	a 31.12±1.87	c 13.67±1.50	b 22.79±1.44	d 11.38±1.79	
Ca absorption rate (%)	a 93.05±4.43	b 37.73±5.27	a 92.66±3.59	b 38.50±4.82	
Ca retention rate (%)	a 91.49±4.46	b 38.72±3.91	a 90.79±3.66	b 37.01±6.61	
Bone resistance (N)	a 53.34±7.76	b 36.67±2.08	b 34.22±4.16	c 25.90±2.79	

Figure 2. Effect of chia ingestion in calcium bioavailability in *Wistar* rats ($n = 8$) for 35 days. SD + CC: standard diet + calcium carbonate; SD+C: standard diet + chia; HFD+CC: high fat diet + calcium carbonate; HFD+C: high fat diet + chia; Ca: calcium, N: newton. Average scores on the lines followed by different letters differ by Newman Keuls test ($p < 0.05$).

The rats fed chia (SD+C and HFD+C) showed lower total cholesterol, VLDL, and LDL cholesterol levels ($p < 0.05$) than did the calcium carbonate (SD+CC and HFD+CC). In contrast, glucose, HDL, AST, urea, and creatinine levels did not differ among the four diet groups ($p > 0.05$). Nonetheless, the ALT concentration was higher ($p \leq 0.05$) in the two groups that consumed the high-fat diet (HFD+C and HFD+CC). Triglycerides levels were lower ($p \leq 0.05$) in the animals that were fed the standard diet with chia (SD+C) than in the animals that were fed the high-fat diet without chia (HFD+CC) (Table 4).

Table 4. Effects of chia consumption for 35 days on the biochemical variables in *Wistar* rats (n = 8)

Groups	SD+CC	SD+C	HFD+CC	HFD+C
Glucose (mg·dL ⁻¹)	188.25 ± 47.35 ^a	186.39 ± 10.39 ^a	197.63 ± 20.46 ^a	190.14 ± 20.01 ^a
HDL (mg·dL ⁻¹)	20.67 ± 2.94 ^a	24.57 ± 3.40 ^a	21.88 ± 4.36 ^a	27.00 ± 4.24 ^a
TC (mg·dL ⁻¹)	56.00 ± 9.73 ^a	43.02 ± 8.89 ^b	61.03 ± 9.16 ^a	46.00 ± 5.87 ^b
TGL (mg·dL ⁻¹)	53.26 ± 7.79 ^{ab}	43.56 ± 8.83 ^b	61.53 ± 6.22 ^a	52.38 ± 6.20 ^{ab}
AST (U·L ⁻¹)	154.40 ± 32.03 ^a	137.88 ± 9.57 ^a	161.83 ± 17.15 ^a	155.43 ± 20.12 ^a
ALT (U·L ⁻¹)	45.83 ± 7.39 ^b	46.00 ± 6.12 ^b	55.67 ± 6.41 ^a	55.71 ± 1.80 ^a
VLDL (mg·dL ⁻¹)	10.65 ± 1.56 ^a	8.71 ± 1.89 ^b	12.31 ± 1.24 ^a	10.12 ± 1.47 ^b
LDL (mg·dL ⁻¹)	27.80 ± 5.86 ^a	10.34 ± 1.97 ^b	27.19 ± 3.90 ^a	11.72 ± 2.19 ^b
Urea (mg·dL ⁻¹)	1.90 ± 0.21 ^a	1.87 ± 0.47 ^a	1.91 ± 0.44 ^a	1.92 ± 0.26 ^a
Creatinine (mg·dL ⁻¹)	0.36 ± 0.04 ^a	0.35 ± 0.06 ^a	0.31 ± 0.03 ^a	0.32 ± 0.04 ^a

SD + CC: standard diet + calcium carbonate; SD+C: standard diet + chia; HFD+CC: high fat diet + calcium carbonate; HFD+C: high fat diet + chia; HDL: high-density lipoprotein; TC: total cholesterol; TGL: triacylglyceride; AST: alanine aminotransferase; ALT: aspartate aminotransferase; VLDL: very-low density lipoprotein; LDL: low-density lipoprotein. Average values in the same row followed by different superscript letters differ significantly according to the Newman–Keuls test ($p < 0.05$).

SOD mRNA expression did not differ among experimental groups ($p > 0.05$). Nevertheless, the animals fed the high-fat diet with chia (HFD+C) manifested a higher ($p \leq 0.05$) SOD activity in the liver than did the animals fed the high-fat diet without chia (HFD+CC), with values comparable to those of rats fed the standard diet with chia (SD+C) (Table 5). Consumption of the high-fat diet for 35 days did not alter the total antioxidant capacity of the liver and plasma ($p > 0.05$) in young *Wistar* rats (Table 5). In addition, chia

consumption in rats fed the standard or high-fat diet did not improve these parameters. Nevertheless, the plasma catalase concentration was higher ($p \leq 0.05$) in the rats that were fed chia. The concentration of MDA in the liver and concentration of NO in the liver and plasma did not differ among the different experimental groups ($p > 0.05$) (Table 5). PPAR- α mRNA expression (Table 5) was higher ($p \leq 0.05$) in the rats fed the high-fat diet containing chia (HFD+C) than in the other diet groups. Nonetheless, PPAR- α protein levels were higher ($p \leq 0.05$) in the rats that were fed chia, regardless of the diet type (i.e., in SD+C and HFD+C rats; Table 5). The mRNA expression and the protein levels of NF κ B were lower ($p \leq 0.05$) in the rats fed chia (SD+C and HFD+C) and in the rats fed the standard diet with calcium carbonate (SD+CC; Table 5). Furthermore, mRNA expression of TNF- α and IL-10 were lower ($p \leq 0.05$) in the animals fed chia (SD+C and HFD+C).

Table 5. Effects of chia consumption for 35 days on the inflammation and oxidative stress in *Wistar* rats (n = 8).

Groups	SD+CC	SD+C	HFD+CC	HFD+C
SOD (U SOD/ mg protein)	5.12 ± 0.23 ^a	4.84 ± 0.48 ^{ab}	4.31 ± 0.16 ^c	4.60 ± 0.33 ^b
TAC (mM trolox)	0.67 ± 0.05 ^a	0.71 ± 0.05 ^a	0.67 ± 0.07 ^a	0.75 ± 0.05 ^a
LAC (mM trolox)	0.14 ± 0.00 ^a	0.12 ± 0.04 ^a	0.13 ± 0.01 ^a	0.13 ± 0.04 ^a
CAT (μmol/min/mL)	4.59 ± 0.77 ^{ab}	5.54 ± 0.89 ^a	3.96 ± 0.48 ^b	4.98 ± 0.86 ^a
MDA (nmol/mg protein)	3.47 ± 0.18 ^a	3.49 ± 0.44 ^a	3.27 ± 0.18 ^a	3.31 ± 0.52 ^a
Hepatic nitric oxide (μmol/mg protein)	4.72 × 10 ⁻⁶ ± 2.40 × 10 ^{-7a}	4.95 × 10 ⁻⁶ ± 4.11 × 10 ^{-7a}	4.14 × 10 ⁻⁶ ± 3.81 × 10 ^{-7a}	4.57 × 10 ⁻⁶ ± 1.02 × 10 ^{-6a}
Plasma nitric oxide (μmol)	2.82 ± 0.48 ^a	3.27 ± 0.65 ^a	2.54 ± 0.51 ^a	2.92 ± 0.46 ^a
mRNA PPAR-α/mRNA GAPDH	1.00 ± 0.00 ^b	0.45 ± 0.01 ^b	0.40 ± 0.10 ^b	1.94 ± 0.59 ^a
Hepatic PPAR-α (ng/mL)	30.28 ± 0.38 ^b	48.92 ± 0.50 ^a	36.47 ± 0.87 ^b	58.70 ± 0.59 ^a
mRNA NFκB/mRNA GAPDH	1.00 ± 0.00 ^b	0.75 ± 0.18 ^b	7.40 ± 1.27 ^a	2.28 ± 0.51 ^b
Hepatic NFκB (ng/mL)	1.80 ± 0.82 ^b	1.38 ± 0.12 ^b	2.79 ± 0.47 ^a	1.96 ± 0.35 ^b
mRNA TNF-α/mRNA GAPDH	1.00 ± 0.00 ^a	0.76 ± 0.12 ^b	1.13 ± 0.10 ^a	0.13 ± 0.02 ^b
mRNA IL-10/mRNA GAPDH	1.00 ± 0.00 ^a	0.44 ± 0.12 ^b	1.65 ± 0.51 ^a	0.33 ± 0.10 ^b

SD + CC: standard diet + calcium carbonate; SD+C: standard diet + chia; HFD+CC: high fat diet + calcium carbonate; HFD+C: high fat diet + chia; MDA: malondialdehyde. SOD: superoxide dismutase; TAC: total antioxidant capacity; LAC: liver antioxidant capacity; CAT: catalase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; PPAR-α: peroxisome proliferator-activated receptor alpha; NFκB: factor nuclear kappa B; TNF-α: tumor necrosis factor alpha; IL-10: interleukin 10. Average scores in the same row followed by different superscript letters differ significantly according to the Newman–Keuls test (p < 0.05).

4. Discussion

Chia is a good source of calcium; however, it has not been previously reported its bioavailability associated with inflammatory condition. Thus, the present study focused on the bioavailability of calcium in chia for rats fed a standard or a high-fat diet in part to determine whether a typical Western diet can reduce the bioavailability of calcium. In addition, the study

allowed us to test whether chia consumption can improve calcium absorption, the lipid profile, oxidative stress, and inflammatory status in young rats.

The lower food intake and the lower phytic acid and phenolic consumption observed in animals fed the high-fat diet can be attributed to higher energy density and greater satiety during the experimental period, as found other studies (Natal et al., 2017; Silva et al., 2016). Chia intake reduced the amount of saturated fatty acid and increased the amount of polyunsaturated fatty acids, owing to the composition of unsaturated fatty acids present in chia seeds, e.g., n-3 and n-6 fatty acids (Silva et al., 2017). A significant body weight difference among the groups was observed only in the last week of the experiment. On the other hand, it is known that weight is a less sensitive variable, and that changes in weight are typically observed later than changes in biochemical or cellular markers (Natal et al., 2017).

Despite the increase in liver weight in our HFD group owing to greater accumulation of fat globules, the levels of the ALT and AST liver enzymes were within the normal range for rodents (Spinelli et al., 2014). Nevertheless, ALT concentration was higher in the animals fed the high-fat diet, thus indicating the beginning of a proinflammatory state, which can alter liver homeostasis and cause liver injury (Warner et al., 2017). The cecal weight and cecal index did not differ among the experimental groups, likely because these diets were balanced in terms of macronutrients and dietary fiber. The same result was observed in a another study that evaluated inflammation modulation in adult *Wistar* rats with high-fat diet-induced obesity ; in that study, the high-fat diet did not promote differences in cecum weight between test and standard groups (Natal et al., 2016).

Because the body can regulate calcium metabolism via specific mechanisms, calcium concentration in the femur and blood did not differ among the experimental groups. When calcium intake is low there is an increase in calcium absorption and a reduction in its elimination (Fleet, 2017). This fact is supported by the urinary calcium concentration observed in the present study because the calcium content was lower in the rats that were fed the standard diet with chia (SD+C) and the high-fat diet (HFD+C and HFD+CC).

Furthermore, the data of bone resistance allows us to infer that high fat diet was able to reduce the bone resistance and the presence of chia did not improve this effect. In addition, all the evaluated parameters associated with calcium bioavailability were lower in the rats fed chia, thus, showing that the calcium present in chia is not well absorbed, metabolized, and/or utilized by the body when compared to a standard calcium source. The reduction in all the parameters associated with calcium bioavailability can be explained by two mechanisms: 1)

the increased fecal calcium excretion observed in the animals that consumed chia and 2) chia is a food of plant origin, rich in phenolic compounds, tannins, and phytate (Silva et al., 2017) and can chelate calcium, forming insoluble molecules, thus reducing its bioavailability (Amalraj & Pius, 2015). It is worth mentioning that the calcium supplied in both the chia and calcium carbonate-containing diets was 50% of the recommended intake for rats. In the present study, we observed that chia consumption was able to improve the rat's lipid homeostasis. The same was observed in other studies (Fortino et al., 2017; Lucero et al., 2017; Poudyal et al., 2012; Sierra et al., 2015; Silva et al., 2016b; Tenore et al., 2018). This result can be attributed to the higher polyunsaturated fatty acid intake by the rats fed chia as compared to the rats that consumed diets without chia. Chia is an oilseed that is rich in polyunsaturated fatty acids, such as n-3, n-6, and n-9 fatty acids, which exert positive effects on the blood lipid profile. It was shown that the high concentrations of n-3 fatty acids present in chia are associated with an improved lipoprotein profile in Wistar rats during a 48-day feeding period (Lucero et al., 2017). In addition, chia seeds contain high concentrations of dietary fiber, including insoluble fiber (Silva et al., 2017) and phenolic compounds, such as caffeic and rosmarinic acids, myricetin, quercetin, and kaempferol (Oliveira-Alves et al., 2017), which also have positive effects on the lipid profile (Natal et al., 2016; Noratto et al., 2015; Silva et al., 2016b; Yang et al., 2011; Yung et al., 2013). In a recent study, our research group demonstrated that chia consumption can reduce glucose, triacylglyceride, LDL cholesterol, and VLDL cholesterol concentrations in the blood of *Wistar* rats fed chia for 28 days (Silva et al., 2016b). The same result was obtained in a study that evaluated the potential effects of chia seeds on plasma triglyceride levels of healthy subjects with moderate dyslipidemia (Tenore et al., 2018).

Although SOD mRNA expression did not differ among experimental groups, SOD quantification was lower in group that received high fat diet with calcium carbonated suggesting greater generation of free radicals. Chia intake increased the concentration of SOD and catalase, without altering the oxidative stress (MDA and NO) and maintained the total antioxidant activity of plasma and liver, increasing the activity of antioxidants enzymes which have ability to defend the body against the oxidative stress. The same effect was observed by Marineli et al (2015) and Rincón-Cervera et al (2016) that conclude chia intake was able to increase SOD concentrations in *Wistar* rats. Plasma catalase concentration was higher in the animals that consumed chia in our study, owing to a positive effect of the compounds present

in this food on the activity of the antioxidant enzyme capable of decomposing hydrogen peroxide.

In our study, PPAR- α negatively regulates inflammation since its ligands inhibited pro-inflammatory cytokines, interfering with the NF- κ B signaling pathway once NF κ B and TNF- α are major transcription factors involved in inflammatory responses (Fountain et al., 2017). Chia consumption was able to inhibit the activation of NF- κ B and TNF- α , decreasing inflammatory cytokine, as TNF- α , leading to improve in anti-inflammatory body capacity in standard diet and high fat diet that subsequently cause a low-grade inflammatory state (Catrysse & Van Loo, 2017). Thus, based on these results, chia consumption had a beneficial effect on one of the pathways of inflammation in *Wistar* rats, regardless of diet type (standard or high fat). The same effect was observed in other studies that analyzed chia seeds and the omega-3 unsaturated fatty acids resulting from chia consumption in rats feed a high fat diet (Poudyal et al., 2012a; Poudyal et al., 2012b; Poudyal et al., 2013). This phenomenon could be due the fatty acid, phenolic compounds, and other bioactive compounds present in the food, such as vitamins, minerals, and antioxidant substances (Silva et al., 2017) that increased PPAR- α expression and reduced TNF- α expression in our study, which may, in turn, control secreted factors, gene expression, and cell signaling pathways, thereby reducing inflammation (Natal et al., 2016).

IL-10 mRNA expression diminished in the chia groups because the proinflammatory factors (mRNA expression of NF κ B and TNF- α) decreased in these same groups. This finding is only logical because of modulation of the cascade of molecular events leading to a reduction in the production of anti-inflammatory substances, which occurs owing to lower expression of proinflammatory genes in order to maintain a homeostasis in the body (Tunon et al., 2009).

In addition, it is known that inflammation leads to lower bone absorption and enhanced bone resorption because of increased production of proinflammatory cytokines, which downregulates osteoblastogenic pathways while activating osteoclastogenic processes (Wong et al., 2016). In our study, the consumption of the high-fat diet resulted in increased inflammation, reduced the bone resistance, that was not accompanied by a reduction in bone calcium concentration. This finding suggested that long-term inflammation may interfere with calcium bioavailability in animals fed a high-fat diet. Therefore, this food should not be offered as an isolated, bioavailable source of calcium. Finally, the results of the molecular analyses and gene and protein expression assays as well as the evaluated biochemistry

parameters were in accordance with the expected results, namely, low bioavailability of calcium in chia and the beneficial effect of chia consumption on the lipid profile and inflammatory processes in young *Wistar* rats fed standard or high fat diet, receiving 50% of calcium requirement on the diet.

5. Conclusions

Chia as a unique source of calcium, showed low calcium bioavailability regardless of the type of diet consumed. Nonetheless, chia consumption reduced inflammatory processes, improved the lipid profile and had no effect on oxidative stress in young *Wistar* rats. We believe that additional longer experimental studies on adult rats are needed to confirm these results.

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Compliance with Ethical Standards

All the procedures performed in this study involving animals were in accordance with the ethical standards of the Federal University of Viçosa and with the U.K. Animals (Scientific Procedures) Act, 1986.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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PAPER 2: Effects of chia (*Salvia hispanica* L.) on oxidative stress and inflammation in ovariectomized adult female *Wistar* rats

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Effects of chia (*Salvia hispanica* L.) on oxidative stress and inflammation in ovariectomized adult female *Wistar* rats

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The present study investigated the influence of chia consumption on inflammation, oxidative stress, and lipid profiles in adult female ovariectomized rats fed a high-fat diet. Forty ovariectomized and 40 intact (SHAM) rats were allocated into 8 groups ($n = 10$), and each rat received one of the following four diets: standard diet (ST); standard diet + chia (STC); high-fat diet (HF); and high-fat diet + chia (HFC) for 126 days. Biochemical parameters and biomarkers of lipid peroxidation, inflammation, and oxidative stress were evaluated. The mRNA expression levels of PPAR- α , NF- κ B, TNF- α and Zn-SOD1 were analyzed, as well as those of TNF- α and IL-1 β . Chia intake increased HDL cholesterol (HDL-c) and reduced LDL cholesterol (LDL-c) levels. Plasma catalase activity was elevated in the STC group. Concentrations of TBARS were higher in all groups fed HF. PPAR- α mRNA expression was elevated, and levels of NF- κ B mRNA expression were reduced in the STC group. mRNA expression and protein levels of TNF- α were lower in rats fed the standard diet. Protein levels of IL-1 β were reduced in rats fed the standard diet, and the high fat diet with chia. In general, ovariectomy did not influence the inflammatory and oxidative stress parameters. Chia intake improved antioxidant activity by increasing SOD expression, PPAR- α expression, catalase activity, and HDL-c levels. In addition, chia consumption decreased the concentrations of the inflammatory markers IL-1 β and LDL-c.

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Introduction

Obesity is a chronic disease, which can be defined as excessive accumulation of body fat¹. The prevalence of obesity has increased considerably in recent years, reaching 15% of the adult Brazilian population². Consumption of a high fat diet is correlated with inflammation, by promoting resulting in adipocyte hypertrophy and subsequent macrophage infiltration, and culminating in activation of specific signaling pathways³. These pathways lead to production of inflammatory substances, including nuclear factor kappa B (NF- κ B), interleukin 1 beta (IL-1 β), and tumor necrosis factor alpha (TNF- α)⁴. Thus, consumption of a high-fat diet contributes to the activation of inflammatory pathways, deregulation of lipid metabolism, alteration in protein expression, and increased oxidative stress.

Imbalance between the production of reactive oxygen and nitrogen species and their elimination by protective mechanisms leads to oxidative stress⁵. This response occurs in certain pathophysiological conditions, such as inflammation and obesity, and leads to alterations in cellular signaling⁶. In this way, oxidative stress reduces biological activities, and increases inflammation⁷.

Changes in lipid profile, body composition, and inflammatory and oxidative processes can be triggered by hormonal loss⁸. Although associations between inflammatory biomarkers and high-fat intake have been investigated, the association between inflammatory biomarkers and high-fat intake in adult female ovariectomized *Wistar* rats has not been reported yet. In addition, among species of the genus *Salvia*, Chia (*Salvia hispanica* L.) is currently widely used as a dietary source of phenolic compounds, vitamins, minerals (including iron and calcium), dietary fiber, and polyunsaturated lipids such as n-3 and n-6, which confer high nutritional and functional value to these seeds⁹. These compounds have beneficial effects on the human body, including anti-inflammatory and antioxidant effects^{10,11}. The hypothesis of the present study is that chia, as a source of bioactive compounds, can improve lipid homeostasis, and modulate inflammation and oxidative stress in prevention and treatment stages of obesity. The innovation of this study is the elucidation of previously unreported actions of dietary chia on inflammatory markers, oxidative stress, biochemical profiles, and liver histology, and the correlation of these effects with standard and high fat diets, and presence or absence of ovariectomy, using female *Wistar* rats. The objective of this study was to investigate the influence of chia consumption on inflammation, oxidative stress, and blood lipid profiles in ovariectomized and non-ovariectomized adult female *Wistar* rats fed a standard or high-fat diet.

Experimental

Raw materials and preparation of flours

Chia seeds (*Salvia hispanica* L.) grown in the state of Rio Grande do Sul (Brazil) were used for the study. To obtain the flour, the seeds were ground up in three repetitions, using a knife mill (Marconi Equipment, Piracicaba, Brazil) to a particle size of 850 μm . Subsequently, chia flour was packed in polyethylene aluminum bags and stored in a freezer ($-18^{\circ}\text{C} \pm 1^{\circ}\text{C}$) until analysis.

Determination of chemical composition

Analyses of moisture, ash, lipids, carbohydrates, proteins, total dietary fiber, calcium, and total phenolic compounds were performed as previously reported⁹. The results were used to determine the composition of the experimental diets (lipids: 32.2%; carbohydrates: 4.6%; proteins: 18.2%; total dietary fiber: 33.4%; total phenolic: 0.97%), since we supplemented the standard and high-fat diets with the same source of chia. The chemical composition of each diet was determined by the Association of Official Analytical Chemists (AOAC)¹². The total energy value of the chia was estimated using the conversion factors 4 kcal/g⁻¹ for protein or carbohydrate, and 9 kcal/g⁻¹ for lipids.

Animals and diets

Eighty adult female rats (*Rattus norvegicus*, Wistar, albinus variation), 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, and subdivided into 8 groups of 10 animals each, randomized by body weight. The animals were distributed into individual stainless-steel cages in a temperature controlled environment (22°C) with automatically controlled light and dark cycles of 12 hours. The animals received deionized water and their respective experimental diets *ad libitum*. The experimental diets were based either on the standard AIN-93M diet¹³ or on a high-fat diet (Research Diets, New Brunswick, NJ) with modifications. The chemical composition of the diets was determined by the AOAC (2012)¹². The standard diet contained 19% protein, 17% fat, and 64% carbohydrate. The high fat diet contained 51% fat, 15% protein, and 34% carbohydrate^{14,15}. The available calcium in each diet provided 100% of the recommended amount (0.005 kg Ca/kg diet)¹³, with chia contributing with 20% of the total. This established amount was based on human consumption

of 40 g of chia/day. The other ingredients were added in sufficient quantities to provide the planned amounts of lipids, proteins, carbohydrates, fiber, and calories (Table 1).

Table 1. Nutritional composition of experimental diets

<i>Ingredients</i>	Experimental Diets					
	ST	HF	ST	STC	HF	HFC
Calcium carbonate (mg)	6.25	6.25	12.50	10.00	12.50	10.00
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 without calcium (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	78.45	347.50	319.40	151.00	122.90
Cholesterol	-	1.50	-	-	1.50	1.50
<i>Nutritional composition</i>						
Total calories (Kcal)	3778.00 ^b	4971.80 ^a	3759.80 ^b	3647.40 ^b	4728.80 ^a	4616.48 ^a
Caloric density (Kcal/g)	3.78 ^b	4.97 ^a	3.76 ^b	3.65 ^b	4.73 ^a	4.62 ^a

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 the Duncan test.

During the 7 week induction phase, the animals received the standard AIN-93M diet (n=40), or the high fat diet (n=40), according to their group. After that period, 20 rats from each group were subjected to ovariectomy (OVX) and the remaining animals underwent SHAM surgeries (SHAM). Rats were kept on their respective diets for 3 weeks to recover from surgery.

At 10 weeks, the OVX and SHAM animals were redistributed into 8 groups (n=10), and each group received one of the following four diets: standard diet (ST); standard diet + chia (STC); high-fat diet (HF); high-fat diet + chia (HFC). On day 126, after a 12 h fast, the animals were anesthetized with isoflurane (Isoforine, Cristália®, Itapira, Brazil) and then sacrificed. Blood was then collected by cardiac puncture and centrifuged at 4°C for 600 s (Fanem-204, São Paulo, Brazil) and plasma and serum were stored at -80°C. Liver tissue was collected and stored prior to analysis at -80°C.

Body weight gain and food consumption were monitored weekly during the experimental period to determine feed efficiency ratios (FER)¹⁶. The hepatosomatic and cecal indices were calculated by dividing liver or cecum weight by body weight, and multiplying the respective ratios by 100¹⁷. The percentage of adiposity was calculated using the following formula: (visceral + gonadal + retroperitoneal + mesenteric + inguinal adipose tissues) / total body weight x 100¹⁸.

All experimental procedures using 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).

Biochemical analysis

For the determination of biochemical parameters, 0.5 mL of plasma was used. Plasma glucose concentrations, total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), very-low density lipoprotein (VLDL), triacylglycerides (TGL), uric acid, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured by colorimetric methods using commercially available kits (Bioclin®, Belo Horizonte, Brazil). Analyses were conducted on a BS-200 Chemistry Analyzer (Bioclin®, Nanshan, China).

Extraction of mRNA from liver tissue and cDNA synthesis

Liver tissue was macerated in liquid nitrogen under RNase free conditions and the samples were aliquoted for total RNA extraction. Total RNA was extracted with the TRIzol Reagent (Invitrogen, Carlsbad, USA). The extracted mRNA was used to synthesize the cDNA with the M-MLV reverse transcription kit (Invitrogen Corp., Grand Island, NY)¹⁹.

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

mRNA Expression levels of genes in the liver that are involved in inflammation processes was 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, Foster, USA) by means of the measurement system involving SYBR-Green Fluorescence and Primer Express software (Applied Biosystems, Foster, USA). Sense and antisense primer sequences were ordered (Choma Biotechnologies) to amplify PPAR- α (ID: 2404316), NF κ B (ID: 234230511), Zn-SOD1 (ID: 5615763) and TNF- α (ID: 234230513). The relative expression levels of mRNA were normalized to the endogenous control: glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Table 2). All the steps were performed under open conditions with RNase.

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

Genes	Oligonucleotide (5'-3')	
	Forward	Reverse
GAPDH	AGGTTGTCTCCTGTCACTTC	CTGTTGCTGTAGCCATATTC
PPAR- α	CCTGCCTTCCCTGTGAACT	ATCTGCTTCAAGTGGGGAGA
NF κ B	ACCGAAGCAGGAGCTATCAA	GCGTACACATTCTGGGGAGT
Zn-SOD1	GAGCAGAAGGCAAGCGGTGAA	CCACATTGCCCAGGTCTG
TNF- α	ACGGCATGGATCTCAAAGAC	AGATAGCAAATCGGCTGACG

GAPDH: glyceraldehyde 3-phosphate dehydrogenase; PPAR- α : peroxisome proliferator-activated receptor alpha; NF κ B: factor nuclear kappa B; SOD: superoxide dismutase; TNF- α : tumor necrosis factor alpha.

TNF- α and IL-1 β quantification

To determine the concentrations of TNF- α and IL-1 β in the liver, hepatic tissue samples were homogenized using a specify extraction kit reagents (Invitrogen, São Paulo, Brazil). The fractions of TNF- α and IL-1 β were assessed by immunoassay using the Rat TNF- α (Cat#88-7340, Invitrogen) and Rat IL-1 β (Cat#88-7340, Invitrogen), ELISA kits, respectively. The microplates that were provided in the ELISA kits, respectively. The microplates that were provided in the ELISA kits were respectively precoated with anti-TNF-

α and anti- IL-1 β antibodies. The concentrations of TNF- α and IL-1 β were calculated by comparison to the corresponding standard curves.

Lipid peroxidation and oxidative stress levels analysis

Homogenate preparation

To obtain a liver homogenate, 200 mg of liver was mixed with 1000 μ L of phosphate buffer (50 mM) and 1 mM EDTA (pH 7.4). The sample was macerated and centrifuged at 12,000 $\times$ g and 4°C for 10 minutes, and then the supernatant was removed and stored in an ultra-freezer until analysis.

Liver lipid peroxidation (TBARS)

The hepatic lipid peroxidation was quantified by the thiobarbituric acid reactive substances (TBARS) method²⁰. TBARS concentration was calculated via the molar absorptivity coefficient²¹, and the results were expressed in nanomoles of TBARS per milligram of protein (TBARS/PTN). Total protein in the liver homogenate was quantified by the Bradford method²².

Liver superoxide dismutase activity (SOD)

The quantification of SOD was performed in relative units, and one unit was defined as the amount of SOD enzyme that inhibits the pyrogallol oxidation rate by 50%. The analysis was carried out on a spectrophotometer (Multiskan GO, Thermo Scientific, Ratastie, Finland) at 570 nm, and the results were expressed as units of SOD activity per milligram of protein²³.

Liver catalase activity (CAT)

Catalase was analyzed according to the methodology proposed by Aebi²⁴. At 0, 30, and 60 seconds after the reaction was initiated, the absorbance at 240 nm was determined on a spectrophotometer (T70 + UV/VIS Spectrometer, Taylors, USA). Enzymatic activity was recorded in micromoles per milliliter of a sample. Catalase activity was calculated according to Lambert Beer's law.

Liver antioxidant capacity

Aliquots (10 μ L) of the liver homogenate were added to the wells along with 20 μ L of the reagent metmyoglobin and 150 μ L of the ABTS solution (2,2'-azino-bis(3-

ethylbenzothiazoline-6-sulphonic acid). Then, 10 μ L of increasing concentrations of trolox standard were pipetted into the wells, in triplicate, to construct a standard curve. The microtiter plate was incubated at room temperature, and then the absorbance at 405 nm was read by means of a spectrophotometer (Multiskan GO, Thermo Scientific, Ratastie, Finland). The values are expressed in mM Trolox equivalents.

Histological analysis

Semi-serial histological sections of fragments of the liver with 3 μ m thickness were obtained in automatic microtome (Reichert-Jung®, Genossen, Germany) and were stained by hematoxylin and eosin (HE) technique. The slides were examined under an Olympus BX43 light microscope. The samples were semi-quantified in: absence steatosis (0), minimal steatosis (1), mild steatosis (2), moderate steatosis (3) and severe steatosis (4) (Figure 1).

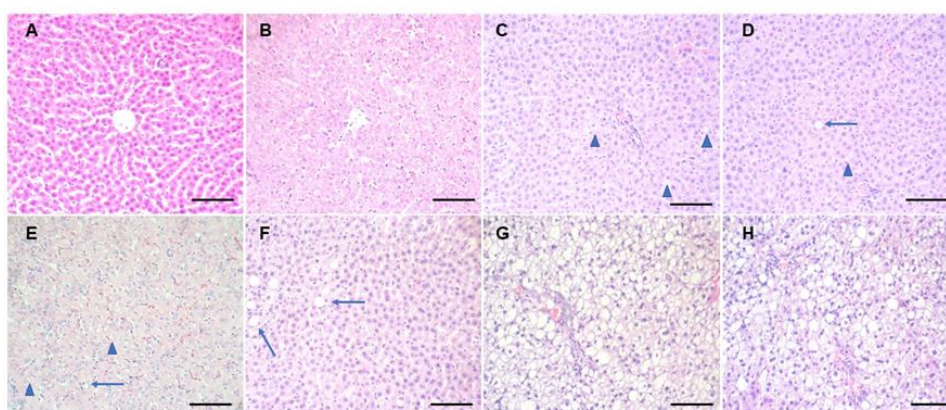


Figure 1. Histological sections used to semi-quantify steatosis lesions: (A) normal hepatic parenchyma, score 0 (group 1, #5); (B) normal hepatic parenchyma, score 0 (group 2, #1); (C) minimal steatosis lesions, microvesicular degeneration (arrowhead), score 1 (group 3, #6); (D) mild steatosis lesions, microvesicular degeneration (arrowhead) and macrovesicular degeneration (arrow), score 2 (group 4, #3); (E) mild steatosis lesions, microvesicular degeneration (arrowhead) and macrovesicular degeneration (arrow), score 2 (group 8, #4); (F) moderate steatosis lesions, macrovesicular degeneration (arrow), score 3 (group 5, #9); (G) severe steatosis lesions, diffuse macrovesicular degeneration, score 4 (group 6, #2); (H) severe steatosis lesions, diffuse macrovesicular degeneration, score 4 (group 7, #8) (bar: 150 μ m); #: animal.

Statistical analyses

Treatments were administered according to a completely randomized design, with ten repetitions. The results were subjected to analysis of variance at 5% probability. To determine "F-value" significance, the post hoc Duncan test was used to compare means among groups with or without ovariectomized rats. T-tests were used to compare groups fed the same diet $\pm$

ovariectomy. Classification results based on histological analyses were assessed by the non-parametric Kruskal-Wallis test and by Fisher's LSD test for post-hoc comparisons between groups. Parametric tests were expressed as means $\pm$ standard deviations, and non-parametric tests were expressed as means. Statistical analyses were performed using SAS software, version 9.0. Data with a p-value ≤ 0.05 were considered statistically significant.

Results

Comparison of the OVX group fed the standard diet (ST) with the OVX group fed the high fat diet (HF) revealed that consumption of the high fat diet increased weight gain, adiposity, FER, consumption of saturated fatty acids, the hepatic index, and the rate of hepatic fat. By contrast, the standard diet (ST) reduced food intake, consumption of polyunsaturated fatty acids, and the cecal index compared to the high fat diet (HF) (Table 3). Animals fed a high fat diet (HF) showed higher glucose, TC, AST, and ALT concentrations, and higher c-LDL levels than those fed the standard diet (ST). Triglycerides, c-VLDL, and urea levels did not differ between groups (Table 4). Concentrations of the inflammatory and oxidative stress markers TBARS, TNF- α , and IL-1 β (Table 5), and expression levels of SOD and TNF- α are higher in animals fed the high fat diet (HF) compared to animals fed the standard diet (ST) (Figure 2A, 2D). The content of LAC, catalase activity, SOD levels (Table 5), NF κ B mRNA expression (Figure 2C) did not differ between ST and HF groups. PPAR- α mRNA expression levels were lower in the HF group compared to the ST group.

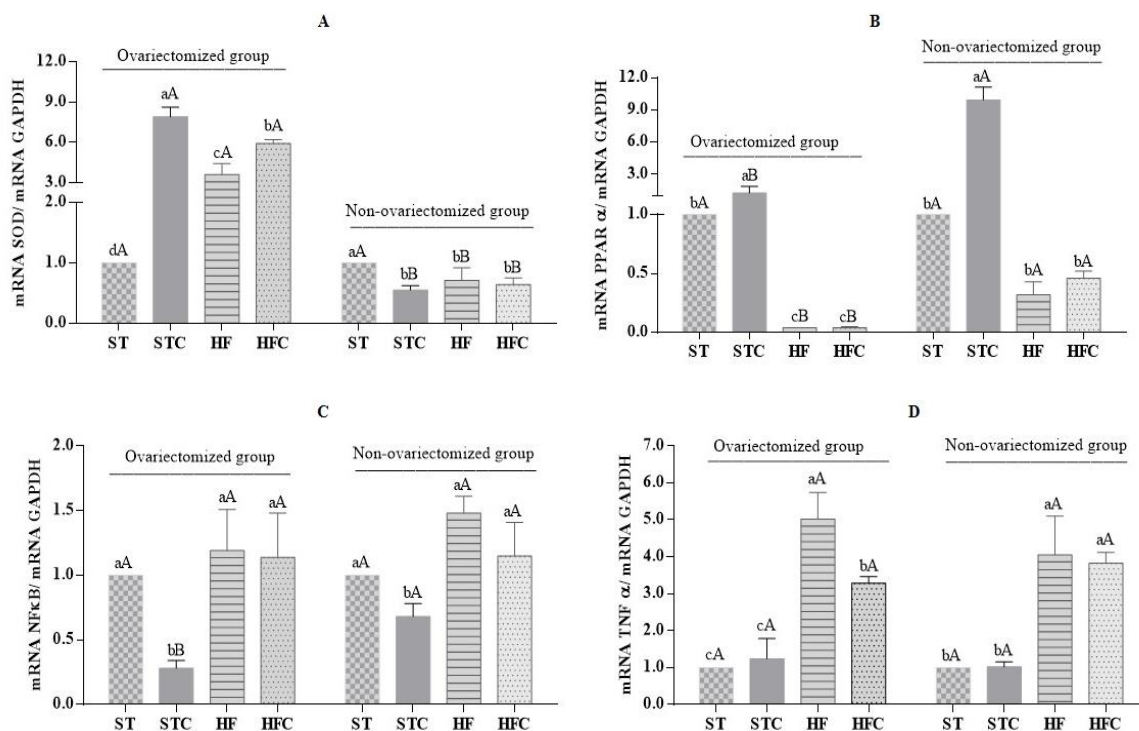


Figure 2. Effect of chia consumption (standard diet and high fat diet) on the gene expression of proteins in liver. RT-qPCR analysis. (A) SOD expression, (B) PPAR- α expression, (C) NF κ B p65 expression, (D) TNF- α expression. ST: standard diet; STC: standard diet + chia; HF: high fat diet; HFC: high fat diet + chia. GAPDH: glyceraldehyde 3-phosphate dehydrogenase; SOD: superoxide dismutase; PPAR- α : peroxisome proliferator-activated receptor alpha; NF κ B: factor nuclear kappa B; TNF- α : tumor necrosis factor alpha. Means followed by the same lowercase letter did not differ significantly according to the Duncan test at the 5% of probability, for each group. Means followed by the same capital letter do not differ from each other, by the T test at 5% probability, comparing the groups that received the same diet, but with or without ovariectomy.

Comparison of the OVX group fed the high fat diet with chia (HFC) and the OVX group fed with high fat diet (HF) revealed that animals fed the high fat diet (HF) consumed higher levels of saturated fatty acids, and had a higher hepatic index and lower cecal index relative to those fed HFC. Weight gain, adiposity, food intake, FER, and liver fat did not differ between these two experimental groups (HF and HFC) (Table 3).

Table 3. Weight gain, food intake, food efficiency ratio (FER), consumption of phenolics, folic acid content and fatty acids intake, indexes and adiposity by the animals (n=10/group) for 126 days

Groups	Ovariectomized				Non-ovariectomized (SHAM)			
	ST	STC	HF	HFC	ST	STC	HF	HFC
Weight gain (g)	191.37±18.74 ^{bA}	197.78±16.85 ^{bA}	261.98±23.61 ^{aA}	262.60±22.10 ^{aA}	175.60±19.92 ^{bA}	178.45±23.49 ^{bA}	202.60±25.61 ^{aB}	206.37±29.51 ^{aB}
Adiposity (%)	6.40±1.16 ^{bA}	7.25±1.60 ^{bA}	14.53±2.70 ^{aA}	12.74±1.95 ^{aA}	6.16±1.56 ^{bA}	7.11±2.20 ^{bA}	10.38±1.76 ^{aB}	10.87±1.85 ^{aB}
Food intake (g/week)	104.29±10.13 ^{aA}	106.21±13.47 ^{aA}	95.63±9.18 ^{bA}	88.54±5.31 ^{bA}	104.17±9.87 ^{aA}	112.82±12.51 ^{aA}	89.21±9.17 ^{bA}	90.50±9.47 ^{bA}
FER	0.30±0.04 ^{bA}	0.29±0.04 ^{bA}	0.36±0.05 ^{aA}	0.37±0.04 ^{aA}	0.26±0.03 ^{bA}	0.24±0.02 ^{bB}	0.31±0.03 ^{aA}	0.31±0.04 ^{aA}
Totals phenolic (mgGAE/kg/day)	-	1.03±0.13 ^{aA}	-	0.86±0.05 ^{bA}	-	1.09±0.12 ^{aA}	-	0.88±0.09 ^{bA}
Phytic acid (g/kg/day)	-	1.01±0.12 ^{aA}	-	0.84±0.05 ^{bA}	-	1.05±0.11 ^{aA}	-	0.85±0.06 ^{bA}
Fatty acids (g/kg/day)								
Saturated	26.91±1.48 ^{cA}	18.01±0.98 ^{dA}	190.15±17.15 ^{aB}	171.72±7.83 ^{bB}	29.56±1.61 ^{cA}	20.82±1.71 ^{dA}	212.50±12.41 ^{aA}	203.80±10.87 ^{bA}
Polyunsaturated	108.20±5.61 ^{bB}	184.47±10.07 ^{aB}	141.86±9.06 ^{bB}	191.21±5.60 ^{aB}	121.55±10.15 ^{bA}	206.32±10.51 ^{aA}	162.24±9.48 ^{bA}	229.74±12.25 ^{aA}
Hepatic index	2.53±0.17 ^{cB}	2.98±0.25 ^{bB}	3.46±0.34 ^{aA}	3.00±0.12 ^{bB}	2.95±0.29 ^{bA}	2.67±0.26 ^{bA}	3.22±0.24 ^{aA}	3.62±0.27 ^{aA}
Hepatic fat*	0.89 ^b	0.89 ^b	2.78 ^a	2.30 ^a	1.56 ^b	1.67 ^b	3.60 ^a	3.00 ^a
Cecum index	1.34±0.16 ^{bA}	1.58±0.25 ^{aA}	1.06±0.22 ^{bA}	1.30±0.35 ^{aA}	1.21±0.25 ^{bA}	1.87±0.39 ^{aA}	1.20±0.28 ^{bA}	1.48±0.32 ^{aA}

ST: standard diet; STC: standard diet + chia; HF: high fat diet; HFC: high fat diet + chia. FER: weight gain/food intake x 100. Indexes: cecum and liver weight/ body weight x 100. Means followed by the same lowercase letter in the row did not differ significantly according to the Duncan test at the 5% threshold of probability, for each group. Means followed by the same capital letter do not differ from each other, by the T test at 5% probability, comparing the groups that received the same diet, but with or without ovariectomy.* The hepatic fat were assessed by Kruskal-Wallis test and Fisher's LSD test for post-hoc comparisons between groups.

Concentrations of biochemical variables glucose, TC, ALT, and c-LDL were lower in the group fed the high fat diet with chia (HFC) relative to the group fed the high fat diet (HF). Concentrations of triglycerides, c-HDL, AST, c-VLDL, and urea did not differ between these groups (Table 4). Concentrations of inflammatory and oxidative stress biomarkers TBARS, LAC, and TNF- α (Table 5), and expression levels of PPAR- α and NF κ B (Figure 2B, 2C) did not differ between these groups. However, chia consumption (HFC) did promote a higher concentration in SOD levels (Table 5) and SOD mRNA expression (Figure 2A) and increased catalase activity (Table 5). Chia intake reduced IL-1 β concentration (Table 5) and TNF- α expression (Figure 2D).

Table 4. Effects of chia consumption for 126 days on the biochemical variables in *Wistar* rats (n=10/group) for 126 days

Groups	Ovariectomized				Non-Ovariectomized (SHAN)			
	ST	STC	HF	HFC	ST	STC	HF	HFC
Glucose (mg·dL ⁻¹)	87.12±5.49 ^{bA}	77.86±4.85 ^{bA}	97.13±4.22 ^{aA}	85.50±7.11 ^{bA}	88.00±7.48 ^{aA}	84.85±6.94 ^{abA}	87.71±5.19 ^{aB}	80.00±3.95 ^{bA}
HDL (mg·dL ⁻¹)	20.63±2.77 ^{cA}	26.75±4.32 ^{aA}	20.86±1.61 ^{bcA}	24.29±1.63 ^{abA}	18.00±5.40 ^{cA}	25.86±3.00 ^{aA}	16.00±2.75 ^{cB}	22.14±2.04 ^{bA}
TC (mg·dL ⁻¹)	65.14±11.82 ^{bA}	51.75±6.50 ^{cA}	86.75±9.93 ^{aA}	66.88±11.38 ^{bA}	53.11±6.80 ^{cB}	56.00±9.53 ^{bcA}	65.80±5.33 ^{aB}	62.44±9.11 ^{abA}
TGL (mg·dL ⁻¹)	110.50±8.81 ^{aA}	120.10±19.00 ^{aA}	106.10±8.06 ^{aB}	113.33±8.14 ^{aA}	120.75±16.79 ^{aA}	116.22±18.19 ^{aA}	117.7±7.93 ^{aA}	122.00±15.10 ^{aA}
AST (U·L ⁻¹)	149.33±18.46 ^{bA}	135.20±25.37 ^{bA}	262.83±20.88 ^{aA}	241.83±31.72 ^{aA}	146.00±24.92 ^{bA}	131.83±20.92 ^{bA}	251.00±16.28 ^{aA}	242.57±27.42 ^{aA}
ALT (U·L ⁻¹)	30.66±5.05 ^{cA}	43.63±11.08 ^{cA}	78.33±13.12 ^{aA}	60.33±18.59 ^{bA}	38.88±9.30 ^{bA}	54.57±10.56 ^{aA}	55.86±9.30 ^{aB}	48.29±11.88 ^{abA}
VLDL (mg·dL ⁻¹)	22.10±1.76 ^{aB}	24.02±3.80 ^{aA}	21.22±1.61 ^{aB}	22.27±1.63 ^{aA}	26.15±3.24 ^{aA}	23.24±3.24 ^{bA}	24.40±1.59 ^{abA}	23.54±3.05 ^{bA}
LDL (mg·dL ⁻¹)	22.83±4.80 ^{bA}	6.70±2.01 ^{cA}	37.66±2.70 ^{aA}	27.53±2.20 ^{bA}	21.53±3.69 ^{bA}	7.17±2.79 ^{cA}	28.65±3.42 ^{aB}	18.90±3.61 ^{bB}
Urea (mg·dL ⁻¹)	0.70±0.12 ^{Aa}	0.73±0.19 ^{Aa}	0.68±0.19 ^{aA}	0.67±0.12 ^{aA}	0.73±0.14 ^{aA}	0.70±0.16 ^{aA}	0.68±0.18 ^{aA}	0.74±0.09 ^{aA}

ST: standard diet; STC: standard diet + chia; HF: high fat diet; HFC: high fat diet + chia; HDL: high-density lipoprotein; TC: total cholesterol; TGL: triacylglyceride; AST: alanine aminotransferase; ALT: aspartate aminotransferase; VLDL: very-low density lipoprotein; LDL: low-density lipoprotein. Means followed by the same lowercase letter in the row did not differ significantly according to the Duncan test at the 5% threshold of probability, for each group. Means followed by the same capital letter do not differ from each other, by the T test at 5% probability, comparing the groups that received the same diet, but with or without ovariectomy.

Table 5. Effects of chia consumption for 126 days on the inflammation and oxidative stress in *Wistar* rats (n = 10) for 126 days.

Groups	Ovariectomized				Non-Ovariectomized (SHAN)			
	ST	STC	HF	HFC	ST	STC	HF	HFC
LAC (mM trolox)	1.09±0.17 ^{aA}	1.02±0.13 ^{aA}	1.13±0.14 ^{aA}	1.02±0.12 ^{aA}	1.13±0.20 ^{aA}	0.99±0.09 ^{aA}	1.13±0.17 ^{aA}	1.08±0.28 ^{aA}
CAT (μmol/min/ mL)	0.52±0.08 ^{bcA}	0.74±0.14 ^{aA}	0.50±0.15 ^{cA}	0.65±0.10 ^{abA}	0.53±0.08 ^{bA}	0.63±0.11 ^{aA}	0.39±0.08 ^{cA}	0.52±0.06 ^{bA}
SOD (U SOD/ mg protein)	2.74±0.69 ^{bB}	4.89±1.10 ^{aA}	3.09±1.14 ^{bA}	5.85±1.37 ^{aA}	4.46±0.27 ^{aA}	2.87±0.81 ^{bA}	2.57±0.82 ^{bB}	2.24±0.78 ^{bB}
TBARS (nmol/mg protein)	0.86±0.10 ^{bA}	0.91±0.07 ^{bA}	1.44±0.20 ^{aA}	1.41±0.17 ^{aA}	0.85±0.06 ^{bA}	0.77±0.10 ^{bA}	1.20±0.13 ^{aB}	1.15±0.12 ^{aB}
TNF-α (pg/mL)	271.61±38.57 ^{bA}	257.44±71.74 ^{bA}	395.78±60.48 ^{aA}	382.07±56.91 ^{aA}	277.44±16.67 ^{bA}	278.00±54.30 ^{bA}	379.67±34.17 ^{aA}	424.22±46.68 ^{aA}
IL-1β (pg/mL)	1233.89 ± 170.98 ^{bA}	1258.33 ± 132.00 ^{bA}	1802.78 ± 107.44 ^{aA}	1388.33 ± 118.02 ^{bA}	1302.78 ± 99.30 ^{bA}	1339.44 ± 104.35 ^{bA}	1557.22 ± 161.66 ^{aA}	1332.78 ± 157.67 ^{bA}

ST: standard diet; STC: standard diet + chia; HF: high fat diet; HFC: high fat diet + chia; TBARS: thiobarbituric acid reactive substances. SOD: superoxide dismutase; LAC: liver antioxidant capacity; CAT: catalase; TNF-α: tumor necrosis factor alpha; IL-1β: interleukin 1 beta. All analyses were performed on liver sample. Means followed by the same lowercase letter in the row did not differ significantly according to the Duncan test at the 5% threshold of probability, for each group. Means followed by the same capital letter do not differ from each other, by the T test at 5% probability, comparing the groups that received the same diet, but with or without ovariectomy.

Comparing OVX animals fed the standard diet (ST) with those fed the standard diet with chia (STC) revealed that chia consumption did not alter the animals' weight gain, adiposity, food intake, FER, or liver fat. However, the STC diet was able to reduce intake of polyunsaturated fatty acids while increasing the cecal and hepatic indices (Table 3). Chia had a reducing effect on TC and c-LDL, and increased c-HDL. Concentrations of glucose, triglycerides, AST, ALT, c-VLDL, and urea did not differ between the ST and STC groups (Table 4). Moreover, chia consumption increased catalase activity, SOD mRNA (Table 5), and SOD activity (Figure 2A) and PPAR- α expression (Figure 2B). In addition, NF κ B expression was reduced in animals fed the STC diet compared to animals fed the ST diet (Figure 2C). The concentration of LAC, TBARS, TNF- α , and IL-1 β (Table 5) as well as TNF- α expression (Figure 2D) did not differ between the two experimental groups.

SHAM rats fed the HF diet had increased consumption, weight gain, adiposity, FER, saturated fatty acids, hepatic index, and liver fat relative to those fed the ST diet. The cecal index did not differ between the two groups (Table 3). Concentrations of the biochemical variables glucose, c-HDL, triglycerides, c-VLDL, and urea did not differ between groups. However, high fat consumption did increase TC, AST, ALT, and c-LDL levels (Table 4). Considering the inflammatory parameters, the consumption of the HF diet also reduced catalase activity, and SOD expression and quantification (Table 5, Figure 2A).

Consumption of the HF diet increased protein concentrations of TBARS, TNF- α , and IL-1 β (Table 5) and increased expression of TNF- α (Figure 2D). However, the mRNA expression levels of PPAR- α and NF κ B did not differ between groups (Figure 2B, 2C). Weight gain, adiposity, food intake, FER, hepatic index, and liver fat did not differ between HF and HFC groups. In addition, consumption of chia in association with the high fat diet reduced the cecal index and the saturated fatty acid content (Table 3). Chia also reduced concentrations of glucose and c-LDL, while increasing c-HDL content. TC, triglycerides, AST, ALT, VLDL, and urea did not differ between groups (Table 4). These groups showed no difference in LAC, SOD, TNF- α , PPAR- α expression, NF κ B expression, or TBARS concentration. However, consumption of chia did increase catalase activity and reduced the content of IL-1 β (Table 5; Figure 2).

The same trends in weight gain, adiposity, food intake, FER, hepatic index, cecum index, and liver fat were found in the HFC group and the STC group (Table 5). Compared with the ST group, mice in the STC group had increased c-HDL and ALT levels, and reduced concentrations of c-VLDL and c-LDL. An increase in catalase activity and PPAR- α

expression was observed in animals fed a standard diet associated with chia (STC). However both SOD concentration and expression were lower in the STC group compared to the ST group. No difference was observed for LAC, TBARS, IL-1 β , TNF- α , or NF κ B. In general, ovariectomy did not influence inflammatory and oxidative stress parameters.

Discussion

Chia is a good source of bioactive compounds, including vitamins, phenolic compounds, and fatty acids. However, its anti-inflammatory activity in adult female *Wistar* rats $\pm$ ovariectomy has not been previously reported. It is important to highlight that the changes in lipid profile, body composition, and inflammatory and oxidative processes happen due to hormonal loss⁸. Therefore, we chose an animal model (ovariectomized rats) that could imitate this condition associated with consumption of a high fat diet. Adult rats were chosen to mimic this model of postmenopause. The present study showed that chia intake spanning 126 days increased catalase activity, cecal index, and HDL-c levels, and reduced c-LDL levels in adult female rats $\pm$ ovariectomy, fed a standard or high-fat diet. In addition, standard diet consumption, with or without chia, decreased hepatic fat, liver index, saturated fatty acid consumption, AST concentration, TBARS levels, TNF- α concentration, and TNF- α mRNA expression in animals $\pm$ ovariectomy. In the OVX group, chia intake also promoted an increase in SOD concentration and mRNA expression. Additionally, in the present study, ovariectomy did not influence parameters related to inflammation and oxidative stress

TNF- α mRNA expression levels and quantification, and IL-1 β quantification levels were higher in the high fat diet group. Thus, we proved that a high fat diet increases inflammation in the animals that consume it. The increases in both TNF- α mRNA and protein levels, in addition to the increase in IL-1 β indicate NF κ B activation. Consumption of chia associated with HF diet had a protective effect, decreasing IL-1 β levels, but did not change the level of TNF- α despite a decrease in its mRNA in the OVX group. Although chia did not reduce NF κ B expression, we suggest that, even without altering all inflammatory markers, chia may reduce inflammation resulting from an HF diet. This benefit is consistent with antioxidants found in chia seeds, including phenolic compounds, vitamins, minerals, dietary fiber, and polyunsaturated lipids⁹. The increased expression and quantification of these inflammatory cytokines may occur due to hypertrophy and hyperplasia of adipose tissue, culminating in activation of pro-inflammatory pathways through TLR-4²⁵. This receptor stimulates NF κ B expression resulting in translocation of the p65 subunit to the nucleus,

culminating in production of inflammatory cytokines that induce transcription of target genes³. Results similar to our study were observed by Tapia et al.²⁶, who noticed that HF-fed mice increased NFκB expression relative to animals fed a standard diet.

Ovariectomized animals fed diets with chia had higher mRNA and protein levels of SOD, showing the benefits of the compounds present in the seed to increase levels of this antioxidant enzyme. The same result was observed by Marineli et al.²⁷ and Rincón-Cervera et al.²⁸ in *Wistar* rats fed chia. However, the SHAM animals fed chia had lower SOD mRNA and protein levels compared with the ST group. Thus, chia intake did not influence this variable. The groups fed with high fat diet, whether ovariectomized or not, had lower PPAR-α expression. In addition, addition of chia to standard diet increased PPAR-α expression in both OVX and SHAM groups.

PPAR-α is a transcription factor that regulates lipid metabolism in the liver, and negatively regulates inflammation²⁹. Moreover, PPAR-α ligands inhibit the action of pro-inflammatory cytokines by interfering with the NFκB signaling pathway. In addition to inflammation, PPAR-α plays a role in oxidative stress²⁹. Results similar to our study were observed by Tapia et al.²⁶, who noticed that HF-fed mice had reduced PPAR-α expression relative to animals fed the standard diet.

Chia intake increased catalase activity in the standard diet probably due to compounds present in the seeds including phenolic compounds and antioxidants. The main phenolic compounds found in chia are rosmarinic acid, quercetin, myricetin, kaempferol, caffeic acid, and galic acid^{29,30,31,32}. These compounds provide benefits to the human body due to the presence of hydroxyl groups that are readily oxidized to produce the corresponding O-quinones, which are effective scavengers of reactive oxygen species^{33,34}. Phenolic compounds can also alter recruitment of inflammatory cells, decreasing production of pro-inflammatory mediators. Although it contains high concentrations of bioactive compounds, chia intake is not able to increase the hepatic antioxidant profile, as can be observed in a study that added a fruit rich in antioxidant compounds to the diet of rabbits fed a high-cholesterol diet³⁵. Expectedly, high fat diet consumption increased malondialdehyde levels, increasing oxidative stress and free radical accumulation, resulting in lipid decomposition³⁶, corroborating the results of our biochemical studies conducted on the liver. In addition, the accumulation of lipids in hepatic tissue, as observed in our histological results, can induce this higher inflammatory state, increasing production of substances correlated with the inflammatory process.

The lower food intake observed in animals fed the high-fat diet can be attributed to higher satiety during the experimental period, as found in other studies^{16,37,38}. However, despite the lower dietary intake, higher weight gain and higher percent adiposity were observed in the high fat diet group, due to increased concentration and energy density of lipids. Chia reduced intake of saturated fatty acids and increased intake of polyunsaturated fatty acids, owing to the fatty acid composition of chia seeds, e.g., n-3 and n-6 fatty acids⁹. The increase in liver weight and hepatosomatic index in animals fed the HF diet can be explained by accumulation of lipids in the liver, corroborating our histological results. It is important to note that chia consumption in both diets reduced fat accumulation in the animals' liver, demonstrating a hepatoprotective action. This result can be attributed to the presence of polyunsaturated fatty acids in chia, which underlie the reduction of hepatic steatosis and inflammation, and the maintenance of hepatic tissue structure²⁶. Furthermore, the chia mediated increase in cecal index may be due the composition of dietary fiber present in the matrix, especially the insoluble types¹⁰, which are responsible for increasing fecal volume and cecum weight³⁹.

The consumption of chia reduced LDL cholesterol and total cholesterol, and increased HDL cholesterol levels. In addition, chia consumption did not influence the concentration of triglycerides. Chia consumption reduced glucose levels in OVX and SHAM animals fed the high-fat diet. This improvement in lipid profile has been demonstrated in previous animals studies^{16,33,40,41,42} and in a human study⁴³. However, the effects of chia consumption on the lipid profile of adult female rats was not known. A possible mechanism for the observed improvement in lipid profile is that chia is rich in polyunsaturated fatty acids, such as n-3 and n-6, dietary fiber, including insoluble fiber, and phenolic compounds⁹, which can act to reduce levels of LDL, VLDL, and total cholesterol, and increase HDL cholesterol levels^{16,38,41,42,43,44}. The differences observed in the group fed chia can be explained by the proportion of unsaturated dietary fatty acids present in the food that can affect lipoprotein composition and expression of other pro-inflammatory substances. Moreover, the fatty acid composition can affect oxidative stress, and these events could be involved in endothelial dysfunction^{45,46}.

Studies have shown that osteoporosis and high fat diet consumption may promote chronic inflammation^{15,38,47}. The present study aimed to initiate osteoporosis via ovariectomy, to verify if chia consumption would influence inflammation and oxidative status under these conditions. Chia consumption lead to systemically improved anti-inflammatory capacity in rats fed the standard diet, which typically that subsequently causes low-grade inflammation.

Further studies investigating the influence of calcium intake on OVX and SHAM rats should be performed to verify the action of this mineral in the development of osteoporosis in this experimental model.

Conclusions

Chia intake improved lipid profile and increased liver and cecum indices. Chia consumption associated with the standard diet provided to OVX or SHAM rats for 126 days improved antioxidant activity, increased SOD and PPAR- α expression and catalase activity, while at the same time it reduced NF κ B expression. However, chia intake associated with the high fat diet in OVX rats reduced IL-1 β levels and TNF- α expression, increased SOD mRNA and protein levels, and increased catalase activity. In general, ovariectomy did not influence inflammatory and oxidative stress parameters.

Conflicts of interest

There are no conflicts to declare.

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PAPER 3: Soluble Extracts from Chia Seed (*Salvia hispanica* L.) Affect Brush Border Membrane Functionality, Morphology and Intestinal Bacterial Populations *in Vivo* (*Gallus gallus*)

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Soluble Extracts from Chia Seed (*Salvia hispanica* L.) Affect Brush Border Membrane Functionality, Morphology and Intestinal Bacterial Populations *In Vivo* (*Gallus gallus*)

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Abstract: This study assessed and compared the effects of the intra-amniotic administration of various concentrations of soluble extracts from chia seed (*Salvia hispanica* L.) on the Fe and Zn status, brush border membrane functionality, intestinal morphology, and intestinal bacterial populations, *in vivo*. The hypothesis was that chia seed soluble extracts will affect the intestinal morphology, functionality and intestinal bacterial populations. By using the *Gallus gallus* model and the intra-amniotic administration approach, seven treatment groups (non-injected, 18 Ω H₂O, 40 mg/mL inulin, non-injected, 5 mg/mL, 10 mg/mL, 25 mg/mL and 50 mg/mL of chia seed soluble extracts) were utilized. At hatch, the cecum, duodenum, liver, pectoral muscle and blood samples were collected for assessment of the relative abundance of the gut microflora, relative expression of Fe- and Zn-related genes and brush border membrane functionality and morphology, relative expression of lipids-related genes, glycogen, and hemoglobin levels, respectively. This study demonstrated that the intra-amniotic administration of chia seed soluble extracts increased ($p < 0.05$) the villus surface area, villus length, villus width and the number of goblet cells. Further, we observed an increase ($p < 0.05$) in zinc transporter 1 (ZnT1) and duodenal cytochrome b (Dcytb) proteins gene expression. Our results suggest that the dietary consumption of chia seeds may improve intestinal health and functionality and may indirectly improve iron and zinc intestinal absorption.

1. Introduction

Micronutrient deficiency affects approximately two billion people worldwide. Iron (Fe) and zinc (Zn) deficiencies are the most prevalent, affecting approximately 45% and 17%, respectively, of the world population [1–3]. Both mineral deficiencies are more prevalent in Africa, South East Asia and Latin America [4,5]. Among the dietary factors that contribute to Fe and Zn deficiencies is their low bioavailability due to dietary potential inhibitors, such as phytic acid and phenolic compounds [2,6,7]. Dietary Fe and Zn deficiencies affect normal cell division and differentiation, as well as growth and development, impair physical and cognitive development, and increase the risk of infection [4,7,8].

We have previously established the *Gallus gallus* as a model to assess dietary Fe and Zn bioavailability [9–15]. In addition, this experimental model presents a complex gut microbiota [16], as the phylum level was shown to be similar to humans [17,18]. Further, the intra amniotic administration method has been widely used and demonstrates the potential prebiotic effects of soluble fibers from beans, chickpeas, lentil and wheat, with demonstrated effects on the intestinal functionality, morphology, and microbial populations [10,13,15].

Prebiotics are dietary substrates that selectively promote the proliferation and/or activity of health-promoting bacterial populations in the colon [19,20]. The soluble extracts are obtained by the isolation process of the prebiotics of the food matrix and are composed for the most part of soluble fiber. The most commonly used prebiotics, as inulin, raffinose and stachyose, are dietary fibers with a well-investigated and proven ability to promote the abundance of intestinal bacterial populations, which may provide additional health benefit to the host [21]. It is known that soluble extracts are responsible for improving gastrointestinal motility [22,23], intestinal functionality and intestinal morphology [10,13,24,25], and improving mineral absorption [10,26]. Recent Studies have shown that the consumption of plant seed origin soluble extracts can up regulate the gene expression of brush border membrane (BBM) proteins that contribute to the digestion and absorption of nutrients, such as sucrase-isomaltase, aminopeptidase and sodium glucose cotransporter-1 [10,11,13]. Further, soluble extracts can positively affect intestinal health by increasing mucus production, goblet cell number, goblet cell diameter, villus surface area, villus height, villus width, and crypt depth [10,13,15,27,28]. These functional and morphological effects appears to occur due to the increased motility of the digestive tract by the soluble extracts, leading to hyperplasia and/or hypertrophy of muscle cells [29]. In addition, plant origin soluble extract (with high fiber content and, therefore, potential prebiotic properties) administration may act, directly or

indirectly, as a factor that increases iron and zinc bioavailability [30–32]. This event occurs due the lower intestine (colon) fiber fermentation process and the bacterial production of short-chain fatty acids (SCFAs) that reduce the intestinal pH, inhibiting the growth of potentially pathogenic bacterial populations and increasing the solubility and, therefore, the absorption of minerals [10,26]. The SCFAs can increase the proliferation of epithelial cells, which, in return, increases the absorptive surface area, which contributes to the absorption of dietary minerals [33]. Also, it was previously shown that the consumption of soluble extracts has a synergistic effect, as it promotes the metabolic interactions within the gastrointestinal microbial community via the production of organic acids, which provide an acidic environment in the colon, indirectly suppressing the growth of pathogens [34].

The use of iron- and zinc-rich foods may be a good strategy aimed to reduce the prevalence of iron and zinc deficiencies, respectively. Chia (*Salvia hispanica* L.) is an herbaceous plant with good nutritional and functional value with high concentrations of bioactive compounds such as dietary fiber and minerals, including iron and zinc [35]. Although iron and zinc are present in high concentrations, it is important to take into account the bioavailability of these minerals [36]. In the present study, chia was chosen as the soluble extract source, since the consumption of chia became extensively common worldwide, and specifically consumed with increasing amounts in Mexico, Argentina, Chile, New Zealand, Japan, USA, Canada and Australia [37], as in some of these geographical regions (e.g., South America), dietary Fe and Zn deficiencies are a major health concern [4,5]. Thus, the primary objective of this study was to assess the effects of the intra-amniotic administration of chia soluble extracts with a putative prebiotic effect on Fe and Zn status and brush border membrane functionality, in vivo. A secondary objective was to evaluate the effects of the tested extracts on intestinal bacterial populations. The third objective was to evaluate the effects of the chia soluble extracts on intestinal morphology. We hypothesized that the chia soluble extracts will affect the intestinal morphology, functionality and bacterial populations.

2. Material and Methods

2.1. Sample Preparation

Chia seeds (*Salvia hispanica* L.) grown in the state of Mato Grosso (Brazil) were used for this study. To obtain the flour, the seeds were ground up in three replicates, using a knife mill (Marconi Equipment, Algodual, Brazil), to a particle size of 850 μm . Subsequently, chia

flour was packed in polyethylene aluminum bags and stored in a freezer (-20°C) until analysis.

2.2. Polyphenols Analysis

2.2.1. Chia Sample Preparation

A volume of 5 mL of methanol:water (50:50 v/v) was added to 0.5 g of chia flour. The resulting slurry was vortexed for 1 min before incubation in a 24°C sonication water bath for 20 min at room temperature. Samples were again vortexed and placed on a rocker at room temperature for 60 min before centrifuging at $4000\times g$ for 15 min. Supernatants were filtered with a $0.2\text{ }\mu\text{m}$ PTFE syringe filter and stored at -20°C for later use.

2.2.2. Liquid chromatography–mass spectrometry (LC-MS) Analysis

Extracts and standards were analyzed by an Agilent 1220 Infinity Liquid Chromatograph (LC; Agilent Technologies, Inc., Santa Clara, CA, USA) coupled to an Advion expressionL® compact mass spectrometer (CMS; Advion Inc., Ithaca, NY, USA). Ten-microliter samples were injected and passed through an XBridge Shield RP18 $3.5\text{ }\mu\text{m}$ $2.1\times 100\text{ mm}$ column (Waters, Milford, MA, USA) at 0.6 mL/min . The column was temperature-controlled at 40°C . The mobile phase consisted of ultra-pure water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). Polyphenols were eluted using linear gradients of 94.0 to 84.4% A in 1.50 min, 84.4 to 81.5% A in 2.25 min, 81.5 to 77.0% A in 6.25 min, 77.0 to 55.0% in 1.25 min, 55.0 to 46.0% in 2.25 min, 46.0 to 94.0% in 2.25 min and hold at 94.0% A for 2.25 min for a total run time of 18 min. From the column, the flow was directed into a variable wavelength Ultraviolet (UV) detector set at 280 nm. The flow was then directed into the source of an Advion expressionL® CMS, and Electrospray ionization (ESI) mass spectrometry was performed in the negative ionization mode using selected ion monitoring with a scan time of 200 ms. The capillary temperature and voltages were 250°C and 180 volts, respectively. The ESI source voltage and gas temperature were 2.5 kilovolts and 250°C , respectively. The desolvation gas flow was 240 L/h. Advion Mass Express™ software (Advion, Ithaca, USA) was used to control the LC and compact mass spectrometers (CMS) instrumentation and data acquisition. Individual polyphenols were identified and confirmed by comparison of m/z and LC retention times with authentic standards. The analysis of MS and UV data was performed using Advion Data Express™ software (Advion, Ithaca, USA).

2.3. Extraction of Soluble Extracts from Chia

The extraction of prebiotics was performed according to Tako et al. [14], Hou et al. [13] and Pacific et al. [10]. Chia flour samples were dissolved in distilled water (50 g/L) (60 °C, 60 min) and centrifuged at 3000 rpm (4°C) for 25 min, and then the supernatant was collected. The supernatant was then dialyzed (MWCO 12–14 kDa) (48 h) against distilled water. The dialysate was collected and lyophilized to yield a fine off-white powder [12].

2.4. Phytate, Dietary Fiber, Iron and Zinc Analysis in Chia Seeds and Chia Extract

Dietary phytic acid (phytate)/total phosphorous was measured as phosphorus released by phytase and alkaline phosphatase, according to manufacturer's instructions (n = 5) (K-PHYT 12/12. Megazyme International, Bray, Ireland). The determination of total fiber and soluble and insoluble fractions was performed by the enzymatic-gravimetric method according to AOAC [38], using enzymatic hydrolysis for a heat-resistant amylase, protease and amyloglucosidase (Total dietary fiber assay Kiyonaga, Sigma®, Kawasaki, Japan). For the determination of iron and zinc, chia seed and chia extract (0.5 g) were treated with 3.0 mL of a 60:40 HNO₃ and HClO₄ mixture in a Pyrex glass tube and left overnight to destroy organic matter. The analyses were performed using an inductively coupled plasma atomic emission spectrometer (ICP-AES) (Thermo iCAP 6500 series, Thermo Jarrell Ash Corp., Franklin, MA, USA) [12,28].

2.5. Animals and Design

Cornish-cross fertile broiler eggs (n = 105) were obtained from a commercial hatchery (Moyer's chicks, Quakertown, PA, USA). The eggs were incubated under optimal conditions at the Cornell University Animal Science poultry farm incubator. All animal protocols were approved by the Cornell University Institutional Animal Care and Use committee (ethic approval code: 2007-0129).

Intra Amniotic Administration Lyophilized soluble extracts were diluted in 18 Ω H₂O and for sample osmolarity determination (≤320 OSM). At 17 days of embryonic incubation, eggs containing viable embryos were weighed and divided into 7 groups (n = 15). All treatment groups were assigned eggs of a similar weight frequency distribution. Each group was then injected with the specified solution (1 mL per egg), using a 21 gauge needle into the amniotic fluid, which was identified by candling. The 7 groups were assigned as follows: (1) non-injected; (2) 18 Ω H₂O; (3) inulin (40 mg/mL); (4) chia seed extract 0.5% (5 mg/mL); (5)

chia seed extract 1% (10 mg/mL); (6) chia seed extract 2.5%; (7) chia seed extract 5% (50 mg/mL). After the injections, the holes were sealed with cellophane tape and the eggs were placed in hatching baskets. Immediately after hatch (21 days), the chicks were euthanized by CO₂ exposure and their small intestine, blood, pectoral muscle, cecum and liver were collected.

2.6. *Iron and Zinc Content in Serum and Liver*

Liver (0.5 g) and serum (50 µL) were treated with 3.0 mL of a 60:40 HNO₃ and HClO₄ mixture in a Pyrex glass tube and were incubated overnight. The mixture was then heated to 120 °C for two hours and 0.25 mL of 40 µg/g Yttrium was added as an internal standard. Next, the temperature of the heating block was raised to 145 °C for 2 h. Then, for 10 min, the temperature of the heating block was raised to 190 °C. The cooled samples were then diluted to 20 mL, vortexed and transferred into autosampler tubes to be analyzed via inductively coupled plasma atomic emission spectrometer (ICP-AES). (Thermo Jarrell Ash Corp., Franklin, MA, USA) [12,28].

2.7. *Isolation of Total RNA from Duodenum and Liver*

Total RNA was extracted from 30 mg of the proximal duodenal tissue or liver tissue (n = 10) using Qiagen RNeasy Mini Kit (RNeasy Mini Kit, Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. Total RNA was eluted in 50 µL of RNase-free water. All steps were carried out under RNase-free conditions. RNA was quantified by absorbance at A 260/280 and the integrity of the 18S ribosomal RNAs was verified by 1.5% agarose gel electrophoresis followed by ethidium bromide staining. RNA was stored at -80°C.

2.8. *Real Time Polymerase Chain Reaction (RT-PCR)*

To create the cDNA, a 20 µL reverse transcriptase (RT) reaction was completed 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 and 280 nm using an extinction coefficient of 33 (for single stranded DNA). Genomic DNA contamination was assessed by a real-time RT-PCR assay for the reference gene samples [12].

2.9. Primer Design

The primers used in the real-time qPCR were designed based on 13 gene sequences from the Genbank database, using Real-Time Primer Design Tool software (IDT DNA, Coralville, IA, USA). The sequences and the description of the primers used in this work are summarized in Table 1. 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. Results obtained from the qPCR system were used to normalize those obtained from the specific systems as described below.

Table 1. The sequences of the primers used in this study.

Analyte	Forward Primer (5'–3')	Reverse Primer (5'–3')	Base Pair	GI Identifier
DMT1	TTGATTCAGAGCCTCCCATTAG	GCGAGGAGTAGGCTTGTATTT	101	206597489
Ferroportin	CTCAGCAATCACTGGCATCA	ACTGGGCAACTCCAGAAATAAG	98	61098365
Dcytb	CATGTGCATTCTCTTCCAAAGTC	CTCCTTGGTGACCGCATTAT	103	20380692
Hepcidin *	GAGCAAGCCATGTCAAGATTTC	GTCTGGGCCAAGTCTGTTATAG	132	8056490
ZnT1	GGTAACAGAGCTGCCTTAACT	GGTAACAGAGCTGCCTTAACT	105	54109718
SI	CCAGCAATGCCAGCATATTG	CGGTTTCTCCTTACCACTTCTT	95	2246388
AP	CGTCAGCCAGTTTGACTATGTA	CTCTCAAAGAAGCTGAGGATGG	138	45382360
SGLT1	GCATCCTTACTCTGTGGTACTG	TATCCGCACATCACACATCC	106	8346783
LPL *	TGCTCAGATGCCCTACAAAG	TCTCGTCTAGAGTGCCATACA	119	396219
CEL *	ATGCTGCTGACATCGACTAC	TTCTGAAGTGGACGGTTGATAG	97	417165
18S rRNA *	GCAAGACGAACTAAAGCGAAAG	TCGGAAGTACGACGGTATCT	100	7262899

DMT1, Divalent metal transporter 1; Dcytb, Duodenal cytochrome b; Znt 1, Zinc transporter 1; SI, Sucrose isomaltase; AP, Amino peptidase; SGLT1, Sodium-Glucose transport protein 1; LPL, Lipoprotein lipase; CEL, Carboxyl ester lipase; 18S rRNA, 18S Ribosomal subunit. * liver analyses.

2.10. Real-Time qPCR

Design All procedures were conducted as previously described [10–13]. The specific primers that were used are shown in Table 1.

2.11. Collection of Microbial Samples and Intestinal Content DNA Isolation

The cecum contents were removed under sterile conditions, placed into a sterile tube containing 9 mL of Phosphate buffered saline (PBS) and homogenized by vortexing with glass beads for 3 min [27,39]. All procedures were conducted as previously described [10–14].

2.12. Primer Design and PCR Amplification of Bacterial 16S rDNA

Primers for *Lactobacillus*, *Bifidobacterium*, *Clostridium* and *Escherichia coli* were used [16,39]. The universal primers were designed with the invariant region in the 16S rRNA of bacteria and were used as internal standards. The proportions of each bacterial group are presented. The PCR products were loaded on 2% agarose gel stained with ethidium bromide and quantified by Quantity One 1-D analysis software (Bio-Rad, Hercules, CA, USA) [12]. The evaluation of the relative abundance of each examined bacterium was conducted as previously described [10–14].

2.13. Glycogen Analysis

At hatch, the pectoral muscle (20 mg) was collected for glycogen analysis. The tissue samples were homogenized in 8% perchloric acid, and glycogen concentration was determined as previously described [40]. After homogenization, the samples were centrifuged at 12,000 rpm at 4 °C for 15 min. The supernatant was removed, and 1.0 mL of petroleum ether was added. After mixing, the petroleum ether fraction was removed, and samples from the bottom layer were transferred to a new tube containing 300 µL of color reagent. All samples were read at a wavelength of 450 nm in an ELISA reader and the amount of glycogen was calculated according to a standard curve. The amount of glycogen present in pectoral sample was determined by multiplying the weight of the tissue by the amount of glycogen per 1 g of wet tissue.

2.14. Morphological Examination

As previously described [10,41], liver and intestine samples were collected at the conclusion of this study. Samples were fixed in 4% (v/v) buffered formaldehyde, dehydrated, cleared, and embedded in paraffin. Serial sections of 5 µm were obtained and were deparaffinized in xylene, rehydrated in a different concentration of alcohol, stained with hematoxylin/eosin or Alcian Blue/Periodic acid-Schiff, and examined by light microscopy.

The following variables were measured in the intestine: villus height, villus width, depth of crypts, goblet cell number and goblet cell diameter in each segment, performed with a light microscope using EPIX XCAP software (Standard version, Olympus, Waltham, MA, USA). Four segments for each biological sample and five biological samples per treatment group were used. Villi height was measured using the lamina propria as the base; villi width, depth of the crypt and the number of goblet cell were measured per side of a longitudinal section through the villus; goblet cell size was measured as the diameter of the goblet cells (μm^2). Villi surface area was calculated from the villus height and width at half height as according to Uni et al. [42]. For the Alcian Blue and Periodic acid-Schiff stain, the segments were only counted for the types of goblet cells in the villi epithelium, goblet cells within the crypts and the mucus layer thickness. Goblet cells were enumerated on 10 villi/sample, and the means were utilized for statistical analysis. The liver was stained with hematoxylin-eosin (H&E) for standard microscopy and visualized using the same light microscope. Mean adipocyte diameter was determined by random, utilizing the EPIX XCAP software (standard version, Olympus, Waltham, MA, USA), by enumerating 10 adipocytes/segment/sample, and the means were utilized for statistical analysis.

2.15. Statistical Analysis

All values are expressed as the means and standard deviations. Experimental treatments for the *in ovo* assay were arranged in a completely randomized design. The results were analyzed by ANOVA. For significant “p-value”, post hoc Duncan test was used to compare test groups. Statistical analysis was carried out using SPSS version 20.0 software (IBM, Armonk, USA). The level of significance was established at $p < 0.05$.

3. Results

3.1. Concentration of Iron, Zinc, Phytic Acid and Dietary Fiber and the Phytate:Iron Ratio in Chia Flour and in Chia Extract

The iron and zinc concentrations, insoluble fiber content, phytic acid and the phytate:iron ratio were higher ($p < 0.05$) in the chia seed compared to the chia extract (Table 2). However, the content of soluble fiber was significantly greater ($p < 0.05$) in the chia extract relative to chia seed.

Table 2. Concentration of iron, zinc, dietary fiber and phytic acid in chia flour and in chia extract.

Treatment Group	Iron (ppm)	Zinc (ppm)	Insoluble Fiber (g/100g)	Soluble Fiber (g/100g)	Phytic Acid (g/100g)	Phytic Acid : Iron ratio
Chia seed	110.25 ± 4.97 ^a	57.82 ± 0.40 ^a	34.67 ± 1.84 ^a	4.01 ± 0.21 ^b	0.71 ± 0.02 ^a	5.47 ^a
Chia extract	41.46 ± 0.89 ^b	31.29 ± 0.89 ^b	23.53 ± 1.74 ^b	19.68 ± 0.76 ^a	0.08 ± 0.00 ^b	1.60 ^b

Values are means ± SEM, n=5. ^{a,b} Treatment groups not indicated by the same letter are significantly different (p < 0.05).

3.2. Polyphenol Profile in Chia Flour

The concentration of the five most prevalent polyphenolic compounds found in chia is presented in Table 3. Chia presented high concentrations of rosmarinic acid and rosmarinyl glucoside. In addition, we observed the presence of ferulic acid, caffeic acid and protocatechuic acid.

Table 3. Polyphenol profile present in chia flour.

Polyphenolic Compounds	Mean Peak Area (mAU-min/10 ⁶)
Rosmarinic acid	42.30 ± 1.90
Rosmarinyl glucoside	57.70 ± 0.02
Ferulic acid	1.19 ± 0.06
Caffeic acid	0.76 ± 0.38
Protocatechuic acid	0.21 ± 0.03

Values are the means ± SEM, n = 10. mAU: milli absorbance unit; min: minutes.

3.3. In Ovo Assay (*Gallus Gallus* Model)

3.3.1. Hb Concentration

The Hb values were significantly (p < 0.05) higher in the “2.5% chia” extract treatment group compared to the 18 Ω H₂O and non-inject group. The other treatments did not differ from each other (Table 4).

Table 4. Blood hemoglobin (Hb) concentrations (g/dL).

Treatment Group	Hb (g/dL)
Non-injected	5.93 ± 0.00 ^b
18Ω H ₂ O	5.52 ± 1.49 ^b
Inulin	7.76 ± 1.16 ^{a,b}
0.5% Chia	7.08 ± 1.16 ^{a,b}
1.0% Chia	9.51 ± 1.34 ^{a,b}
2.5% Chia	10.41 ± 1.37 ^a
5.0% Chia	10.06 ± 2.48 ^{a,b}

Values are the means ± SEM, $n = 10$. ^{a,b} Treatment groups not indicated by the same letter are significantly different ($p < 0.05$).

3.3.2. Iron and Zinc Concentration in Liver and Serum

As shown in Table 5, there were no significant ($p > 0.05$) differences in liver iron concentration and serum zinc concentration between treatment groups. However, “1% chia” extract treatment increased ($p < 0.05$) the zinc liver content compared to non-inject treatment. In addition, we observed that “5% chia” extract treatment showed a lower ($p < 0.05$) serum iron concentration when compared to the 18Ω H₂O and inulin groups. In general, different concentrations of chia extract did not affect iron and zinc concentrations in liver and serum.

Table 5. Iron and zinc concentrations (ppm).

Treatment Group	Liver		Serum	
	Iron (µg/g)	Zinc (µg/g)	Iron (µg/g)	Zinc (µg/g)
Non-injected	35.28 ± 2.52 ^a	14.77 ± 1.26 ^b	3.14 ± 0.25 ^{a,b,c}	0.001 ± 0.000 ^a
18Ω H ₂ O	41.00 ± 3.24 ^a	16.10 ± 1.57 ^{a,b}	4.04 ± 0.52 ^{a,b}	0.002 ± 0.000 ^a
Inulin	40.92 ± 3.32 ^a	16.39 ± 2.43 ^{a,b}	4.24 ± 0.96 ^a	0.001 ± 0.000 ^a
0.5% Chia	35.57 ± 3.16 ^a	18.45 ± 1.13 ^{a,b}	2.99 ± 0.44 ^{a,b,c}	0.002 ± 0.000 ^a
1.0% Chia	43.17 ± 4.08 ^a	21.63 ± 2.59 ^a	2.36 ± 0.24 ^{a,b}	0.003 ± 0.001 ^a
2.5% Chia	33.52 ± 1.67 ^a	16.60 ± 1.41 ^{a,b}	3.23 ± 0.63 ^{a,b,c}	0.001 ± 0.000 ^a
5.0% Chia	35.88 ± 2.81 ^a	17.87 ± 2.52 ^{a,b}	1.59 ± 0.29 ^c	0.002 ± 0.000 ^a

Values are the means ± SEM, $n = 10$. ^{a,b,c} Treatment groups not indicated by the same letter are significantly different ($p < 0.05$).

3.3.3. Gene Expression of Fe- and Zn-Related Genes

The gene expression of DMT1 was lower ($p < 0.05$) in the group treated with 2.5% chia soluble extract compared to the inulin and 18 Ω H₂O groups (Figure 1). However, other various concentrations of chia soluble extract did not affect the expression of DMT1 ($p > 0.05$). The relative expression of DcytB and hepcidin was significantly up-regulated ($p < 0.05$) in the 1%, 2.5% and 5% chia extract. The groups treated with 1%, 2.5% and 5% chia extract showed lower ($p > 0.05$) ferroportin gene expression compared to the 18 Ω H₂O injected group. However, no differences ($p > 0.05$) were observed between chia treatment groups. The relative expression of ZnT1 was significantly up-regulated ($p < 0.05$) in the 1%, 2.5% and 5% chia extract.

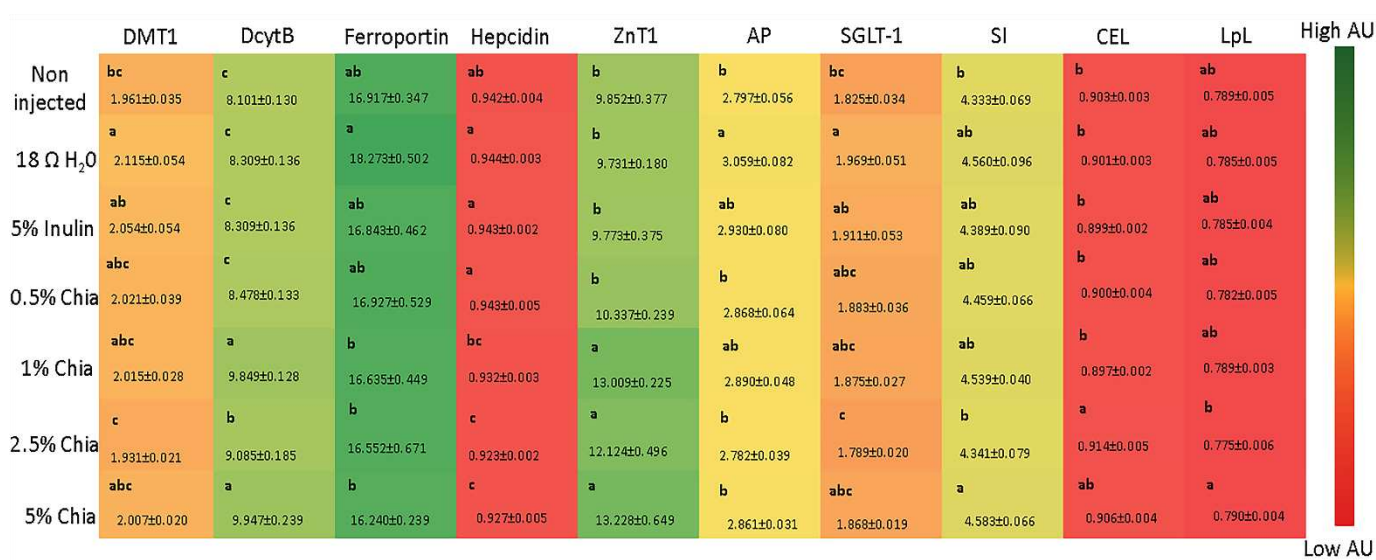


Figure 1. Effect of the intra-amniotic administration of experimental solutions on intestinal and liver gene expression. Values are the means $\pm$ SEM, $n = 10$. ^{a-c} Per gene, treatments groups not indicated by the same letter are significantly different ($p < 0.05$). DMT1, Divalent metal transporter 1; DcytB, Duodenal cytochrome b; ZnT1, Zinc transporter 1; AP, Amino peptidase; SGLT1, Sodium-Glucose transport protein 1; SI, Sucrose isomaltase; CEL, Carboxyl ester lipase; LpL, Lipoprotein lipase.

3.3.4. Gene Expression of BBM Functional Proteins

The gene expression of aminopeptidase (AP), sodium-glucose transport protein 1 (SGLT1) and sucrase isomaltase (SI) are used as biomarkers of brush border membrane digestive and absorptive functions. AP and SGLT1 gene expression did not differ ($p > 0.05$) between groups treated with chia extract. However, the gene expression of SI was higher ($p < 0.05$) in “5% chia” extract treatment group compared to the “2.5% chia” extract treatment group (Figure 1).

3.3.5. Gene Expression of Lipids Metabolism Protein

The gene expressions of carboxyl ester lipase (CEL) and lipoprotein lipase (LpL) are used as biomarkers of lipid metabolism. As shown in Figure 1, the “2.5% chia” extract treatment group presented higher ($p > 0.05$) CEL expression compared to the control groups. However, the gene expression of LpL did not differ between chia extract groups and control groups ($p < 0.05$).

3.3.6. Cecum-to-Body-Weight Ratio

As shown in Figure 2, the chia soluble extract treatment groups showed a higher ($p < 0.05$) cecum weight (B) and cecum weight/body weight ratio (C) compared to control groups ($p < 0.05$). However, no significant difference ($p > 0.05$) was observed in body weight (A) among treatment groups and controls.

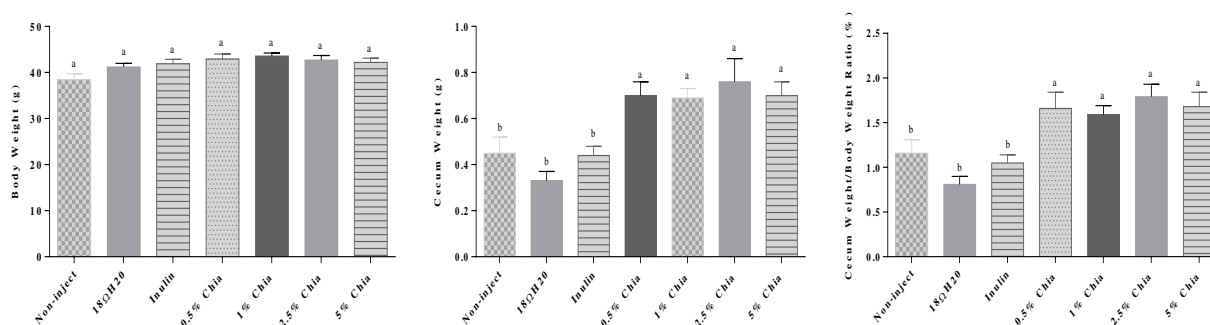


Figure 2. The effect of chia on the: (A) body weight; (B) cecum weight; and (C) cecum weight/body weight ratio (%). Values are the means $\pm$ SEM $n = 15$. ^{a,b} Within a column, means without a common letter are significantly different ($p < 0.05$).

3.3.7. Microbial Analysis

As shown in Figure 3, the relative abundance of both *Bifidobacterium* and *Lactobacillus* genera, increased ($p < 0.05$) in the “0.5% chia” treatment, relative to the 18 Ω H₂O group and non-injected group. The “5% chia” treatment group showed a lower ($p < 0.05$) concentration of these bacterial populations compared to the other groups. The relative abundance of *E. coli* significantly decreased ($p < 0.05$) in the 1%, 2.5% and 5% chia extract treatment groups compared to the control groups. The relative abundance of *Clostridium* was significantly ($p < 0.05$) lower in the non-inject group, 18Ω H₂O group and “5% chia” treatment group. These results suggest that a lower concentration of chia extract may positively affect gut health.

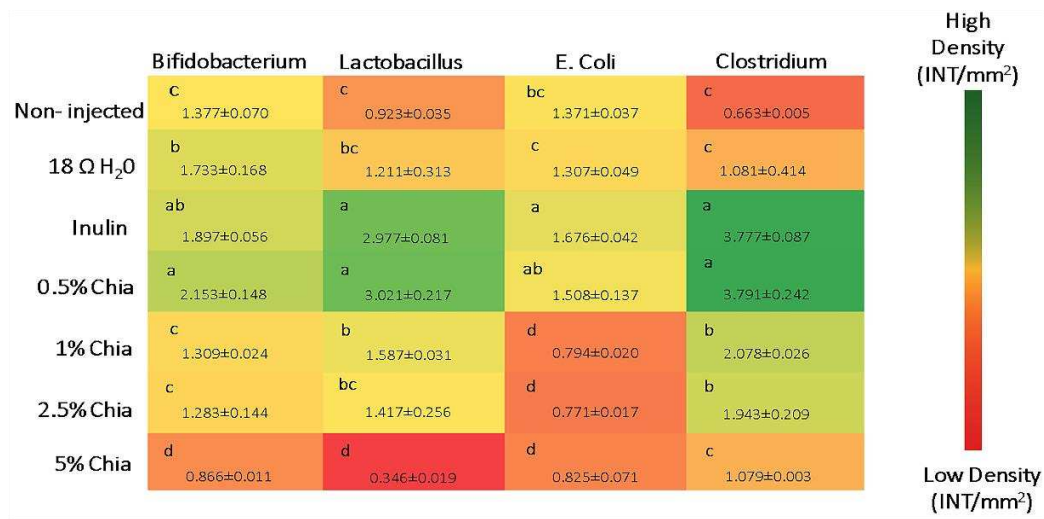


Figure 3. Genera- and species-level bacterial populations (AU) from cecal contents measured on the day of hatch. Values are the means $\pm$ SEM, $n = 10$. ^{a-d} Per bacterial category, treatment groups not indicated by the same letter are significantly different.

3.3.8. Glycogen Analysis

No significant difference was observed in pectoral muscle glycogen content between groups (Table 6, $p > 0.05$).

Table 6. Concentration of glycogen in pectoral muscle.

Treatment Group	Glycogen Concentration (mg/g)
Non-injected	0.17 $\pm$ 0.04 ^a
18Ω H ₂ O	0.21 $\pm$ 0.05 ^a
Inulin	0.29 $\pm$ 0.06 ^a
0.5% Chia	0.13 $\pm$ 0.03 ^a
1.0% Chia	0.31 $\pm$ 0.06 ^a
2.5% Chia	0.26 $\pm$ 0.08 ^a
5.0% Chia	0.29 $\pm$ 0.15 ^a

Values are the means $\pm$ SEM, $n = 10$. ^a Treatment groups not indicated by the same letter are significantly different ($p < 0.05$).

3.3.9. Morphometric Data for Villi, Depth of Crypts and Goblet Cell

The villus surface areas, villi length, width and the number of goblet cells were significantly ($p < 0.05$) higher in all chia extract treatment groups compare to controls (Tables 7 and 8), indicating that soluble extracts from chia had a positive effect on intestinal development, through the proliferation of enterocytes, and the increased number in mucus-producing cells. However, there were no significant ($p > 0.05$) differences in crypt depth and mucus layer width between treatment groups. Further, all chia extract treatments increased (p

< 0.05) the diameter of goblet cells compared to controls. In relation to the types of goblet cells observed (acidic, neutral, mixed), we can note that the administration of 2.5% chia soluble extracts reduced ($p < 0.05$) the number of neutrals goblet cells compared to the control groups. In addition, the administration of 2.5% and 5% chia soluble extracts increased ($p < 0.05$) the number of acidic goblet cells, whereas the administration of 1% and 2.5% chia extract caused an increase ($p < 0.05$) in mixed goblet cells, compared to controls. In relation to the types of goblet cells in the crypt epithelium, the administration of 0.5% chia soluble extract increased ($p < 0.05$) the number of neutrals goblet cells compared to controls. In addition, the administration of 2.5% chia extracts increased ($p < 0.05$) the number of mixed goblet cells compared to controls. The number of acid goblet cells did not differ ($p > 0.05$) between groups (Figure 4). No significant differences between treatment groups were measured in fat cell diameter ($p > 0.05$, Figure 5).

Table 7. Effect of the intra-amniotic administration of experimental solutions on the duodenal small intestinal villus and crypt.

Treatment Group	Villus Surface Area (mm ²)	Villus Length (μM)	Villus Width (μM)	Depth of Crypts (μM)	Mucus Layer Width (μM)
Non-injected	170.29 ± 5.33 ^c	248.64 ± 2.83 ^c	43.26 ± 0.42 ^c	12.76 ± 0.10 ^a	2.21 ± 0.27 ^a
18Ω H ₂ O	127.13 ± 8.16 ^c	204.30 ± 3.40 ^d	39.24 ± 0.37 ^d	12.60 ± 0.09 ^a	2.32 ± 0.15 ^a
Inulin	130.00 ± 9.42 ^c	208.90 ± 3.63 ^d	41.20 ± 0.56 ^{c,d}	13.01 ± 0.10 ^a	2.36 ± 0.1 ^a
0.5% Chia	237.53 ± 7.98 ^b	323.85 ± 3.51 ^b	46.42 ± 0.40 ^b	12.49 ± 0.08 ^a	2.41 ± 0.25 ^a
1.0% Chia	234.78 ± 7.36 ^b	298.82 ± 2.43 ^b	49.70 ± 0.51 ^b	13.08 ± 0.09 ^a	2.22 ± 0.10 ^a
2.5% Chia	264.95 ± 2.74 ^b	334.44 ± 5.62 ^b	50.15 ± 0.57 ^b	12.83 ± 0.10 ^a	2.20 ± 0.13 ^a
5.0% Chia	343.93 ± 9.38 ^a	374.47 ± 5.50 ^a	58.18 ± 0.59 ^a	12.71 ± 0.11 ^a	2.15 ± 0.14 ^a

Values are the means ± SEM, $n = 5$. ^{a-d} Treatment groups not indicated by the same letter are significantly different ($p < 0.05$).

Table 8. Effect of the intra-amniotic administration of experimental solutions on the goblet cells.

Treatment Group	Goblet Cell Diameter (μM)	Total Goblet Cell Number (Unit)	Villus Goblet Cell Number (Unit)			Crypts Goblet Cell Number (Unit)		
			Neutral	Acid	Mixed	Neutral	Acid	Mixed
Non-injected	4.20 ± 0.03^c	21.23 ± 0.24^c	$2.50 \pm 0.33^{a,b}$	8.77 ± 0.23^b	9.11 ± 0.33^c	0.01 ± 0.00^b	10.36 ± 0.57^a	0.47 ± 0.15^c
18 Ω H ₂ O	4.10 ± 0.03^c	20.18 ± 0.26^c	2.14 ± 0.24^b	8.05 ± 0.74^c	9.62 ± 0.47^c	0.10 ± 0.00^b	9.69 ± 0.55^a	1.64 ± 0.16^a
Inulin	4.89 ± 0.06^b	24.88 ± 0.20^b	3.90 ± 0.99^a	8.67 ± 0.48^b	$11.02 \pm 1.02^{b,c}$	0.01 ± 0.00^b	10.32 ± 0.36^a	0.43 ± 0.14^c
0.5% Chia	$5.48 \pm 0.03^{a,b}$	28.59 ± 0.32^a	$3.63 \pm 0.25^{a,b}$	$10.76 \pm 0.71^{a,b}$	14.26 ± 0.51^a	0.46 ± 0.12^a	10.02 ± 0.91^a	$0.89 \pm 0.06^{b,c}$
1.0% Chia	$5.36 \pm 0.05^{a,b}$	29.19 ± 0.29^a	2.07 ± 0.17^b	$11.43 \pm 0.61^{a,b}$	15.55 ± 0.71^a	0.09 ± 0.05^b	9.65 ± 1.05^a	$1.30 \pm 0.11^{a,b}$
2.5% Chia	5.61 ± 0.02^a	29.61 ± 0.40^a	1.63 ± 0.16^c	13.70 ± 1.53^a	13.72 ± 1.35^a	0.05 ± 0.02^b	9.45 ± 0.49^a	1.45 ± 0.30^a
5.0% Chia	$5.42 \pm 0.06^{a,b}$	29.91 ± 0.39^a	$2.55 \pm 0.43^{a,b}$	13.13 ± 1.35^a	$13.20 \pm 1.51^{a,b}$	$0.18 \pm 0.06^{a,b}$	9.88 ± 0.13^a	$0.82 \pm 0.21^{b,c}$

Values are the means $\pm$ SEM, $n = 5$. ^{a-c} Treatment groups not indicated by the same letter are significantly different ($p < 0.05$).

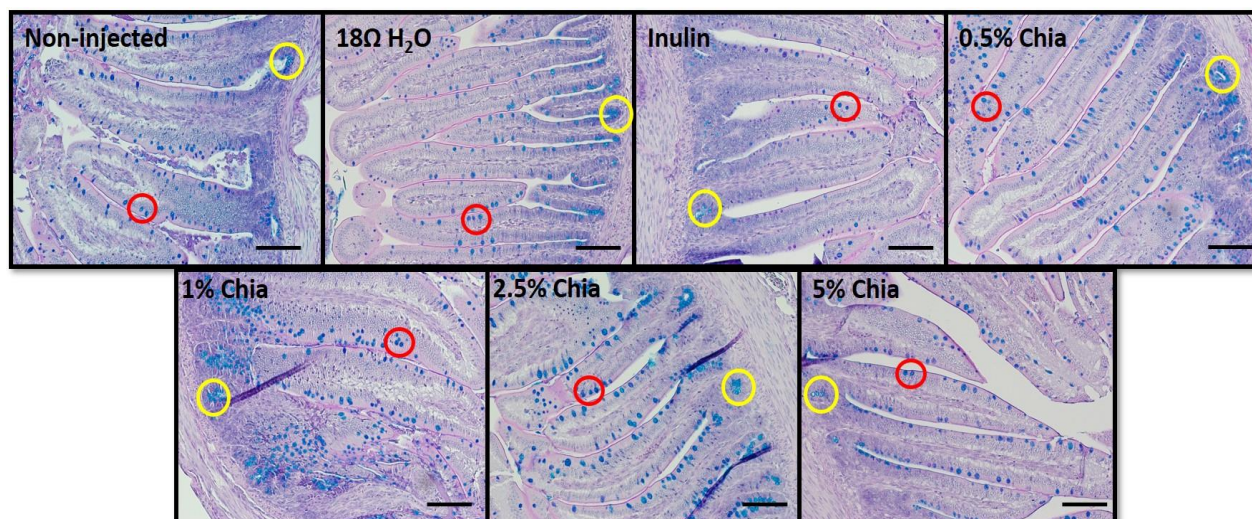


Figure 4. Representations of the intestinal morphology of each treatment group are shown (Alcian Blue and Periodic acid-Schiff Stain). The yellow circles indicate crypts within the villi and the red circles indicate goblet cells on the villi. Bar = 50 μ m.

3.3.10. Hepatic Morphometric Measurement

As shown in Figure 5, no significant differences were observed in hepatic fat cell diameter between all treatment groups ($p > 0.05$).

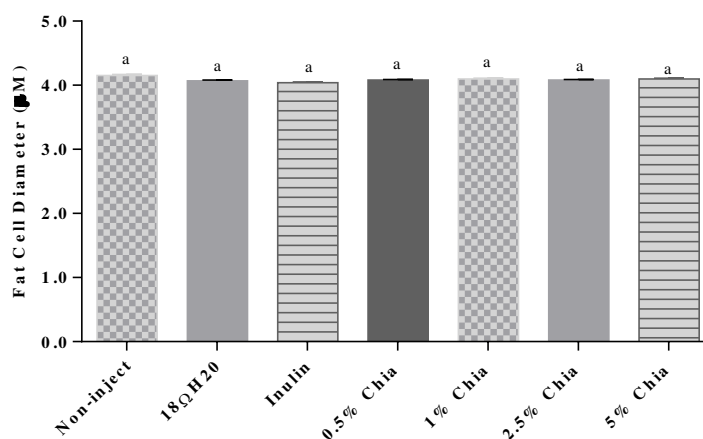


Figure 5. Fat cell diameter. Values are the means $\pm$ SEM, $n = 5$. ^a Treatment groups not indicated by the same letter are significantly different.

4. Discussion

Chia is a good source of dietary fiber, which was demonstrated to have a beneficial effect on intestinal health [29]. However, until now, the potential effects of soluble extracts

from chia seed on the intestinal microbiota, intestinal morphology and mineral bioavailability, such as iron and zinc, were not investigated. Further, it is important to highlight that the alterations in microbiota populations, due the consumption of dietary fiber, may be associated, directly or indirectly, to the increased dietary bioavailability of iron and zinc in vulnerable populations [13,15,18,27]. The present study indicates that the *in ovo* administration of soluble extracts from chia seed increased the intestinal villus surface area, villi length, villi width, goblet cell number and goblet cell size (diameter), as well as cecum weight (used as biomarker of microbial presence and activity). In addition, the administration of chia seed soluble extracts up-regulated the expression of proteins related to zinc metabolism. Further, the chia soluble extract (0.5%) increased the *Bifidobacterium* and *Lactobacillus* relative abundance in cecum content.

According to our results, the hemoglobin concentration results corroborate with our findings of serum iron. We did not observe a change in liver iron concentrations, due to the short time of exposure of the soluble extracts, which was not sufficient to cause a modification in hepatic iron storage. This was in agreement with previous observations that evaluated the effects of intra-amniotic raffinose and stachyose administration on Fe status, as the results showed no significant differences in hemoglobin values between treatment groups [10]. Further, another study that assessed the effect of the intra-amniotic administration of bean soluble extracts on iron status indicated that bean extracts did not affect serum or liver iron concentrations [12]. A similar result was observed post intra-amniotic administration of wheat extracts [14]. In addition, a BBM Fe metabolism-related gene expression analysis of DcytB, DMT, ferroportin and hepcidin was conducted. DcytB is the protein responsible for reducing Fe³⁺ to Fe²⁺ in the apical membrane of the enterocyte [10,43]. DMT1 plays a key role in Fe²⁺ transport into the enterocyte, being considered the major Fe intestinal transporter [10,43], whereas ferroportin is the protein that transports Fe²⁺ from the enterocyte into the bloodstream [10,43]. In the current study, the administration of 1%, 2.5% and 5% chia soluble extract solutions up-regulated the expression of DcytB, which in return may increase the transportation of Fe by DMT1 into the enterocyte, and as previously demonstrated, this effect can potentially increase iron absorption efficiency in a long-term feed trial [12]. Further, we investigated hepcidin gene expression as the key iron-regulatory hormone that controls systemic iron homeostasis, as hepcidin is able to down regulate the expression of ferroportin [44,45]. Further, the increase in hepcidin production is stimulated by iron loading and inflammation [46,47]. In the present study, hepcidin gene expression was lower ($p < 0.05$) in

the 1%, 2.5% and 5% chia soluble extract groups compared to the inulin and water groups, which suggests that in a long-term feeding trial, the dietary inclusion of chia may have a positive effect on Fe-related proteins.

ZnT1 is the only transporter of the ZnT transporters family that is localized on the enterocyte's basolateral membrane and functions by exporting cytosolic zinc into the extracellular space [48], an up-regulation in ZnT1 mRNA gene expression may occur under increased cellular zinc levels [49]. In the current study, the groups treated with chia seed soluble extract (1%, 2.5% and 5%) shown a gene expression up-regulation ($p < 0.05$) of ZnT1 compared to the other groups, although the zinc serum concentrations did not differ between experimental groups.

Previous studies demonstrated the potential beneficial effects of soluble extract from various sources and plant origin compounds (such as raffinose, stachyose, diadzein, bean, and wheat) on BBM functionality and intestinal bacterial populations [10–13,27]. In the current study, the expression of BBM functional genes (AP, SI and SGLT1) was not affected by the chia seed soluble extract administration, due to the short exposure time. However, in relation to microbial populations, there was an increasing abundance of *Lactobacillus* ($p < 0.05$), and *Bifidobacterium* ($p < 0.05$) in the cecal contents of animals received 0.5% chia soluble extracts compared to the 18Ω H₂O and non-injected group. Further, we observed an increased abundance in *Lactobacillus* ($p < 0.05$), *Bifidobacterium* ($p < 0.05$), *E. Coli* ($p < 0.05$) and *Clostridium* ($p < 0.05$) in the cecal contents of the animals that received 0.5% chia seed soluble extracts compared to other groups treated with chia seed extract. It is important to highlight that the increase in *Lactobacillus* and *Bifidobacterium* abundance, due the consumption of dietary fiber, may further contribute, directly or indirectly, to the increased bioavailability of iron and zinc in vulnerable populations, as these bacterial genera produce short-chain fatty acids (SCFAs), which reduce the intestinal pH, and therefore, may increase mineral (as Fe and Zn) solubility and therefore absorption [50]. *Bifidobacterium* and *Lactobacillus* can break down non-digestible fiber (prebiotics), due to their 1,2-glycosidase activity, leading to greater SCFA production [16,27,39], culminating with the increase in the absorption of iron and zinc.

The morphological parameters described in the current study, including villi development parameters and the crypt depth, are used as indicators of intestinal health, functionality and development [51]. The administration of chia seed soluble extracts, regardless of the concentration used, increased all parameters related to intestinal villi. These

values (villus surface area, villus length and width) were significantly higher ($p < 0.05$) in the 5.0% chia group and relative to all other groups. This can be explained by the potential increased proliferation of intestinal cells in the short term, due the presence of soluble fiber, leading to hyperplasia and/or hypertrophy of intestinal cells and potentially enhancing the absorptive and digestive capacity of the villi BBM [29]. Another explanation is that the tested extracts had potentially increased butyrate production, which may lead to enterocyte proliferation [52]. Added to these factors, the soluble extract of chia seed contains a high concentration of phenolic compounds, among them are rosmarinic acid and rosmarinyl glucoside, which present the ability to affect intestinal morphology [53], increasing the villus height, crypt depth ratio, and muscularis thickness, as observed in the study that evaluated the administration of dietary polyphenol concentrate previously performed in *Gallus gallus* [54]. The morphological results agree with our cecum weight and cecum weight/body weight ratio observations. All experimental groups showed a higher ($p > 0.05$) cecal weight (Figure 2B) post intra-amniotic soluble extract administration, indicating, and as previously suggested, increased cecal bacterial populations activity [10,12,13]. As for crypt depth, no differences between the experimental groups were observed, since duodenal crypts require a longer time to allow cellular proliferation. However, the intestinal crypts are meager and are able to rise to the surface of the villus, increasing the number of enterocytes in intestinal villi [52]—a phenomenon that was observed in the current study. Additionally, we observed increased goblet cell number and goblet cell diameter, which suggests an increased production of mucus that coats the intestinal lumen. As previously suggested, this may increase the intestinal BBM digestive and absorptive capabilities, and may indirectly increase the bioavailability of dietary components as suggested by the effects observed on the morphometric parameters [55–57]. The increase in “acidic goblet cells”, containing acidic mucin due to the administration of 2.5% and 5% chia soluble extracts, may contribute to the reduction of intestinal pH, which in the long term, may lead to increased solubilization and uptake of iron and zinc and affect intestinal microbial profile [14,39]. The increase in “acidic goblet cells” was previously observed in a study that evaluated the effects of the intra amniotic administration of carbohydrate solution (containing maltose, sucrose and dextrin) on mucin content, goblet cell development, and levels of mucin mRNA in the *Gallus gallus* small intestine [58].

In general, previous studies showed a positive effect of prebiotic administration on intestinal morphology [10,13,25,51,52], for example, the intra-amniotic administration of raffinose and stachyose increased villus surface area compared to the control [10]. Similar

results were observed by Hou et al. [13] who evaluated the effect of chickpea and lentil prebiotics administration *in ovo*. In another study, the authors evaluated the development of morphological parameters in *Gallus gallus*, and the results showed that the administration of a synthetic prebiotic increased the villus width and crypt depth. The prebiotic had no impact on villus height, villus surface area, and muscular thickness compared to the animals that received saline solution administration [51]. Bogucka et al. [52] evaluated the effect of inulin administration on the development of the intestinal villi and the number of goblet cells in the small intestine on the 1st and the 4th day post hatch (*Gallus gallus*) and the study indicated that on day one, the villus height did not differ among experimental groups. However, the villus width, villus surface area and crypt depth were lower in the prebiotic group. On day four, the inulin group showed a lower villus width, villus surface area and crypt depth [52]. Another study that evaluated the effect of the intra amniotic administration of wheat bran prebiotic extract indicated increased villus height, goblet cell diameter and number in all treatment groups [11]. Further, Mista et al. [25] evaluated the effect of intra amniotic administered prebiotics on the development of the small intestine (*Gallus gallus*) and found that prebiotics did not affect the villus length, but did increase the crypt depth.

The observations described in the current study suggest that dietary chia seed consumption may be an effective strategy to reduce dietary iron and zinc deficiency and to potentially improve intestinal health. Overall, the up-regulation of Zn gene expression and the DcytB-Fe metabolism protein, the increase in villus surface area, villus length, villus width, goblet cell number and goblet cell diameter as well as cecum weight suggest that chia is a promising food ingredient that may improve mineral bioavailability and intestinal morphology. Hence, long-term feeding trials assessing the dietary effects of chia are now warranted.

5. Conclusions

The intra-amniotic (*in ovo*) administration of chia seed soluble extracts with putative prebiotic effects improved the intestinal morphology and up-regulated Zn-related protein gene expression. Further, chia seed soluble extract administration affected the intestinal microbiota and iron-related gene expression. The current study is the first to investigate the effects of chia seed soluble extracts with a potential prebiotic effect *in vivo*; thus, future studies aimed to assess dietary chia seed in a long-term feeding trials should be conducted, since chia may

be a viable dietary ingredient that may improve intestinal health and contribute to intestinal mineral absorption.

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7. GENERAL CONCLUSIONS

In this study, it has demonstrated how chia can affect calcium, zinc and iron bioavailability, as well as the effect of chia consumption on inflammation, oxidative stress and intestinal health, that include gut microbiota, intestinal morphology and intestinal gene expression.

Our results demonstrated that animals (male *Wistar* rats) that consumed chia as a unique source of calcium showed low calcium bioavailability. Nonetheless, chia consumption reduced inflammatory processes and improved the lipid profile in young male *Wistar* rats.

Also, chia intake improved lipid profile and increased liver and cecum indices in female *Wistar* rats submitted or not to ovariectomy. Chia consumption associated with the standard diet provided to rats improved antioxidant activity, increased SOD and PPAR- α expression and catalase activity, while at the same time it reduced NF κ B expression. In addition, chia intake associated with the high fat diet in ovariectomized rats reduced IL-1 β levels and TNF- α expression, increased SOD mRNA and protein levels, and increased catalase activity. In general, ovariectomy did not influence inflammatory and oxidative stress parameters.

In addition, and for the first time, was evaluated the effect of intra-amniotic (*in ovo*) administration of chia seed soluble extracts on intestinal morphology and minerals bioavailability. It was observed that chia improves the intestinal morphology and is able to up-regulated Zn-related protein gene expression, affected the intestinal microbiota and iron-related gene expression.

Overall, our findings showed that chia consumption improved the inflammation, oxidative stress and intestinal morphology *in vivo*. Besides this, we observed how chia consumption could affect the calcium, zinc and iron nutritional status in animal's model. Thus, we can conclude that chia consumption is a good strategy for improving inflammation and oxidative stress as well as intestinal function and structure. Also, all these studies build a scientific basis for the release of chia consumption to the general population.

8. FINAL CONSIDERATIONS

We suggest that a long-term feeding trials are performed to evaluate the action of dietary chia seed on mineral absorption and intestinal health, since chia may be a viable dietary ingredient that may improve the intestinal morphology, intestinal functionality and contribute to improve mineral bioavailability. In addition, future studies relating chia consumption to intestinal microbiota composition should be performed. Finally, it would be interesting to evaluate the effect of chia consumption on adipose tissue in animals fed a high-fat diet.

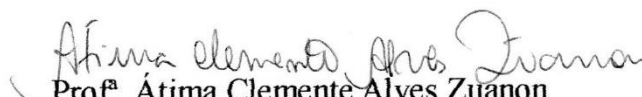
APPENDIX I

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 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.


Prof. Átima Clemente Alves Zuanon
Presidente

Comissão de Ética no Uso de Animais – CEUA/UFV