BÁRBARA PEREIRA DA SILVA

CONCENTRATION OF NUTRIENTS AND BIOACTIVE COMPOUNDS IN CHIA (*SALVIA HISPANICA* L.), PROTEIN QUALITY AND IRON BIOAVAILABILITY IN *WISTAR* RATS

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

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BIOGRAPHY

Bárbara Pereira da Silva, daughter of Fernando Antônio Pereira da Silva and Regina Célia Pereira da Silva was born on May 28, 1990, in Belo Horizonte, Minas Gerais.

In March 2008, she joined the Nutrition Course of the Federal University of Viçosa (UFV), graduating Nutritionist in September 2013.

In March 2014, she began in the Graduate Program in Science of Nutrition (UFV) in Master level, submitting to the defense of the dissertation in February 2016.

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GENERAL METHODOLOGY

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LIST OF ABBREVIATIONS AND ACRONYMS

AA	Ascorbic Ácid
ABS	Maximum absorbance
ALT	Alanine aminotransferase
ARR	Antirradical activity
AST	Alanine aminoaspartase
ANOVA	Analysis of variance
BHT	Butylated hydroxytoluene
cDNA	DNA complementary
DcytB	Duodenal cytochrome b
DMT-1	Divalent metal transporter
DNAse	Deoxyribonuclease
DPPH	1,1- difenil-2-picrilhidrazila
EDTA	Acid etilen diamino tetraacetic acid
EqAG	Gallic acid equivalente
FER	Food efficiency ratio
Fe^{+2}	Ferrous iron
Fe^{+3}	Ferric iron
G	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLU	Glucose
HPLC	High-performance liquid chromatography
H_2SO_4	Sulfuric acid
H_3CO_3	Boric acid
HCl	Hydrochloric acid
HDL	High density lipoprotein cholesterol
HFD+C	High fat diet + chia
HFD+FS	High fat diet + ferrous sulfate
HG	Hemoglobin gain
HRE%	Hemoglobin regeneration efficiency
IRP1	Iron - responsive element - binding protein 1

IRP2	Iron - responsive element - binding protein 2
Kcal	Kilocalorie
Kg	Kilogram
LDL	Low density lipoprotein cholesterol
M	Molar
Min	Minutes
Mg	Milligram
Mg	Microgram
MT	Mato Grosso
mRNA	Messenger RNA
µm	Micrometres
µL	Microliter
N	Nitrogen
NPR	Net protein ratio
NaCl	Sodium chloride
Na_2SO_4	Sodium sulfate
NF-kB	Nuclear factor kappa B
Nm	Nanometer
PBS	Buffered saline solution with phosphate
PER	Protein efficiency ratio
PPAR-α	Peroxisome proliferator-activated receptor
Ppm	Part per million
RBV-HRE	Biological relative value of HRE
RS	Rio Grande do Sul
RT-PCR	Polymerase chain reaction in real time
SD	Standard deviation
SD+C	Standard diet + chia
SD+FS	Standard diet + ferrous sulfate
TC	Total cholesterol
TD	True digestibility
TGL	Triacylglycerides
V	Volume

VLDL	Very low density lipoprotein cholesterol
w-3	Omega 3
w-6	Omega 6
3-DXAs	3- Deoxyanthocyanidins

SYMBOL LIST

® - Registered Mark

α - Alpha

β - Beta

γ - Gamma

δ - Delta

RESUMO

SILVA, Bárbara Pereira da, M.Sc., Universidade Federal de Viçosa, Fevereiro de 2016. **Concentração de nutrientes e compostos bioativos em chia (*Salvia hispanica* L.), qualidade proteica e biodisponibilidade de ferro em ratos *Wistar*.** Orientadora: Helena Maria Pinheiro Sant'Ana. Coorientadoras: Ceres Mattos Della Lucia e Hércia Stampini Duarte Martino.

A chia (*Salvia hispanica* L.) vem sendo consumida pela população mundial devido às suas propriedades funcionais e alta concentração de ácidos graxos essenciais, fibras e proteínas. No entanto, é necessário caracterizar a concentração de nutrientes nas sementes de chia cultivadas no Brasil. Assim, o presente estudo objetivou analisar a ocorrência e concentração de macro e micronutrientes, carotenoides, flavonoides, ácido fítico e tanino em sementes de chia cultivadas em dois locais distintos, assim como avaliar a qualidade proteica e a biodisponibilidade de ferro nas sementes, uma vez que estudos nesse linha de pesquisa são escassos. Foram utilizadas sementes de chia cultivados em Catuípe, Rio Grande do Sul (RS) e Jaciara, Mato Grosso (MT). As sementes foram moídas e armazenadas em sacos de polietileno recobertos com papel alumínio em freezer até o momento das análises. Foi determinada a concentração de umidade, lipídios, proteínas, carboidratos, fibra alimentar total, cinzas totais e minerais presentes nas sementes de chia. A investigação de carotenoides (luteína e zeaxantina), vitamina C (ácido ascórbico), flavonoides (3-deoxiantocianidinas - 3-DXAs, flavonas e flavanonas) foi realizada por cromatografia líquida de alta eficiência (CLAE), com detecção por arranjo de diodos, enquanto a análise de vitamina E (α, β, γ, δ tocoferóis e tocotrienois) foi feita por CLAE, com detecção por fluorescência. A capacidade antioxidante, concentração de fenólicos totais, fitato e taninos foram realizados por espectrofotometria. Além disso, foi avaliada em ratos *Wistar*, a qualidade proteica da semente e farinha de chia por meio do coeficiente de eficiência alimentar (CEA), coeficiente de eficiência proteica (PER), razão proteica líquida (NPR) e digestibilidade verdadeira (DV) e a biodisponibilidade de ferro da farinha, utilizando o método de depleção/repleção de hemoglobina. Para análise estatística dos compostos presentes nas sementes na chia, foi utilizado o teste t para comparação de duas amostras. Para a análise da qualidade proteica, os dados obtidos foram submetidos à ANOVA. As médias dos grupos testes foram comparadas pelo teste de Duncan. No ensaio de biodisponibilidade de ferro, para avaliar se houve diferença entre os grupos experimentais quanto ao ganho de peso, CEA, ganho de

hemoglobina e marcadores moleculares, foram realizados a ANOVA e o teste de Newman Keulls. O nível de significância estabelecido para todos os testes foi 5%. Os dados foram analisados com auxílio do software estatístico *SPSS*, versão 20.0. A chia apresentou alta concentração de lipídios (31,2 g.100 g^{-1}, em média), proteínas (18,9 g.100 g^{-1}, em média) e fibra alimentar total (35,3 g.100 g^{-1}, em média). A chia cultivada no RS apresentou maior ($p \leq 0,05$) concentração de ferro, manganês, boro, chumbo, alumínio, nitrogênio e fósforo. A concentração de vitamina E total nas sementes de chia foi alta (7038,43 μg.100 g^{-1} e 7024,59 μg.100 g^{-1} para a sementes cultivada no RS e MT, respectivamente), sendo γ-tocoferol o componente principal encontrado (7031,51 ± 129,54 μg.100 g^{-1}, em média). Os carotenoides foram identificados apenas na semente cultivada no RS e a ocorrência de vitamina C e 3-DXAs não foi observada nas duas sementes. Observou-se valores semelhantes de compostos fenólicos totais e ácido fítico nas sementes de chia ($p > 0,05$). A concentração de taninos foi superior ($p \leq 0,05$) na chia cultivada no Mato Grosso (19,08 ± 1,08 eq. catequina/g amostra) em relação à cultivada no Rio Grande do Sul (14,93 ± 0,24 eq. catequina/g amostra). A chia cultivada no RS apresentou a maior ($p \leq 0,05$) atividade antioxidante (478,2 ± 0,02 μmol TEAC/g de amostra) em relação à cultivada no MT. Os valores de PER, NPR e DV não diferiram ($p > 0,05$) entre os animais alimentados com diferentes formas de tratamento da chia e foram inferiores ao grupo controle (caseína) ($p \leq 0,05$). Os animais que foram alimentados com dietas testes apresentaram concentrações de glicose, triacilglicerídeos (TGL) e lipoproteína de muito baixa densidade (VLDL) inferiores ($p \leq 0,05$) e lipoproteína de alta densidade HDL superior ($p \leq 0,05$) aos animais que consumiram caseína. O peso do fígado dos animais alimentados com chia não diferiu ($p > 0,05$) e foi inferior ($p \leq 0,05$) ao grupo controle. A espessura das camadas musculares do intestino e a profundidade das criptas foram superiores ($p \leq 0,05$) nos grupos alimentados com chia. No experimento biodisponibilidade do ferro observou-se consumo de ferro e ingestão total menores nos animais que receberam dieta rica em gordura ($p \leq 0,05$). O ganho de peso corporal, concentração de hemoglobina, aumento de hemoglobina, eficiência de regeneração da hemoglobina (HRE%) e valor biológico da eficiência de regeneração da hemoglobina (RBV-HRE) não diferiu ($p > 0,05$) entre os grupos experimentais ($p > 0,05$). O grupo alimentado com dieta padrão + chia (SD + C) apresentou menor expressão de transferrina, quando comparado com o grupo de controle (dieta padrão + sulfato ferroso) ($p \leq 0,05$). A expressão da ferritina foi inferior ($p \leq 0,05$) em todos os grupos experimentais quando comparados ao controle. A expressão do gene peroxissoma proliferador ativado por receptor α (PPAR-α) em animais alimentados com SD + C foi maior

do que no grupo de controle ($p \leq 0,05$). A expressão gênica de citocromo b duodenal (DcytB) e transportador de metal divalente (DMT-1) foi maior ($p \leq 0,05$) no grupo alimentado com dieta hiperlipídica + chia (HFD + C). No entanto, a expressão de hephaestina foi inferior ($p \leq 0,05$) em todos os grupos experimentais em comparação com o grupo controle e a expressão gênica de ferroportina foi inferior ($p \leq 0,05$) nos animais alimentados com chia. Animais alimentados com a dieta rica em gordura apresentaram biodisponibilidade do ferro da chia semelhante aos animais alimentados com uma dieta padrão. Em conclusão, a chia apresentou elevada concentração de vitamina E, ácidos graxos poli-insaturados, fibra alimentar, atividade antioxidante, ferro, cálcio, manganês e zinco. Além disso, o consumo de chia por ratos resultou em boa digestibilidade proteica, efeito hipoglicêmico e melhora no perfil lipídico, reduzindo, ainda, a deposição de gordura no fígado dos animais em um curto período de tempo (28 dias), e também promoveu alterações no tecido intestinal, aumentando a sua funcionalidade. Animais alimentados com dieta rica em gordura apresentaram biodisponibilidade do ferro da chia semelhante aos animais alimentados com dieta padrão.

ABSTRACT

SILVA, Bárbara Pereira da, M.Sc., Universidade Federal de Viçosa, February, 2016. **Concentration of nutrients and bioactive compounds in chia (*Salvia hispanica* l.), protein quality and iron bioavailability in *Wistar* rats.** Adviser: Helena Maria Pinheiro Sant'Ana. Co-Advisers: Ceres Mattos Della Lucia and Hércia Stampini Duarte Martino.

Chia (S*alvia hispanica* L.) has been consumed by the world population due to their functional properties and high concentration of essential fatty acids, dietary fiber and protein. However, it is necessary to characterize the nutrient concentration in chia seeds grown in Brazil. The present study aimed to analyze the occurrence and concentration of macro and micronutrients, carotenoids, flavonoids, phytic acid and tannin in chia seeds grown in two different places, as well as evaluating the protein quality and bioavailability of iron in the seeds, since studies in this research field are scarce. Chia seeds used were grown in Catuípe, Rio Grande do Sul (RS) and Jaciara, Mato Grosso (MT). The seeds were ground and stored in polyethylene bags covered with foil in a freezer until the moment of analysis. The concentration of moisture, lipids, proteins, carbohydrates, dietary fiber, minerals and total ash present in chia seeds was determined. The carotenoid investigation (lutein and zeaxanthin), vitamin C (ascorbic acid), flavonoids (3-deoxiantocianidins - 3-DXAs, flavones, flavanones) was performed by high-performance liquid chromatography (HPLC) with detection by diode array, while vitamin E analysis (α, β, γ, δ tocopherols and tocotrienol) was made by HPLC with fluorescence detection. The antioxidant capacity, concentration of total phenolics, phytate and tannins were carried out by spectrophotometry. Furthermore, was available in Wistar mice the protein quality of chia seed and chia flour by feed efficiency ratio (FER), protein efficiency ratio (PER), net protein ratio (NPR) and true digestibility (TD) and iron bioavailability was evaluated using the hemoglobin depletion/ repletion method in animals fed with standard diet and high fat diet. For the analysis of compounds present in chia seeds, we used the t test to compare two samples. For statistical analysis of protein quality, data were submitted to ANOVA. The average test groups were compared by Duncan test. The iron bioavailability experiment, to evaluate the differences between the groups on weight gain, CEA, hemoglobin gain and molecular analysis it were performed ANOVA and the Newman Keulls tests. The significance level for all tests was 5%. Data were analyzed using the statistical software SPSS, version 20.0. There were high lipid (31.2 g.100 g^{-1}, on average), proteins (18.9 g.100 g^{-1}, on average) and total dietary fiber (35.3 g.100 g^{-1}, on average)

concentrations in chia seeds. The chia that has grown in RS had a higher ($p<0.05$) concentration of iron, manganese, boron, lead, aluminum, nitrogen and phosphorus. The concentration of total vitamin E in the chia seeds was high (7038.43 μg.100 g^{-1} and 7024.59 μg.100 g^{-1} for the seed grown in RS and MT, respectively), being γ-tocopherol the major component found (7031.51 ± 129.54 μg.100 g^{-1}, on average). The carotenoids were identified only in the chia grown in RS and the occurrence of vitamin C and 3-DXAS was not observed in the chia seed grown in RS and MT. Similar values of total phenolic compounds and phytic acid were observed in the chia seeds ($p>0.05$). The concentration of tannins was higher ($p<0.05$) in the seed grown in Mato Grosso (19.08 ±1.08 eq. catequina/g sample) than in the seed grown in Rio Grande do Sul (14.93 ± 0.24 eq. catequina/g sample). Chia grown in RS showed the highest ($p<0.05$) antioxidant activity (478.2 ± 0.02 μmol TEAC/g sample) compared to that grown in MT. In the study of protein quality it were used chia seeds and chia flour with and without heat treatment. The values of PER, NPR and DV did not differed ($p>0.05$) among the animals that were fed with chia and were lower than the control group (casein). The animals fed with tests diets showed lower concentrations of glucose, triacylglycerides (TGL) and very low density lipoprotein (VLDL) and higher concentrations of high-density lipoprotein (HDL) ($p<0.05$) than the control group. The liver weights of animals that were fed with chia seed and chia flour did not differed ($p>0.05$) and were lower ($p<0.05$) than the control group. Crypt depth and thickness of intestinal muscle layers were higher ($p<0.05$) in groups that were fed with chia seed and chia flour. The iron bioavailability experiment of chia flour showed that the animals fed with high fat diet had similar iron bioavailability of chia compared to animals fed with standard diet. The total consumption and iron intake were lower in animals which received high fat diet ($p<0.05$). Body weight gain, hemoglobin concentrations, hemoglobin gain, hemoglobin regeneration efficiency (HRE%) and biological value of hemoglobin regeneration efficiency (RBV-HRE) did not differed among the experimental groups ($p>0.05$). The standard diet + chia (SD + C) group showed lower expression of transferrin when compared to the control group (standard diet + ferrous sulfate) ($p<0.05$). Ferritin expression was lower ($p<0.05$) in all experimental groups when compared to the control. The peroxisome proliferator-activated receptor-α (PPAR-α) gene expression in animals fed with SD + C was higher than in the control group ($p\leq0.05$). The mRNA expression of duodenal cytochrome b (DcytB) and divalent metal transporter (DMT-1) was higher ($p<0.05$) in the high fat diet + chia (HFD + C) group. However, hephaestin expression was lower ($p<0.05$) in all experimental groups compared to the control group and

the gene expression of ferroportin was lower ($p<0.05$) in the groups fed with chia flour. In conclusion, chia exhibits high concentration of vitamin E, polyunsaturated fatty acids, dietary fiber, antioxidant activity, iron, calcium, manganese and zinc. Moreover, the consumption of chia showed good protein digestibility, hypoglycemic effect, and improved the lipid profile, reduced fat deposition in the liver of animals in a short period of time (28 days), and also promoted alterations in the intestinal tissue, which increased its functionality. Animals fed with high fat diet showed similar iron bioavailability of chia compared to animals fed with standard diet.

1. GENERAL INTRODUCTION

The genus Salvia, native from southern Mexico and northern Guatemala, includes approximately 900 species of *Lamiaceae* seeds (IXTAINA; NOLASCO; TOMÁS, 2008; LU; FOO, 2002). From these species, 47 are endemic to Brazil and 61 are grown in the northeast (Bahia), central west (Goiás, Mato Grosso and Mato Grosso do Sul), southeast (Espírito Santo, Minas Gerais, Rio de Janeiro and São Paulo) and south (Paraná, Rio Grande do Sul and Santa Catarina) of the country.

Chia (*Salvia hispanica* L.) is a pseudo cereal that has been consumed by the world population due to their protective, functional and antioxidant effects (AYERZA; COATES, 2011; GALLI; MARANGONI, 2006), attributed to the presence of lipids, dietary fiber, protein, phenolic compounds, vitamins and minerals (CVETKOVIKJ *et al.*, 2013; CAPITANI *et al.*, 2012; MOHD ALI *et al.*, 2012; AYERZA; COATES, 2011). It is known that chia has hypoglycemic (VUKSAN *et al.*, 2010), anti-inflammatory (HOSSEINZADEH; MOTAMEDSHARIATY; ZIAEE, 2009), antioxidant (TOSCO, 2004), anti-hypertensive (TOSCANO *et al.*, 2014) and cardio protective (AYERZA; COATES, 2005) actions.

The chemical composition and nutritional value of chia seed varies according to species, climate conditions, the growing site and soil (AYERZA; COATES, 2009), and the energy value of the seed corresponding to 486 kcal / 100 g (USDA, 2015).

Some studies have examined the occurrence and concentration of bioactive compounds in Mexican, Argentinian and Chilean chia seeds (BORNEO; AGUIRRE; LEÓN, 2010; AYERZA, RICARDO; COATES, 2009; REYES-CAUDILLO; TECANTE; VALDIVIA-LÓPEZ, 2008). However, one study (FERREIRA, 2013) evaluated the presence of bioactive compounds such as carotenoids, vitamin E, vitamin C, flavonoids (flavones, flavanones and anthocyanins) and bioactive compounds in Brazilian seeds.

Moreover, there are no studies that evaluated the digestibility and protein quality of chia seeds and the iron bioavailability of this food *in vivo*. Therefore, it is necessary to evaluate the nutrients and bioactive compounds in chia seeds grown in Brazil, as well as to evaluate the protein quality and the bioavailability of iron *in vivo* in these seeds.

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2. LITERATURE REVIEW

2.1. Nutritional value of chia and the functional effects of chia consumption

The chemical composition and the nutritional value of the chia seeds vary according to the species, climatic condition, growing site and type of soil (AYERZA; COATES, 2009). The energetic value of the seed corresponds to 486 kcal/100g (USDA, 2015) and its main components are lipids and dietary fibers, although it is also highlighted in protein and bioactive compounds as phenolics, carotenoids and vitamins.

The concentration of lipids in the chia seeds vary from 30 to 38%, from which about 70% correspond to linoleic acid (w-3) and 19% to linoleic acid (w-6) (FERREIRA, 2013; BHATTY, 1993). Chia is source of dietary fiber (about 30%), mainly of the insoluble type, which correspond to approximately 95% of the total fiber concentration (FERREIRA, 2013; VÁZQUEZ-OVANDO *et al.*, 2009; MONROY-TORRES *et al.*, 2008; CAHILL, 2003). Moreover, the seed has high protein concentration (18 to 25%) (AYERZA; COATES, 2004).

Regarding the micronutrients concentration, chia is rich in vitamin E and complex B vitamins, such as riboflavin (0.17mg/100g), niacin (8.82mg/100g) ad thiamine (0.62mg/100g). In addition, chia has high calcium concentration (631mg/100g), phosphorus (860mg/100g), potassium (407mg/100g), magnesium (335 mg/100g), iron (7.72 mg/100g) and zinc (4.58 mg/100g) (USDA, 2015).

The regular ingestion of chia seed can accelerate the intestinal movement due to the quantity of insoluble fibers, which increases the volume of the faecal mass and provides satiety, preventing the obesity and colon cancer in humans. Furthermore, the presence of insoluble fiber is related to the control of diabetes (YUAN *et al.*, 2014; CAPITANI *et al.*, 2012; VÁZQUEZ-OVANDO *et al.*, 2009).

2.2. Protein quality

The proteins are the main structural compounds in all the cells in the human body and are important to the construction and maintenance of tissues, enzyme formation, hormones, antibodies, in the regulation of metabolic processes, besides providing energy (MAIHARA *et al.*, 2006).

Proteins provide amino acids that are classified in three categories: the essentials (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and

valine) that should be consumed through the ingestion of food; the non-essentials, that are supplied by the organism in normal physiological conditions (cysteine, tyrosine, glycine, arginine, glutamine, proline, serine) and the conditionally essentials, for which the food ingestion is essential in certain stages of life such as the growth phase, pregnancy or in specific physiological conditions (pathologies or compromised immunological condition (KIRBY; DANNER, 2009).

Although chia stands out regarding its polyunsaturated fatty acids concentration and profile and dietary fiber, the amino acids profile present in chia does not has limiting factor in relation to adults diet (BUSHWAY; BELYEA; BUSHWAY, 1981). The same is not observed in children, once the limiting amino acids are threonine, lysine and leucine (WEBER; GENTRY; KOHLHEPP, 1991). Chia protein digestibility related to the percentage of this nutrient that is hydrolyzed by the digestive enzymes and absorbed by the organism in form of amino acids or any other nitrogen form (PIRES *et al*., 2006). The protein nutritional quality varies according to its source, once the vegetables has low biological value, mainly because they are poor in some essential amino acids (BOAVENTURA *et al.*, 2000).

Studies *in vivo* that analyzed chia protein quality are scarce. Thus, it is of major interest to evaluate the protein quality of this food because it is a relevant aspect for human nutrition.

2.3. Iron bioavailability

Iron is the fourth more abundant element in nature, constituting about 4.7% of earth surface. Despite this abundance, the iron deficiency is the most prevalent nutritional deficiency in the world, reaching more than 2 billion people. The iron deficiency anemia is highly prevalent in underdeveloped countries, mas also continues to be a problem in developed countries, where other malnutrition forms have been already eradicated (SILVA; MURA, 2011; STOLTZFUS; DREYFUSS, 2002).

This mineral has many functions such as: participation in protein formation, including hemoglobin and myoglobin, participation in the process of regulation and differentiation of growth cells, neurological functioning and myelin formation (ANGULO-KINZLER *et al.*, 2002; EVSTATIEV; GASCHE, 2010).

The term 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; JACKSON, 1997). 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).

There are two types of iron in the diet: the non-heme iron that is present in foods of plant and animal origin and the heme iron, found in foods of animal origin. The non-heme iron is the main form present in the diet, mas its bioavailability depends on the presence of other dietary factors that act increasing or inhibiting its absorption in the duodenum (HURRELL; EGLI, 2010).

The non-heme iron is found in the form of Fe^{+3} (ferric). For it to be absorbed, it has to be reduced to the ferrous form (Fe^{+2}), through the action of duodenal β-cytochrome (Dcytb) that is located in the apical membrane of the enterocytes or by the presence of exogenous dietary reductive agents, such as the ascorbic acid. The reduced iron is carried to the interior of the enterocyte through the divalent metal transporter-1 (DMT1) (ANDREWS, 2002; GROTTO, 2008; GUNSHIN *et al.*, 1997).

Reaching the interior of the cell, the iron is release from the protoporphyrin by the heme oxygenase, and after being released, will be part of the same non-heme iron pool, being stored in the form of ferritin or released from the enterocyte to the blood through the ferroportin. The ferroportin is an iron exporter that is located in the basolateral membrane of the cells (ABBOUD; HAILE, 2000). The iron in its ferrous form is re-oxidized by the ferroxidase hephaestin to the ferric form, thus being transported in the bloodstream to the liver, spleen and medule by the transferrin. The iron is stored in the form of ferritin and hemosiderin, and the last one corresponds to the degraded ferritin (GROTTO, 2008; VULPE *et al.*, 1999).

The iron homeostasis occurs due to the presence of two key mechanisms. The first one if the intracellular mechanism that controls the input and output of iron according to the quantity of this mineral that the cell has. To avoid the excess of free iron or the lack of it inside the cell, iron regulatory proteins (IRP1 and IRP2) control the post-transcriptional expression of the capture and storage modulatory genes (GROTTO, 2008).

On the other hand, the systemic mechanism has as its essential hormone the hepcidin that control the use and storage of iron according to the ingestion of this mineral.

Hepcidin is synthetized by the liver in the form of 84 amino acid (aa) pro-peptide and subsequently processed and secreted in the bloodstream (GANZ, 2006; GROTTO, 2008).

Ferroportin is the receptor of Hepcidin and the interaction Hepcidin-ferroportin controls the iron levels in the enterocytes, hepatocytes and macrophages. The Hepcidin-ferroportin complex is internalized in the domains of the basolateral membrane of macrophages and the ferroportin is degraded, blocking the release of iron for these cells (NEMETH *et al.*, 2004). The reduction of iron flow to the plasma results in a low saturation of transferrin and less iron is release to the development of the erythroblast (GROTTO, 2008).

Studies that assess the bioavailability of iron have used the expression of mRNA and DMT-1, Dcytb, ferroportin and hephaestin to verify the iron absorption by the organism, thus allowing a better understanding of the mechanisms related to the homeostasis of this mineral (GANZ, 2006; NICOLAS *et al.*, 2002; SONNWEBER, 2011; TAKO; BLAIR; GLAHN, 2011; TAKO *et al.*, 2013).

There are no study that assessed the iron bioavailability in the chia seeds, thus being important to determinate it in order to contribute with data regarding the benefits of this food to human health.

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3. OBJECTIVES

3.1. General Objective

To compare chia seeds (*Salvia hispanica* L.) grown in two diferents places in Brazil regarding the occurrence and the concentration of macro and micronutrients, as well as to determine its protein quality and iron bioavailability in *Wistar* rats.

3.2. Specific Objectives

3.2.1. To evaluate the chemical composition (moisture, lipids, carbohydrates, dietary fiber, ash and protein) in chia seeds;

3.2.2. To determine the occurrence and concentration of minerals (iron, zinc, calcium, magnesium, manganese, copper, boron, lead, cadmium, chromium, sodium, potassium, sulfur, aluminum and nickel) in chia seeds;

3.2.3. To determine the occurrence and concentration of carotenoids (lutein and zeaxanthin) in chia seeds;

3.2.4. To analyze the occurrence and concentration of vitamin E (α, β, γ, δ tocopherols and tocotrienols) and vitamin C (ascorbic acid-AA) in chia seeds;

3.2.5. To determine the occurrence and concentration of flavonoids: flavones (luteolin, apigenin), flavanones (eriodictyol, naringenin), 3-deoxyantocianidins (luteolinidina, apigeninidina, 7-methoxy-apigeninidina and 5-methoxy-luteolinidina); total phenolic compounds; antioxidant activity; tannins and phytate in chia seeds;

3.2.6. To assess the protein quality of untreated and thermally treated chia seed and flour, as well as its action in the homeostasis of lipid and glucose and in the hepatic and intestinal integrity in *Wistar* rats for 28 days;

3.2.7. To assess the iron bioavailability of Brazilian chia flour, as well as the genic expression of proteins concerned in the metabolism of iron in *Wistar* rats fed with high-fat and standard diet.

4. GENERAL METHODOLOGY

This study was divided into three stages which are: nutritional characterization and bioactive compounds of chia, evaluation of protein quality of chia seed and chia flour and evaluation of iron bioavailability in chia.

4.1. Working Sites

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

- Vitamin Analysis Laboratory (Nutrition and Health Department/UFV): analysis of vitamins, carotenoids and bioactive compounds;
- Food Analysis Laboratory (Nutrition and Health Department/UFV): analysis of macronutrients;
- Experimental Nutrition Laboratory (Nutrition and Health Department/UFV): analysis of protein quality and iron bioavailability *in vivo*;
- Soil Analysis Laboratory (Soil Department/UFV): analysis of minerals;
- Structural Biology Laboratory (General Biology Department/UFV): histological analysis;
- Lipids Laboratory (Food Department/UFV): analysis of fatty acids;
- Monogastric Nutrition Laboratory (Nutrition and Health Department/UFV): analysis lipids;
- Clinical Analysis Laboratory (Health Division/UFV): biochemical analysis.

4.2. Raw material

Chia seeds (*Salvia hispanica* L.) produced in Brazil and grown in two different locations: Catuípe, Rio Grande do Sul (RS) and Jaciara, Mato Grosso (MT) were used. The analyses were performed on individual basis for each seed. In the laboratory the seeds were ground in a knife mill with a particle size of 850 micrometers. Subsequently, the flours were packed in polyethylene bags covered with aluminum foil and stored in a freezer (-18 ± 1° C) until the time of analysis.

4.3. Analysis of the chemical composition

The determination of lipids was performed in triplicate and followed the methodology proposed by AOCS (2009), while determination of ash, protein, moisture, total dietary fiber and carbohydrates were performed in triplicate according to the methodology proposed by the AOAC (2012).

4.3.1. Lipids

For the determination of lipid concentration it was used a fat extractor device (AOCS, 2009). It was weighed 1g of sample and it was wrapped in filter bags. The bags were sealed in specific sealer and placed in incubator (New Ética® 400/6ND, Brazil) for drying for 3 hours at 105° C. Then the bags were placed in the extraction equipment (extractor XT15, Ankom). The extraction occurred with filter bag sealed using petroleum ether and elevated temperature (90-100°C). At the end of the analysis, the samples were removed from the equipment, dried in an oven (New Ética® 400/6ND, Sao Paulo, Brazil) for 30 minutes at 105° C, and subsequently weighed.

The amount of lipid in the sample was calculated using the following formula:

$$\% \text{ Lipids} = \frac{100 \times (W_2 - W_3)}{W_1}$$

Where:

W_1 = Weight of the sample

W_2 = Weight of fresh sample + bag filter

W_3 = Weight of dried sample + bag filter

4.3.1.1. Derivatization of lipids and chromatographic conditions for fatty acids analysis

150 mg of sample was weighed into a 30 mL test tube (Pyrex 9826). It was added 1mL of internal standard (triacylglycerol the tridecanoic acid, 5 mg/mL chloroform), 50 mg of pyrogallic acid and 1mL of ethanol 95%. The samples were subjected to acid hydrolysis with 5 mL of HCl 8.3M and stirred in thermostatically controlled bath (Marconi, MA093, Brazil), at 75°C temperature for 40 min. After cooling at room

temperature, 12 mL of ethyl ether was added and each tube was shaken on vortex shaker for 1 min.

Then 12 mL of petroleum ether was added and again stirred. The tubes were centrifuged (centrifuge Hermle®, Z216MK model, Germany) at 2865 g for 10 min and the upper phase (ether) was transferred to another tube. The solvent was slowly evaporated in thermostatic bath (Marconi, MA093, Brazil) at temperature below 40°C using nitrogen gas. Then it was added 1 mL of boron trifluoride 7% in methanol and 0.5 mL of toluene. The tubes were well covered and placed in boiling bath for 45 min.

After cooling at room temperature 2.5 mL of water, 1 mL of hexane and 0.5 g of anhydrous Na_2SO_4 were added. After mixing, the tubes were left to stand until phase separation and then the upper phase was transferred to a vial. One microliter of the samples was analyzed by gas chromatography (GC 2010 Plus, Shimadzu Workstation GC solution) equipped with autosampler AOC-20, flame ionization detector (FID) and a capillary column of fused silica (Supelco, SP-2560, cyanopropyl polysiloxane bis 100 mm x 0.25 mm of internal diameter and 0.25 mm of thickness of stationary phase).

The column temperature programming was isothermal at 140°C for 5 min, heating at 4°C/ min to 240°C, remaining in this temperature for 20 min. The temperatures of the injector and detector were 250°C and 260°C, respectively. The fatty acid retention times were compared with the standard and the calculations were based on area and concentration of the internal standard, using the theoretical response factors of FID, of the method 1j-07 Ce AOCS.

4.3.2. Total ash

The determination of the ash concentration was held at 550° C temperature. Porcelain crucibles in preheated oven at 550° C were weighed in analytical balance. Then, 5 g of the sample was added to sample crucibles. A slow carbonization of the sample was held on a hot plate, to exhalation of the vapor, increasing the temperature by 50° C every 5 min intervals until reaching 300° C, being kept at this temperature for 30 min. The samples were brought into oven at 550° C until obtaining constant weight and clear or slightly gray ash.

The samples were cooled in a desiccator containing silica gel at room temperature for 30 min for later weighing in an analytical balance (Gehaka, BG2000, Brazil). The ash concentration was obtained by the following formula:

$$\text{Concentration of ash (\%)} = \frac{(\text{crucible weight + ash}) - \text{crucible weight}}{(\text{crucible weight + sample}) - \text{crucible weight}} \times 100$$

4.3.3. Proteins

The protein determination was carried out using the digestion, distillation and titration equipment of Kjeldahl. In analytical balance (Gehaka, BG2000, Brazil) 0.5 g of pentahydrate copper sulfate ($CuSO_4.5H_2O$), 1 g of chia and 10 g of sodium sulfate (Na_2SO_4) were weighed.

All materials were transferred for digestion tubes, and in the exhaust hood, 20 mL of sulfuric acid (H_2SO_4) concentrate was added. After sealed, the tubes were taken to the digester block. The digestion and exhaustion systems were activated, increasing the temperature every 10min by 50° up to 350°C.

After digestion, the tube containing the sample was brought to the distillation apparatus. In this step, 50 mL of sodium hydroxide (NaOH) solution 50% was slowly added in the machine hopper, until the mixture shows a black coloration.

In an Erlenmeyer flask (250 mL), 50 mL of boric acid (H_3BO_3) was added. The distillation was performed until obtaining 75 mL of blue dye solution. The solution obtained during distillation was titrated with sulfuric acid (H_2SO_4) 0.05 mol/L until the turning point of a phenolphthalein indicator.

The factor 6.25 was used in the calculation of the conversion of nitrogen to proteins, according to the following formula:

$$\text{\% Protein} = \text{\% Nitrogen} \times 6.25$$

4.3.4. Moisture

The moisture of chia was determined by gravimetric technique with heat employment in an oven with forced air circulation at 105°C (New Ética®, 400/6ND, Brazil). 10 g of sample was weighed in petri dish previously dried in an oven,. The plates

containing the samples were subjected to the heat from the oven, until obtaining of constant weight, using an analytical balance (Gehaka, BG2000, Brazil). Moisture was obtained from the following formula:

$$\text{Total solids (\%)} = \frac{\text{(Plate weight + dry sample) – Plate weight x 100}}{\text{(Plate weight + wet sample) – Plate weight}}$$

$$\text{Moisture (\%)} = 100 - \text{total solids}$$

4.3.5. Total dietary fiber analysis

The total dietary fiber analysis was conducted in 5 replicates, by gravimetric enzymatic method (AOAC, 2012). The crucibles used in this analysis were left soak overnight in a neutral detergent (concentration 20%) and then washed with distilled water until removal of all the soap. Then, the crucibles were oven dried (New Ética® 400/6ND, Brazil) and placed in an oven at 525°C for 5 hours.

After this step, the crucibles were washed 5 times with 20 mL of 0.5N HCl in the normal direction, neutralizing 2 times with 10 mL of distilled water in the same direction and 3 times with 20 mL of distilled water in the opposite direction. The crucibles were oven dried at 105°C (New Ética® 400/6ND, Brazil) for 12 hours, cooled and weighed to obtain the tare zero. Then, in each crucible was weighed 1 g of celite and these were placed in an oven at 105ºC (New Ética® 400/6ND, Brazil) overnight to obtain the tara1.

After this step the enzymatic hydrolysis of samples was made as follows: 4 beakers (600 mL) were prepared for each sample, and therein 0.5 g of sample weighed. 50 mL of phosphate buffer (pH 6.0) was added and the pH of the samples was adjusted to 6.0. Subsequently, it was added 0.1 mL of thermoresistant alpha-amylase and the beakers were placed in a water bath (97ºC) for 30 min. After this period, the beakers were cooled, adjusted the pH to 7.5 and added to 0.2 mL of protease, then incubating them at 60°C in a water bath for 30 min with constant agitation. After this process, the beakers were again cooled, the pH adjusted to 4.3 and it was added 0.1 mL of amyloglucosidase, placing them in a water bath at 60°C for 30 min.

The insoluble dietary fiber was determined as follows: the crucibles prepared were coupled to the vacuum pump and the contents of the beakers poured therein, and washed with 30 mL of distilled water. The content was transferred back to the beaker and 320 mL

of 98% ethanol heated to 60°C was added. This fraction corresponds to the soluble fiber crucible and the residue to the insoluble fiber. The crucibles were washed twice with 20 mL of 95% ethanol and twice with 20 mL of acetone and placed in an oven at 105ºC (New Ética® 400/6ND, Brazil) overnight for subsequent weighing.

The residue in the crucible was washed 2 times with 20 mL of 78% ethanol (v/v), 1 time with 10 mL of 95% ethanol and 1 time with 10 mL of acetone, and placed in an oven at 105ºC (New Ética® 400/6ND, Brazil) overnight, and cooled for 2 hours in a desiccator. Subsequently, data were analyzed for total dietary fiber determination using the following equation:

Total dietary fiber = 100 x (Wr-(P+A)/100 x Wr)/Ws;

Where:
Wr = mg residue;
P = % protein in the residue;
A = % ash in the residue;
Ws = mg sample.

4.3.6. Calculation of carbohydrates

The carbohydrates were calculated by difference using the following formula:

Carbohydrate = 100 - (% moisture + % ether extract + % protein fraction + % fraction dietary fiber + % ash).

4.3.7. Calculation of total energy

To calculate the energy value or total energy intake it were used the conversion factors of 9 kcal/g for lipids, 4 kcal/g for protein and 4 kcal/g for carbohydrates (MERRIL, WATT, 1955).

4.4. Determination of mineral composition

It was determined in triplicate the concentration of: iron (Fe), zinc (Zn), calcium (Ca), magnesium (Mg), manganese (Mn), copper (Cu), boron (B), lead (Pb), cadmium

(Cd), chromium (Cr), sodium (Na), potassium (K), sulfur (S), aluminum (Al) and nitrogen (N) in chia seed. All material used in the analysis was previously demineralized with nitric acid solution 10% for 24 hours and washed with deionized water (3 times).

The digestion and analysis of the minerals was performed according to Gomes *et al.* (2003). 2 g of chia flour was weighed on an analytical balance (Gehaka, BG2000, Brazil) in greaseproof paper and transferred to a digestion tube. Then, 10 mL of nitric acid at room temperature was added to the tube. The tubes were held in a digester block at 150° C for 16 hours. When necessary, 5 mL of nitric acid was added to the tube.

After digestion, the tubes were cooled at room temperature. Then the samples were transferred to a 50 mL volumetric flask, and the tubes were rinsed with deionized water and stirred by vortexing for 3 times. For analysis, the extracts were diluted appropriately using deionized water. In the solutions which were used for the determination of calcium and magnesium were added 5 mL of strontium chloride hexahydrate solution in order to avoid underestimation.

The minerals were determined by atomic emission spectrometry in inductively coupled plasma (ICP-AES) (Perkin Elmer Optima 8300), with inductively coupled argon plasma source, and with the following conditions: power of 1300 W, cooling air flow of 15 L/min, auxiliary air flow of 0.7 L/min, air flow charger of 0.5 L/min, sample introduction rate of 1.5 mL/min and nebulizer Perkin Elmer.

4.5. Extraction and analysis of carotenoids, vitamins and flavonoids

Extraction and analysis of carotenoids, vitamin E, vitamin C and flavonoids in chia seed were determined in five replicates. During the extraction and analysis, samples and extracts were protected from sunlight and artificial light using amber glassware, aluminum foil and blackout curtains, and protected from oxygen using hermetically sealed containers and nitrogen gas environment.

4.5.1. Carotenoids

The occurrence and concentration of carotenoids (lutein and zeaxanthin) were investigated. Carotenoids were extracted in acetone and transferred to petroleum ether according to Rodriguez-Amaya (1996), with modifications proposed by Cardoso *et al.* (2014). 5 g of chia flour was added of 20 mL of acetone chilled and homogenized in a

micro grinder for 5 minutes. The resulting suspension was vacuum filtered on a Buchner funnel and the residue remaining in the extraction tube. Then, the extraction was repeated with subsequent homogenization and vacuum filtration.

Subsequently, the split of the carotenoids from the acetone to the petroleum ether was performed. The filtrate was transferred into two fractions to a separation funnel containing 20 mL of cold petroleum ether, being each fraction washed with distilled water for complete removal of acetone. It was added anhydrous sodium sulfate to the extract in petroleum ether to remove any residual water that may have. Then, the extract was concentrated on a rotary evaporator (Tecnal, TE 211, Brazil) and transferred to a 25 mL volumetric flask, and the volume completed with petroleum ether.

For carotenoids analyses, 25 mL of extract was evaporated under a flow of nitrogen gas, having the dry residue being resumed in 1 mL of acetone (HPLC grade). Then, the extract was filtered in filtering unities with porosity of 0.45 μm. The carotenoids were analyzed using a high efficiency liquid chromatography system (HPLC) (Shimadzu, SCL 10AT VP, Japan) equipped with high pressure pump (Shimadzu, LC-10AT VP, Japan), loop of 500 μL (Shimadzu, SIL-10AF, Japan), diode array detector (DAD) (Shimadzu, SPD-M10A, Japan). The chromatographic condition used for the analysis was as follows: chromatographic column Si 100 (Phenomenex Luna®, 250mm x 4mm), equipped with a pre-column Si 100 (C18) (Phenomenex ODS, 4mm x 3mm), mobile phase composed by hexane: isopropanol (HPLC grade, Tedia, Brazil), with the proportions of 95:5, with 1.5 $mL.min^{-1}$ flow and injection volume of 50 μL (PPINHEIRO-SANT'ANA *et al.*, 1998). The chromatograms were obtained at 450nm.

4.5.2. Vitamin E

The occurrence and concentration of the eight components of vitamin E (α, β, γ and δ tocopherols and tocotrienols) in chia seed were investigated. The extraction and analysis of the components were performed according to Pinheiro-Sant'Ana *et al.* (2011) with some modifications. 4 g of chia flour were added to 4 mL of ultrapure heated water (80 ± 1°C). After this stage, 10 mL of isopropanol, 1 mL of hexane containing 0.05% BHT, 5.0 g of anhydrous sodium sulfate and 25 mL of extraction solvent mixture (hexane: ethyl acetate, 85:15, v/v) was added. The suspension was homogenized using micro grinder for one minute and vacuum filtered on Buchner funnel using filter paper and the keeping the

residue in the extraction tube. Then, the extract was concentrated on a rotary evaporator (Tecnal, TE 211, Brazil) at 70 ± 1°C for 2 minutes, transferred to a volumetric flask and the volume was completed until 25 mL with solvent mixture.

For analysis, an aliquot of 5 mL of extract was evaporated under a nitrogen gas flow, and then taken up in 2 mL of hexane HPLC grade (Tedia, Brazil) and filtered through a membrane with porosity of 0.45 micrometer (Millipore, Brazil). The chromatographic conditions used were: HPLC system (Shimadzu Model SCL 10AT VP, Japan); fluorescence detector (Shimadzu RF10AXL) operating at 290 nm excitation and 330 nm emission); chromatographic column (Phenomenex Luna Si100 (250 x 4 mm, 5 um) coupled with a guard column (Phenomenex Si100 4 x 3 mm); mobile phase - hexane: isopropanol: glacial acetic acid (HPLC grade, Tedia, Brazil) (98.9: 0.6: 0.5 v/v/v). flow of 1 mL/min and 22 min of run time. The identification of components of vitamin E was carried out by comparing the retention time of commercial standards with those obtained for the samples analyzed under the same conditions.

4.5.3. Vitamin C

The extraction and analysis of vitamin C (as AA) was performed according to the conditions proposed by Campos *et al.* (2009) with modifications. 5 g of chia flour were homogenized for five minutes with 15 mL of extraction solution (3% metaphosphoric acid, acetic acid 8%, H_2SO_4 0.3 N and 1 mM EDTA) using a micro grinder (Marconi, MA102, Brazil). The extract was centrifuged (Fanem, Baby 206, Brazil) at 2865 g for 15 min and filtered through Buchner funnel using filter paper. The filtrate was transferred to a 25 mL volumetric flask and the volume was completed with ultrapure water.

The extracts were filtered through filter units with porosity of 0.45 μm (Millipore, Brazil). For the analysis it was used a HPLC-DAD system (Shimadzu, model SCL 10AT VP, Japan). The chromatographic conditions for AA were: chromatographic column (Phenomenex Synergi Hydro 250 x 4 mm, 4μm) coupled to guard column (Phenomenex C18, 4 mm x 3 mm), mobile phase comprising of ultrapure water containing NaH_2PO_4 1 mM, 1 mM EDTA, pH adjusted to 3.0 using H_3PO_4; 1.0 mL/min flow and injection volume of 50 μL. Chromatograms were obtained at 245 nm.

4.5.4. Determination of flavonoids

The main 3-DXAs (luteolinidin, apigeninidinm 7-methoxy-apigeninidin and 5-methoxy-luteliolinidin), flavones (luteolin and apigenin) and flavanones (naringenin and eriodictyol) were investigated in chia seed. The extraction occurred after the addition of 2 g of chia to 20 mL of methanol: HCl (99: 1, v/v) solution. The analyzes were performed on HPLC system (Shimadzu, SCL 10AT VP, Japan) equipped with DAD (Shimadzu SPD-M10A, Japan), high pressure pump (Shimadzu LC-10AT VP, Japan), loop 500 µL (Shimadzu SIL-10AF, Japan), and a degassing system with helium gas according to Yang *et al.* (2012).

The flavonoids were determined by HPLC, using the following chromatographic conditions: C-18 column (Kinetix, 150 x 4.6 mm ID, 5 µm) equipped with a C-18 guard column (Phenomenex, Torrance, CA, 4mm x 3 mm), column temperature at 35°C and injection volume of 100 µL. The mobile phase consisted of 2% formic acid in ultrapure water (line A) and 2% formic acid in acetonitrile (line B).

The elution gradient of B was: 0-3 minutes isocratic, 10%; 3-4 min, 10-12%; 4-5 min isocratic 12%; 5-8 min, 12-18%; 8-10 minutes isocratic 18%; 10-12 min, 18-19%; 12-14 min, isocratic 19%; 14-18 min, 19-21%; 18-22 min, 21-26%; 22-28 min, 26-28%; 28-32 min, 28-40%; 32-34 min, 40-60%; 34-36 min, isocratic 60%; 36-38 min, 60-10%; 38-45 min, isocratic at 10%. The mobile phase was degassed with helium gas at 50 kPa before and during the analyses, the mobile phase flow gradient was: 0-36 min, 1.0 mL/min; 36-38 min, 1.0 to 2 mL/min, 38-44 min, 2.0 mL/min; 44-45 minutes, 2.0-1.0 mL/min. The chromatograms were obtained at 480 nm, 360 nm, 280 nm for 3-deoxyantocianidins, flavones and flavanones, respectively.

4.5.5. Preparation and evaluation of the purity of carotenoids, vitamins and flavonoids standards

Solutions for each of the investigated components in chia seeds (lutein, zeaxanthin, AA, α-tocopherol, α-tocotrienol, β-tocopherol, β-tocotrienol, γ-tocopherol, γ-tocotrienol, δ-tocopherol, δ-tocotrienol, luteolinidin, apigeninidin, 7-methoxy-apigeninidin, 5-methoxy-luteliolinidin, luteolin, apigenin, naringenin and eriodictyol) were prepared.

The purity of the standards was evaluated by HPLC and quantification performed by spectrophotometry, based on the maximum absorbance according to the Lambert-Beer

law. To perform the actual quantification of the standards the following equation was used: C (μg/mL) = ABS x 10^4 / $E^{1\%}_{1cm,}$, where C = concentration; ABS = maximum absorbance; $E^{1\%}_{1cm}$ = molar absorptivity coefficient.

4.5.6. Identification and quantification of carotenoids, vitamins and flavonoids standards

The qualitative identification of the investigated compounds was performed by HPLC, by comparing the retention times obtained for the standards with the interest peaks obtained for the samples, analyzed under the same conditions. Furthermore, the carotenoids, flavonoids and AA were identified by comparing the absorption spectra of the standard and the peaks of interest in the samples, using the DAD.

For the quantification of the compounds found in chia, standard curves were used. The construction of calibration curves was performed by injection in duplicate in five increasing concentrations of the standard solutions in the range from 0.177 to 2.840 μg to lutein; 0.40 to 3.20 μg to zeaxanthin; 0.06 to 5.88 μg AA; 1.02 to 104.21 ng to α-tocopherol, 2.01 to 204.12 ng to α-tocotrienol; 0.44 to 167.89 to β-tocopherol; 0.32 to 154.32 to β-tocotrienol; 2.22 to 107.6 ng for γ-tocopherol; 3.21 to 157.6 ng for γ-tocotrienol; 0.78 to 80.62 ng to δ-tocopherol and 0.79 to 104.56 ng to δ-tocotrienol, 0.70 to 105.65 ng to luteolinidin; 0.19 to 114.08 ng to apigeninidina; 0.19 to 114.08 ng to 7-methoxy-apigeninidin; 0.70 to 105.65 ng to 5-methoxy-luteliolinidina; 0.8 to 40.0 ng for luteolin; 0.8 to 40.0 ng for apigenin; 46.4 to 0.928 ng to naringenin and 0.80 to 80.00 ng to eriodictyol. Thus, a linear correlation between the peak areas and the injected concentration of each compound was done.

The quantification of the compounds in chia was performed from standard curves constructed and from the regression equation obtained for lutein (y = 31.547,09x + 4662.43; R^2 = 0.99); zeaxanthin (y = 23.326,28x + 5644.83; R^2 = 1.00); AA (y = 2.967.843,75x - 263,100.04; R^2 = 1.00), α-tocopherol (y = 7.284.877,24x + 58673.13; R^2 = 0.98), α-tocotrienol (y = 67.799.527,73x - 2449.21; R^2 = 1.0), β-tocopherol (y = 109.337.012,29x - 96.92; R^2 = 1.00), β-tocotrienol (y = x 1,052,509.7795 + 1838.1232; R^2 = 0.99), γ-tocopherol (y = 52.176.064,10x + 5,556,148.10; R^2 = 0.98), γ-tocotrienol (y = 10,541,950.6116 + 15790.3141 ; R^2 = 0.98), δ-tocopherol (y = 49.747.176,34x + 6091.48; R^2 = 1.00), δ-tocotrienol (y = 10,541,950.6116 - 15790.3141; R^2 = 1.00) luteolinidin (y =

8141,5x - 2823.4; R^2 = 0.99), apigeninidin (y = 6345,7x + 8276.4; R^2 = 0.99); 7-methoxy-apigeninidin (y = 6345,7x + 8276.4; R^2 = 0.99) and 5-methoxy-luteliolinidin (y = 7570.4x + 8276.4; R^2 = 0.99), luteolin (y = 4.293,2779x + 7729.2457; R^2 = 0.99); apigenin (y = 6772,2x - 9650.2; R^2 = 0.99), naringenin (y = 2891,4+ 3067.6; R^2 = 0.99) and eriodictyol (1754,7936+ y = 3.9746; R^2 = 1.00). The real concentration was obtained by calculations from the dilutions or concentrations carried out.

4.6. Determination of antioxidant activity

4.6.1. Extract preparation

2g of chia was added to 20 mL of 70% acetone solution. Then, the suspension was stirred automatically (Marconi stirrer, MA093, Brazil) (80 g, 2 hours, 25°C). The suspension was centrifuged (2865 g, 15 minutes) (Fanem, Baby 206, Brazil). The supernatant was transferred to a beaker and the volume was completed to 20 mL with acetone 70%. The extract was placed in amber bottle and stored in a freezer (-18 ± 1°C) until the time of analysis.

4.6.2. Radical removal activity (DPPH)

In a test tube, properly protected from light, 100 µL of the extract obtained in the previous step was added to 1.5 mL of methanolic $DPPH^{\bullet}$ solution (1.1-diphenyl-2-picrilhidrazila) and vortexed for 30 seconds . After 30 minutes of rest, the absorbance of the solution was read in a spectrophotometer (Thermoscientific, Evolution 606, USA) at 517 nm.

The calibration curve was constructed using 50-100 µmol/L of trolox solution. The antiradical activity (AAR) was expressed in µmol trolox equivalent/g of sample (µmol trolox/g) (BLOOR 2001).

4.7. Determination of total phenolics compounds

The total phenolic compounds in the flour were determined using the Folin-Ciocalteu method (SINGLETON, ORTHOFER; LAMUELA-RAVENTOS, 1999). For analysis, 500 µL of prepared extract for the determination of antioxidant capacity was added to 500 µL of Folin-Ciocalteu 20% solution and 500 µL of sodium carbonate

solution 7.5%. Then, the solution was vortexed and left resting for 30 minutes at room temperature (25°C).

The reading of the absorbance was performed on a spectrophotometer (Thermo Scientific Evolution 606, USA) at 765 nm. The quantitation was performed using the analytical curve obtained by reading the absorbance of solutions with different concentrations of Gallic acid. The results were expressed in mg of equivalent Gallic acid per gram of sample (mg EqAG/g).

4.8. Tannin analysis

The tannin determination was performed by the vanillin/HCl reaction (BURNS, 1971) with modifications (MAXSON; ROONEY, 1972; PRICE; SCOYOC; BUTLER, 1978). 200 mg of chia was weighed and 10 mL of 1% HCl solution was added in methanol. The tubes were placed in an automatic shaker (Marconi, MA093, Brazil) (80 g, 20 minutes, 30°C) for extraction of tannin. Then, were centrifuged (Hermle®, Z216MK model, Germany) at 2865 g for 20 minutes. 1 mL aliquots of the supernatant were added to 2.5 mL of 1% vanillin solution in methanol and 2.5 mL of 8% HCl solution in methanol. The absorbance reading was performed in spectrophotometer (MultiskanGo, Thermoscientific, USA) at 500 nm.

The results were expressed as milligrams of catechin per gram of sample according to the analytical curve of catechin. For the construction of the curve, 200 mg of catechin was weighed and the volume of the volumetric flask (200 mL) was completed with methanol. Aliquots were taken from 5, 10, 20, 25 and 50 mL of concentrated solution and placed in a volumetric flask of 100 mL, adjusting the volume with the addition of methanol and the absorbance reading was performed at 500 nm.

4.9. Determination of phytates

The concentration of phytate was determined by spectrophotometry according to Latta and Eskin (1980) method with modifications (ELLIS; MORRIS, 1986). For the extraction of phytate it was weighed 0.1 g of chia flour and added 5 mL of 2.4% HCl, remaining under horizontal stirring for 12 hours at 80 g. The extract containing the phytic acid was centrifuged (Hermle®, Z216MK model, Germany) at 2865 g for 15 minutes and

the supernatant was vacuum filtered in büchnner funnel and purified using ion-exchange column with the stationary phase made of resin Dowex- AGX-4.

The column was preconditioned with NaCl 2 M and the extract obtained from the preceding steps was carefully applied to the same. Inorganic phosphors were eluted with NaCl 0.05 M, followed by elution of phytate retained with NaCl 2 M. The concentration of phytate was determined calorimetrically at 500 nm. It was prepared an analytical curve of phytic acid (Sigma®), at concentrations of 10 to 100 $\mu L/mL^{-1}$, to express the concentration of the phytate in mg of phytic acid/g of chia.

4.10. Evaluation of protein quality of chia seed and chia flour

4.10.1. Raw materials and preparation of flours

Chia seeds (*Salvia hispanica* L.) grown in the state of Rio Grande do Sul (Brazil), and four treatments were used, namely: *in natura* chia seed, chia seed with heat treatment, *in natura* chia flour and chia flour with heat treatment.

The study was conducted using chia grown in Rio Grande do Sul because, through previous studies, this seed had lower concentration of tannins. The thermal treatment was applied to chia seeds and chia flour to check if the heat is capable of inactivating some anti-nutritional factors such as phytate and tannin which complex with the protein, reducing the protein quality of the food.

To obtain the flours, the seeds were grounded using a knife mill (Grinder Vertical Rotor MA 090 CFT, Marconi, Brazil) with a particle size of 850 μm.. For the thermic treatment, the chia seed and flours were exposed to 90°C temperature in an oven with air circulation (New Etica®, model 400/6ND, Brazil) for 20 minutes. Subsequently, seeds and flours were packed in polyethylene bags covered with foil and stored in a freezer (-18 ± 1°C) until the time of analysis.

4.10.2. Analysis of the chemical composition

The chemical composition analysis was performed as described in item 3.3.

4.10.3. Determination of phenolic compounds and phytates

The determination of total phenolic compounds and phytate was performed as described in item 3.7 and 3.9, respectively.

4.10.4. Evaluation of protein quality

The experimental design of the study, including chia seed or chia flour treated or not thermally, was based on *in vitro* studies with modifications (MONROY-TORRES *et al.*, 2008; SANDOVAL-OLIVEROS; PAREDES-LÓPEZ, 2013).

4.10.4.1. Determination of true digestibility (TD)

To determine the digestibility, the diets were labeled with indigo carmine in the proportion of 200 mg/100 g of diet and animal feces were collected from 9^{th} to 13^{th} days and maintained in individual containers under refrigeration. At the end of the experiment, feces were dried in an oven with air circulation (Med Clave, 4, Brazil) at 105°C for 24 hours. Then, the feces were weighed and crushed in multiprocessor (Arno©) to determine the concentration of nitrogen by the semi-micro Kjeldahl method, with samples in triplicate (AOAC, 2012)

The true digestibility was calculated from the amount of nitrogen consumed in the diet (I) and the amount excreted in the feces of the animals of test groups (F) and the fecal loss of nitrogen by the animals that received protein free diet (Fk) (BENDER ; DOELL, 1957).

4.10.4.2. Determination of protein efficiency ratio (PER) and net protein ratio (NPR)

The PER, coefficient which relates the weight gain of animals (g) with the protein intake (g), was determined using the equation proposed by Hegsted (1977). The NPR was determined by the equation proposed by Bender; Doell (1957), taking into account the weight gain of animals (g) from the test group and the weight loss of the animals (g) fed with a free protein diet in relation to the protein intake (g) of the test group.

4.10.5. Biological assay

4.10.5.1. Experimental design

The positive control group received casein diet as protein source and the negative control group received diet without protein. The test groups received chia seed or chia flour, with and without heat treatment, depending on which group they belonged to. So, we used 6 groups, namely:

1- Positive control (casein);

2- Negative control (aproteic);

3- Chia seed grown in Rio Grande do Sul;

4- Chia seed grown in Rio Grande do Sul with heat treatment;

5- Chia flour grown in Rio Grande do Sul;

6- Chia flour grown in Rio Grande do Sul with heat treatment.

4.10.5.2. Experimental animals

Thirty-six male rats (*Rattus norvegicus*, *Wistar*, *albinus* variation), weanling, with 21 days of life were systematically divided into 6 groups with 6 animals each, being each animal considered a repeat, so that the mean difference weights between the groups did not exceed 6 grams. The animals were distributed in individual metabolic cages of stainless steel in controlled temperature environment (21 ± 1°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: positive control (casein); negative control (aproteic); chia seed untreated; chia seed heat treated (90°C for 20 minutes); chia flour untreated and chia flour with heat treatment (90°C for 20 minutes). The heat treatment was applied using an oven with forced air circulation (New Ética®, 400/6ND, Brazil).

After 28 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 2865 g for 10 minutes to separate the plasma. In addition, fragments of liver and cecum were fixed in formaldehyde 10% and kept at room temperature for subsequent histological analysis.

The study was submitted and approved by the Ethics Committee on Animal Research of the Federal University of Viçosa, Brazil (Protocol 97/2014) (Appendix I).

4.10.5.3. Experimental diets

The composition of the diets were based on the AIN-93G diet (REEVES *et al.*, 1993), but with protein concentration around 9.5%. It was prepared six different diets: casein diet (standard), aproteic diet and four diets-tests, whose protein source was chia.

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°C ± 1°C). The protein concentration of each diet was determined by semi-micro Kjeldahl method, using factor of 6.25 to obtain protein concentration (Table 1).

Table 1. Composition of diet AIN 93-G (g Kg^{-1})

	Experimental Groups					
Ingredients (g Kg^{-1})	Casein	Aproteic	Chia seed untreated	Chia seed with HT	Chia flour untreated	Chia flour with HT
Casein	117.2	-	-	-	-	-
Chia seed untreated	-	-	522	-	-	-
Chia seed with HT	-	-	-	522	-	-
Chia flour untreated	-	-	-	-	522	-
Chia flour with HT	-	-	-	-	-	522
Dextrinized Starch	132	132	132	132	132	132
Sucrose	100	100	100	100	100	100
Soybean oil	168.08	168.08	-	-	-	-
Cellulose	154.5	154.5	-	-	-	-
Mineral Mix	35	35	35	35	35	35
Vitamin Mix	10	10	10	10	10	10
L-cystine	3	3	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
Starch	277.72	394.92	195.5	195.5	195.5	195.5
Protein (%)	9.38*	-	9.48	9.48	9.48	9.48
Caloric density (kcal g^{-1})	4.08	4.17	3.93	3.93	3.93	3.93

HT: Heat treatment; * Considering that casein has 80% purity

4.10.5.4. Biochemical analysis

The concentration of plasma glucose, total cholesterol, high density lipoprotein (HDL-cholesterol), low density lipoprotein (LDL-cholesterol), lipoprotein very low density lipoproteins (VLDL-cholesterol), triacylglycerides (TGL), uric acid, creatinine, glutamic oxaloacetic transaminase (AST) and glutamic pyruvic transaminase (ALT) were evaluated by colorimetric methods according to the manufacturer's instructions (Bioclin®) using the Cobas Mira Plus (Roche Diagnostic Systems).

4.10.5.5. Histological analysis

The fragments of liver and cecum, previously fixed in formalin 10% solution, were dehydrated in increasing concentrations of ethanol and, then, immersed in resin (Historesin, Leica) for 24 hours. After this period, the fragments were immersed in pure resin of infiltration for 2 hours for subsequent embedment. This process consisted of immersing the fragments into a pure resin solution (93.17%), added by hardener (6.83%). The material was stored in an oven at 60ºC for 48 hours.

Semi-serial histological sections with 3 mm of thickness were obtained in automatic microtome (Leica RM 2255), using a glass knife, stained by toluidine blue technique. The slides were mounted with Entellan® (Merck) and analyzed in light microscope Olympus CX31. The images were obtained with digital camera SC 020 through Analysis GETIT software, Olympus. The vesicles of fat from the likely areas of hepatic steatosis were accounted for by computational quantification using the software Image Pro-Plus® version 4.5 (Media Cybernetics InC, USA). The overlay of a standard square matrix (336 points) in the photographed field was standardized, being recorded only the fat globules coincident with the intersections (Figure 1).

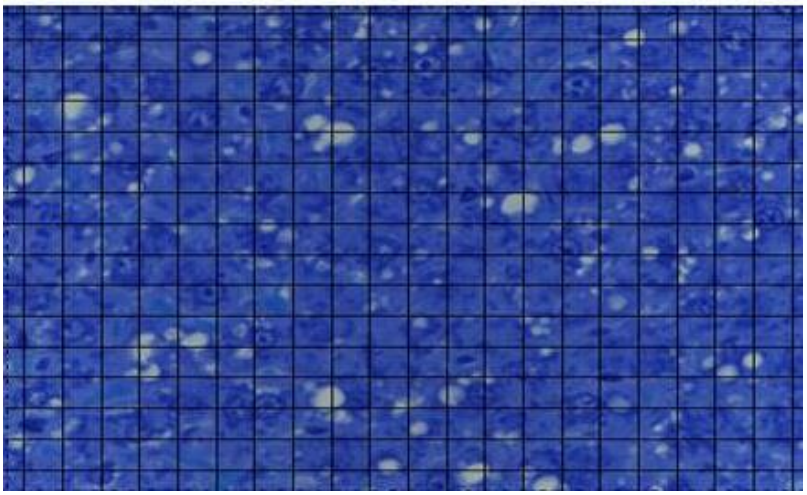

Figure 1: Photomicrography of the histological section of the *Wistar* rat livers, dyed with toluidine blue and overlaid with the standard square matrix (366 points) used for counting the fatty globules.

To measure crypt depth and the thickness of the inner and outer muscle layers it was selected twenty random fields per animal. 120 units of each item were measured per experimental group. Only crypts with rectilinear aspect were used (CAVALIERE, 2013). The images of the intestinal histologic sections were captured with 10X objective and those from the liver with a 40X objective and the measurements were taken with the support of ImagePro-Plus® version 4.5.

4.11. Iron bioavailability evaluation in chia flour

4.11.1 Biological assay

32 male *Wistar* rats (*Rattus norvegicus*, *albinus* variety, *Rodentia* order), weanling, with 21 days old, from the Central Biotery of the Center of Biological Sciences and Health of Federal University of Viçosa, with initial weight between 50 and 60 g were used.

The animals were kept in individual stainless steel cages in an environment with controlled temperature (21 ± 1°C) and light-dark cycle of 12 hours. The method used to access the bioavailability was the hemoglobin depletion/repletion, according to the technique of the AOAC (1998), with modifications.

The iron bioavailability was assessed by bioassay with chia flour, where the control group received ferrous sulfate (standard diet + ferrous sulfate) and the test groups received chia flour as a source of iron and high fat diet (standard diet + chia, high fat diet + ferrous sulfate and high fat diet + chia).

It was used the chia seed grown in Rio Grande do Sul, because it showed higher iron concentration than the chia seed grown in Mato Grosso. Furthermore, the high fat diet was used to verify if increased levels of inflammatory markers could be associated with decreased iron bioavailability. Studies show that the increase of hepcidin is associated to a reduction in iron absorption, since this hormone is capable of cleaving the ferroportin and decrease the expression of duodenal cytochrome B and DMT-1, reducing the bioavailability of this mineral (NICOLAS *et al.*, 2002; GANZ, 2006; DE DOMENICO; WARD; KAPLAN, 2007; MENA *et al.*, 2008; SONNWEBER *et al*, 2011).

Therefore, the study was composed by four experimental groups of 8 animals each, as the following design:

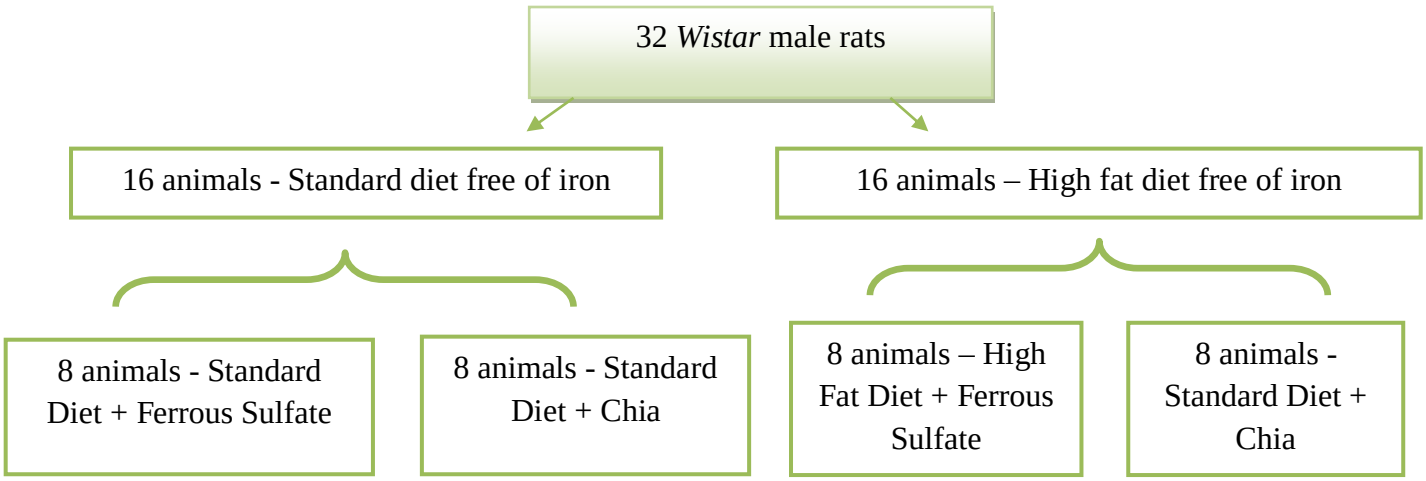

Figure 2: Experimental design of the iron bioavailability study.

4.11.2. Preparation of diets

The standard diets were prepared according to the AIN-93G diet (REEVES *et al.*, 1993) adapted, indicated for animals in the growing stage, comprised of 22% of protein, 15% of lipid and 63% of carbohydrate. The high fat diet was prepared in a proportion of 61% of lipids, 21% of protein and 18% of carbohydrate (Research Diets, New Brunswick, NJ) (Table 2). Albumin was used as a protein source, replacing casein, due to lower concentration of contaminant iron. The ingredients were weighed individually in semi-analytical balance and manually mixed in plastic containers previously washed and rinsed with deionized water. The ingredients were mixed in an industrial mixer (Leme) for 15 minutes.

Table 2. Food and nutritional composition of experimental diets

	Depletion Phase		**Repletion Phase**			
Ingredients (1kg of diet)	**Standard diet without iron**	**High fat diet without iron**	**SD + FS**	**SD + C**	**HFD + FS**	**HFD + C**
Ferrous sulfate (mg)	-	-	59.73	-	59.73	-
Chia (g)	-	-	-	127.80	-	127.80
Albumin (g)	218.22	280.00	218.22	188,36	280.00	250.18
Dextrinized starch (g)	132.00	132.00	132.00	132.00	132.00	132.00
Sucrose (g)	100.00	100.00	100.00	100.00	100.00	100.00
Soybean oil (mL)	70.00	70.00	70.00	28.91	70.00	28.91
Lard (g)	0.00	300.00	00.00	0.00	300.00	300.00
Microcrystalline cellulose (g)	50.00	50.00	50.00	7.36	50.00	7.36
Mineral mix without iron (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)	3.00	3.00	3.00	3.00	3.00	3.00
Choline bitartrate (g)	2.50	2.50	2.50	2.50	2.50	2.50
BHT (g)	0.008	0.008	0.008	0.008	0.008	0.008
Corn starch (g)	379.27	17.49	379.09	365.09	17.43	3.23
Nutritional composition						
Total calories (Kcal)	3847.88	5447.96	4017.80	3864.89	5447.72	5364,67
Caloric density (Kcal/g)	4.15	5.65	4.20	4.17	5.65	5.58
Iron (mg/Kg)*	0.30±0,02[b]	0.28±0,04[b]	18.37±0.25[a]	19.17±0.49[a]	18.63±0.55[a]	18.30±0.53[a]

* Analyzed according to the methodology proposed by Gomes (1996). SD+FS: standard diet + ferrous sulfate; SD+C: standard diet + chia; HFD+FS: high fat diet + ferrous sulfate; HFD+C: high fat diet + chia. Means with different letters in the same line present significant difference (p<0.05) by Newman-Keuls test.

4.11.3. Determination of the iron content

The iron content of albumin and prepared diets was determined by atomic absorption spectrometry. This determination confirmed the concentrations of iron in the diets, thus being an important standard for the method used.

4.11.4. Depletion phase

The depletion phase lasted 21 days. Sixteen animals received standard diet (REEVES *et al.*, 1993) modified, by using mixtures of minerals without iron and deionized water *ad libitum* to induce the iron deficiency (anemia), while the other group (n=16) received high fat diet without iron (61% of fat) (Research Diets, New Brunswick, NJ). The animal weight gain and food consumption were evaluated weekly.

At the end of this period it was performed the hemoglobin dosage of all animals, and these were redistributed so that the average levels of hemoglobin and weight were closest within each group (standard group + ferrous sulfate: average weight corresponding to 135 ± 11.95 g and the hemoglobin mean equal to 5.58 ± 0.99 g/dL; standard group + chia: average weight corresponding to 136 ± 18.04 g and the mean of hemoglobin equal to 5.53 ± 0.75 g/dL; high fat diet group + ferrous sulfate: average weight corresponding to 133 ± 6.59 g and the hemoglobin mean equal to 5.82 ± 1.15 g/dL and high fat diet group + chia: average weight corresponding to 130.37 ± 6.57 g and the hemoglobin mean equal to 5.83 ± 1.19 g/dL).

4.11.5. Repletion phase

In the second stage as a source of iron it was used the ferrous sulfate and chia flour, being used a single level of iron (12 ppm per treatment). The four experimental groups were: standard diet with ferrous sulfate (n=8), high fat diet with ferrous sulfate (n=8), standard diet with chia (n=8) and high fat diet with chia (n=8). The animals received deionized water *ad libitum* and controlled diet weighed daily (18 g), during a period of 14 days.

At this stage it was also monitored the weight gain and food consumption for the calculation of feed efficiency ratio (FER = [weight gain (g) / food intake (g)] x 100). At the end of the repletion phase, it was performed a new hemoglobin dosage to calculate the

hemoglobin gain, by the difference between the values obtained at the end of the repletion period and at the end of the depletion period.

On the 36th day, after fasting for 12 hours, the animals were anesthetized with isoflurane (Isoforine, Cristália®) and euthanized by cardiac puncture. The blood (about 4 mL) was collected in heparinized tubes (16 x 100 mm, BD Vacutainer®) with 10 mL of sodium heparin and centrifuged at 2865 g for 10 minutes (Fanem - 204, Sao Paulo, Brazil) for plasma separation.

4.11.6. Blood Tests

Serum hemoglobin was measured by cyanide methemoglobin method [19], using a colorimetric kit (Bioclin®, Brazil) for in vitro diagnosis. A volume of 20 µL of blood was pipetted and mixed with 5 mL of Drabkin's solution color regent (containing potassium cyanide, and hydrogen cyanide). The reading of absorbance was done in UV-Visible Multiskan (Thermo Scientific, Massachusetts, MA, USA) at a wavelength of 540 nm. The analysis of ferritin and transferrin serum were performed using a specific kit, according to manufacturer's recommendation (Bioclin®, Brazil).

4.11.7. Liver iron concentration

The liver iron concentration was carried out by atomic absorption spectrophotometry, according to Gomes (1996).

4.11.8. Iron bioavailability

The iron bioavailability was calculated according to Hernandez *et al* (2003). The hemoglobin regeneration efficiency (HRE%) was calculated by the expression: HRE% = [(mg Fe final Hb - mg Fe initial Hb) / 100] / mg Fe consumed. The iron in hemoglobin content was estimated by: [Body weight (g)*Hb (g/L)*0.335*6.7]/1000. This variable was calculated assuming the total blood volume equals to 6.7% of the rats' body weight, and the body iron in hemoglobin content as being 0.335. The use of iron was calculated as: [HRE% *% dietary iron]/100, and the absorption of iron was calculated as: [Fe intake - excretion Fe].

4.11.9. Biomolecular analysis

4.11.9.1. Extraction of mRNA in liver and duodenal tissue and cDNA synthesis

The tissues were macerated in liquid nitrogen in RNAse free conditions and samples were aliquoted for total RNA extraction. Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) using the manufacturer's recommendations. 100 mg of tissue was homogenized in 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 of tissue) and the tubes were centrifuged at 10000g (4°C), the RNA was washed with ethanol 75% and centrifuged at 9500 g for 5 minutes (4°C). The pellet was resuspended in 50 ul of Milli-Q water treated with diethylpyrocarbonate (DEPC water).

After extraction, RNA samples were treated with DNase (RQ1 RNase free-DNase Kit; Promega, Madison, WI, USA) using the manufacturers protocol as follows: 7 uL of sample was transferred to a tube and treated with 1 uL of DNAse buffer and 2 uL of DNase (Invitrogen Brazil Ltda) and left at room temperature for 15 minutes, to the action of the enzyme DNAse. After this time, it was added 1 uL of EDTA and the samples were left in a water bath at 65° C for 8 minutes. 2 µL of mRNA extracted was used to synthesize the cDNA using M-MLV reverse transcription kit (Invitrogen Corp., Grand Island, NY) according to the manufacturer's protocol.

4.11.9.2. Determination of gene expression of proteins involved in iron metabolism by reverse transcriptase polymerase chain reaction (RT-qPCR)

Expression of mRNA levels in the duodenal mucosa and the liver of proteins involved in iron metabolism were analyzed by RT-qPCR. The SYBR green PCR master mix from Applied Biosystems (Foster City, CA) was used and analyses were performed on the StepOne™ Real-Time PCR System (Thermo Fisher Scientific) using the measurement system by SYBR-Green Fluorescence and Primer Express software (Applied Biosystems, Foster City, CA). The PCR involved an initial denaturation cycle of 95°C (10 min) and then 40 cycles with 1 min denaturation (94°C), 1 min annealing (56°C) and 2 min elongation (72°C), followed by a standard dissociation curve. Sense and antisense primer sequences (Choma Biotechnologies) were used to amplify protein divalent metal carrier (DMT-1), duodenal cytochrome b (DcytB), ferroportin and hephaestin from duodenum, and proteins ferritin, transferrin and PPAR-α from liver. The relative expression levels of

mRNA were normalized by the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 3). All steps were performed using open conditions with RNase.

4.12. Ethical aspects

The study was approved by the Ethics Committee on Animal Research of the Federal University of Viçosa (Appendix 1).

4.13. Experimental design and statistical analysis of data

For analysis of compounds present in chia seeds it was used t test. For the analysis of protein quality, the data were submitted to ANOVA. The means of the test groups were compared by Duncan test. In the iron bioavailability assay, the results were analyzed by ANOVA. To "F-value" significant, was used the Student Newman-Keuls test to compare the means of all experimental groups. Statistical analyses were performed using SPSS version 20.0, considering a 5% significance level.

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RESULTS

5.1. BOOK CHAPTER

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5.2. ARTIGO 1

BRAZILIAN SEED CHIA (*Salvia hispanica* L.) PRESENTS HIGH CONCENTRATION OF LIPIDS, PROTEINS, TOTAL DIETARY FIBER, MINERALS AND VITAMIN E

ABSTRACT

This study investigated and compared the occurrence and concentration of macronutrients, moisture, ash, dietary fiber, minerals, carotenoids, vitamins, flavonoids, phenolic compounds, antioxidant activity and phytate in Brazilian chia seeds grown in the states of Mato Grosso (MT) and Rio Grande do Sul (RS). There were high lipid (31.2 g.100 g^{-1}, in means), proteins (18.9 g.100 g^{-1}, in means) and dietary fiber (35.3 g.100 g^{-1}, in means) concentrations in chia seeds. The concentration of vitamin E was high (7038.4 μg.100 g^{-1} and 7024.6 μg.100 g^{-1} for chia seed grown in RS and MT, respectively). Similar values of total phenolic compounds and phytic acid in both chia seeds was observed. Chia grown in RS showed higher antioxidant activity than chia grown in MT. The Brazilian chia seeds showed high concentrations of lipids, proteins, total dietary fiber, minerals and vitamin E.

Keywords: *Salvia hispanica* L., bioactive compounds; tocopherols, tocotrienols, antioxidant activity; phytate.

Chemical compounds studied in the article:
Lutein (PubChem CID: 5281243); Zeaxanthin (PubChem CID: 53477763); α-Tocopherol (PubChem CID: 14985); β-Tocopherol (PubChem CID: 6857447); γ-Tocopherol (PubChem CID: 92729); δ-Tocopherol (PubChem CID: 92094); α-Tocotrienol (PubChem CID: 5282347); β-Tocotrienol (PubChem CID: 5282348); γ-Tocotrienol (PubChem CID: 5282349); δ-Tocotrienol (PubChem CID: 5282350).

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1. INTRODUCTION

The genus Salvia, native of southern Mexico and northern Guatemala, includes approximately 900 species of Lamiaceae seeds (Ixtaina, Nolasco, & Tomás, 2008). Of these species, 47 are endemic in Brazil and 61 are grown in the Northeast, Midwest, Southeast and South of the country. The biggest crop seed of this sort occurs in mountainous regions temperate to subtropical (Capitani, Spotorno, Nolasco, & Tomás, 2012; Harley, 2012).

Among the species of the genus Salvia, chia leaves (*Salvia hispanica* L.) is a 4-8 cm long and 3-5 cm wide herbaceous which stands out due to its high nutritional and functional value. The chemical composition and nutritional value of chia seed varies according to the species, climatic conditions, growing location and soil. Studies show that the geographical location and climate can influence the concentration of nutrients in chia seeds (Ayerza & Coates, 2009).

The consumption of chia has increased due to its beneficial effects related to obesity, cardiovascular disease, diabetes and some types of cancer (Poudyal, Panchal, Waanders, Ward, & Brown, 2012; Ixtaina et al., 2011; Vázquez-Ovando, Rosado-Rubio, Chel-Guerrero, & Betancur-Ancona, 2009). These benefits result primarily of the high concentration of essential fatty acids, dietary fiber, antioxidants, flavonoids, anthocyanins, vitamins, carotenoids and minerals present in this seed (Ayerza & Coates, 2011; Reyes-Caudillo, Tecante, & Valdivia-López, 2008).

To date few studies have assessed the chemical characterization and bioactive compounds in Brazilian seed chia. Thus, the purpose of this study was chemically characterize, analyze and compare the occurrence and concentration of minerals, carotenoids, vitamin E, vitamin C, flavonoids, antioxidant activity, total phenolic compounds and phytate in Brazilian chia seeds grown in different places.

2. MATERIALS AND METHODS

2.1. Raw material, storage and preparation of chia flour

Chia seeds (*Salvia hispanica* L.) grown in Brazil were obtained from two distinct regions: Rio Grande do Sul (RS) and Mato Grosso (MT). Samples were stored in hermetically sealed plastic bags and protected from light, under freezing (-18 ± 1°C) until

the time of analysis that occurred within 30 days. All analyzes were performed in chia flour, which was obtained by grinding the grain *in nature*.

2.2. Macronutrients, moisture, ash, total dietary fiber and minerals analysis

The analysis of content of moisture, ash, proteins, lipids and total dietary fiber in chia were performed in three repetitions. Moisture was determined using an oven (Nova Ética®, model 400/6ND, São Paulo, Brazil) at 105°C and ash was quantified using a muffle furnace (Quimis, Q320M model, Brazil) at 550°C. Protein content was determined by the micro-Kjeldhal method, total dietary fiber was determined by the gravimetric non-enzymatic method and lipids were determined by Soxhlet method (AOAC, 2012). Carbohydrates were calculated as the difference, using the equation: [100 – (% moisture + % lipids + % proteins + % total dietary fiber + % ash)]. The total energy value of chia was estimated considering the conversion factors of 4 $kcal \cdot g^{-1}$ for protein or carbohydrate and 9 $kcal \cdot g^{-1}$ for lipid. Concentrations of Fe, Zn, Ca, Mg, Mn, Cu, B, Pb, Cd, Cr, Na, K, S, Al and N were determined according to the methodology proposed by Gomes (1996).

2.2.1. Extraction and analysis of fatty acid composition

To analyze the fatty acids, 150 mg of chia were weighed in a test tube. 1 mL of the internal standard triglyceride tridecanoic acid, 50 mg of pyrogallic acid, 1 mL of 95% ethanol were added and glass beads. The samples were subjected to acid hydrolysis with 5 mL of HCl and stirred in thermostated bath (75°C/40 min). After cooling to room temperature 12 mL of ethyl ether were added and each tube was shaken on a vortex mixer for 1 min. The tubes were centrifuged (2865 g/10 min) (Hermle®, modelo Z216MK, Alemanha) and the supernatant was transferred to another tube. The solvent was slowly evaporated in a thermostat at a temperature below 40°C using N_2 gas; then 1 mL of 7% boron trifluoride in methanol and 0.5 mL toluene were added. The tubes were well covered and placed in boiling bath for 45 min. After cooling to room temperature, 2.5 mL of water, 1 mL hexane and approximately 0.5 g of anhydrous Na_2SO_4 were added. After mixing, the tubes were left to stand until phase separation and then the supernatant was transferred to a vial. One microliter sample was analyzed by gas chromatography GC 2010 Plus (Shimadzu, Workstation GC solution), equipped with an autosampler AOC-20, flame ionization detector (FID) and capillary column of fused silica from Supelco, SP-2560 (Bis

cyanopropyl polisiloxana). The column temperature program was 140°C for 5 min, heat at 4°C/min to 240°C for 20 min; injector and detector temperatures were 250°C and 260°C, respectively; helium carrier gas, flow of 1 mL/min and the splitting ratio 1/50. The fatty acid retention times were compared with the standard and the calculations were based on area and concentration of the internal standard, using theoretical response factors flame ionization detector (FID).

2.3. Extraction and analysis of carotenoids, vitamins and flavonoids

The preparation and analysis of carotenoids, vitamin E, vitamin C and flavonoids content in chia seeds were performed in five repetitions. During analysis, the samples and the extracts were protected from sunlight and artificial light using amber glassware, aluminum foil and blackout curtains, and protected from oxygen using lids and environments with nitrogen gas in glass bottles.

2.3.1. Carotenoids

The occurrence and the concentration of lutein and zeaxanthin in chia seeds were investigated. Carotenoids were extracted according to Rodriguez-Amaya (1996) with modifications. About 5 g of chia flour were homogenized with 30 mL of acetone for approximately 5 minutes in a micro grinder. The extract was vacuum filtered on Buchner funnel using filter paper. Then, the filtrate was transferred to a separatory funnel containing 25 mL of petroleum ether to transfer the pigments from acetone to petroleum ether. Each fraction was washed three times with distilled water to remove all acetone. Anhydrous sodium sulfate was added to the extract to remove any residual water. The pigments were then redissolved in petroleum ether in a 25 mL volumetric flask and stored in amber glass vials at -18 ± 1°C until the time of which occurred in a maximum of one hour.

For analysis, an aliquot of 1 mL of extract was evaporated under nitrogen gas flow, and then recovered in 1 mL of HPLC grade acetone. The extract was filtered through a filter unit with porosity of 0.45 μm (Millipore, Brazil). Carotenoids were analyzed using a high performance liquid chromatography system (HPLC) (Shimadzu, SCL 10AT VP model, Japan) comprised of a high-pressure pump (Shimadzu, LC-10AT VP model, Japan), an autosampler with a loop of 500 μL (Shimadzu, SIL-10AF model, Japan) and a

diode array detector (DAD) (Shimadzu, SPD-M10A model, Japan). The following chromatographic conditions were used: chromatographic column RP-18 (Phenomenex Gemini, 250 mm × 4.6 mm, 5 μm), fitted with a guard column (C18), (Phenomenex ODS 4mm×3mm); mobile phase composed of hexane: isopropanol (HPLC grade, Tedia, Brazil), in the proportions 95:5, with flow rate of 2.0 mL·min-^{1} and injection volume of 50 μL (Pinheiro-Sant'Ana, Stringheta, Brandão, & Azeredo, 1998). The chromatograms were obtained at 450 nm.

2.3.2. Vitamin E

We investigated the occurrence and the concentration of the eight components of vitamin E (α, β, γ and δ tocopherols and tocotrienols) in chia seeds. The extraction of the components was carried out according to Pinheiro-Sant'ana et al. (2011) with modifications. Approximately 4.0 g of chia flour were weighed and added to 4.0 mL of heated ultrapure water (80 ± 1°C). Then 10.0 mL of isopropyl alcohol, 1.0 mL of hexane containing 0.05% BHT, 5 g of anhydrous sodium sulfate and 25 mL of extraction solvent mixture (hexane: ethyl acetate, 85:15 v/v) were added. The sample was homogenized using a micro grinder for 1 minute. The extract was vacuum filtered on Buchner funnel using filter paper. Then, the extract was transported to a rotating evaporator (70 ±1°C/2 min) until it was concentrated. After this procedure, it was transferred to a volumetric flask and the volume was completed to 25 mL using the solvent mixture.

For analysis, an aliquot of 5 mL of extract was evaporated under nitrogen gas flow, and then recovered in 2 mL of HPLC grade hexane (Tedia, Brazil) and filtered through a filter with porosity of 0.45 μm (Millipore, Brazil) and 5μL injected onto the chromatographic column for analysis. The total vitamin E content was calculated by adding the vitamin E identified components.

The chromatographic conditions used included: HPLC system (Shimadzu, SCL 10AT VP model, Japan); fluorescence detector (Shimadzu, RF10AXL); 290 nm excitation and 330 nm emission); chromatographic column Phenomenex Luna Si100 (250x4 mm, 5 um) coupled with a Si100 Phenomenex guard column (4x3 mm); mobile phase - hexane: isopropanol: glacial acetic acid (HPLC grade, Tedia, Brazil) (98.9: 0.6: 0.5, v/v/v); flow rate of 1.0 mL/min and 22 min run time (Guinazi, Miranda Milagres, Pinheiro-Sant'Ana, & Chaves, 2009). The identification of the components of vitamin E was performed by

comparing the retention time of commercial standards with those obtained for the samples analyzed under the same conditions.

2.3.3. Vitamin C

The vitamin C extraction and analysis (in AA form) were carried out according to the conditions proposed by Campos, Ribeiro, Della Lucia, Pinheiro-Sant'Ana, & Stringheta, (2009) with modifications. About 5 g of chia were homogenized for 5 minutes with 15 mL of extraction solution (3% metaphosphoric acid, 8% acetic acid, H_2SO_4 0.3 N and 1 mM EDTA) using a micro grider. The extract was centrifuged (Hermle®, modelo Z216MK, Alemanha) at 2865 g for 15 min, and filtered through Buchner funnel using filter paper. The filtrate was transferred to a 25.0 mL volumetric flask and completed to volume with ultrapure water.

The extracts were filtered through filter units with porosity of 0.45 µm (Millipore, Brazil). For AA analysis chromatographic the following conditions were used: chromatographic column Lichrospher RP 18 (100, 250 x 4mm, 5µm), HPLC system (Shimadzu, SCL 10AT VP model, Japan), DAD, ultrapure water mobile phase containing 1 mM NaH_2PO_4, 1mMde EDTA and adjusted to pH 3.0 with H_3PO_4; flow rate of 1.0 mL/min and injection volume of 50 µL. Chromatograms were obtained at 245 nm.

2.4. Determination of flavonoids

The main 3-DXAs (luteolinidin, apigeninidin, 7-methoxy-apigeninidin and 5-methoxy-luteliolinidin), flavones (luteolin and apigenin) and flavanones (naringenin and eriodictyol) were investigated in chia. The compounds were extracted from 2 g of sample in 20 mL of methanol: HCl (99:1, v:v). Analyzes were performed in a HPLC system (Shimadzu, SCL 10AT VP, Japan) equipped with DAD (Shimadzu, SPD-M10A, Japan), high pressure pump (Shimadzu, LC-10AT VP, Japan), autosampler with a 500 µL loop (Shimadzu, SIL-10AF, Japan), and helium degassing system using the chromatographic conditions described by Yang, Allred, Geera, Allred, & Awika (2012).

Flavonoids were determined by HPLC using the following chromatographic conditions: HPLC system (Shimadzu, SCL 10AT VP model, Japan), Kinetix C-18 column (150 x 4.6 mm id, 5 um) equipped with C-18 guard column (4 mm x 3 mm. Phenomenex, Torrance, CA), column temperature of 35°C, injection volume of 100 µl, with detection at

480 nm, 360 nm, 280 nm for 3-deoxyanthocianidins, flavones and flavanones, respectively. The mobile phase consisted of 2% formic acid in ultrapure water (line A) and 2% formic acid in acetonitrile (line B). The elution gradient of B was 0-3 min isocratic 10%; 3-4 min, 10-12%; 4-5 min isocratic 12%; 5-8 min, 12-18%; 8-10 min, isocratic 18%; 10-12 min, 18-19%; 12-14 min, isocratic 19%; 14-18 min, 19-21%; 18-22 min, 21-26%; 22-28 min, 26-28%; 28-32 min, 28-40%; 32-34 min, 40-60%; 34-36 min, isocratic 60%; 36-38 min, 60-10%; 38-45 min, isocratic 10%. The mobile phase was degassed with helium gas at 50 kPa before and during runs, which were performed using the following flow gradient: 0-36 min, 1.0 mL / min; 36-38 min, 1.0 to 2 mL / min, 38-44 min, 2.0 mL / min; 44-45 min, 2.0-1.0 mL / min. Chromatograms were obtained at 480 nm, 360 nm, 280 nm for 3-deoxiantocianidinas, flavones and flavanones, respectively.

2.5. Preparation and evaluation of the purity of the standards carotenoids, vitamins and flavonoids

Solutions were prepared for each of the investigated components in chia (lutein, zeaxanthin, AA, α-tocopherol, α-tocotrienol, β-tocopherol, β-tocotrienol, γ-tocopherol, γ-tocotrienol, δ-tocopherol, δ- tocotrienol, luteolinidina, apigeninidina, apigeninidina 7-methoxy-5-methoxy-luteliolinidina, luteolin, apigenin, naringenin and eriodictyol).

The purity of the standards was evaluated by HPLC and quantified by spectrophotometry performed, based on the maximum absorbance according to the Lambert-Beer law. To perform the actual quantification of the standards we used the following equation: C (g/mL) x 104 = ABS / E1% 1cm, where C = concentration; ABS = maximum absorbance; E1% 1cm = molar absorptivity coefficient.

2.6. Identification and quantification of patterns of carotenoids, vitamins and flavonoids

The qualitative identification of the investigated compounds was performed by HPLC by comparing the retention times obtained with standards for peaks of interest obtained for samples analyzed under the same conditions. Furthermore, carotenoids, flavonoids and AA were identified by comparing the absorption spectra of the standard and the peaks of interest in the samples, using the DAD.

For the quantification of the compounds found in chia, standard curves were used. The construction of calibration curves was performed by injection in duplicate in five increasing concentrations of the standard solutions.

2.7. Determination of antioxidant activity

2.7.1. Preparation of extracts

Two grams of chia were added to a 20 mL of acetone 70% solution. Then, the suspension was automatically shaken (10 g, 2 hours, 25°C) and centrifuged (2865 g, 15 minutes) (Hermle®, modelo Z216MK, Alemanha). The supernatant was transferred to a beaker and volume was completed to 20 mL with acetone 70%. The extract was placed in amber bottle and stored in a freezer (-18 ± 1°C) until the time of analysis.

2.7.2. Radical removal activity (DPPH)

In a test tube, protected from light, 100 µL of the extract obtained in the previous step was added to 1.5 mL of methanolic DPPH solution (1.1-diphenyl-2-picrylhydrazyl) and stirred by vortex (3000 rpm) for 30 seconds. After 30 minutes of standing, the absorbance of the solution was read in a spectrophotometer (Thermo scientific, 606 Evolution, USA) at 517 nm. The analytical curve was constructed using 50-100 µmol/L trolox solution. The antirradical activity (AAR) was expressed in µmol trolox equivalent/g of sample (µmol trolox/g) (Bloor, 2001).

2.8. Determination of total phenolic

The total phenolic compounds in chia was determined using the Folin-Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventos, 1999). Aliquots of 0.5 mL of extract obtained in item 2.5.1 were added to 0.5 mL of Folin-Ciocalteu reagent (20%). After homogenization, 0.5 mL of sodium carbonate (7.5%) was added. The reaction mixture was homogenized by vortex (2865 g, 10 seconds) and incubated at room temperature (30 minutes).

The reading of absorbance was performed in spectrophotometer (Thermo scientific, Evolution 606, USA) at 765 nm. Analytical curve of gallic acid (0,005-0,10 mg/mL) was used to quantify the compounds. The results were expressed in mg of gallic acid equivalents/g of chia flour (mg GAE/g).

2.9. Determination of phytic acid

The concentration of phytic acid was determined by ion exchange chromatography and spectrometry (Latta & Eskin, 1980) with modifications (Ellis & Morris, 1986). For the extraction of phytic acid, 0.1 g of chia flour was weighed and 5 mL of HCl 2.4% were added, remaining under horizontal shaking for 12 hours at 250 rpm. The extract was centrifuged at 2865 g (Hermle®, modelo Z216MK, Alemanha) for 15 minutes and the supernatant was filtered in vacuo büchnner funnel and purified using ion-exchange column with the stationary phase constituted by resin Dowex- AGX-4.

The column was preconditioned with NaCl 2M and the obtained extract of the previous step was applied. Inorganic phosphors were eluted with NaCl 0.05 M, followed by elution of phytic acid retained with NaCl 2 M. Phytic acid was determined colorimetrically at 500 nm. An analytical curve of phytic acid (Sigma®) was prepared in concentrations of 10 to 100 $\mu L/mL^{-1}$, to express the concentration of phytic acid in g/100 g of chia flour

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2.10. Determination of tannins

The tannin determination was performed by the reaction of vanillin / HCl (Burns, 1971) with modifications (Maxson & Rooney, 1972; Price; Scoyoc & Butler, 1978). Were weighed 200 mg of chia and added a solution of 10 mL 1% HCl in methanol. The tubes were placed in an automatic shaker (Marconi, MA093, Brazil) (80 g, 20 minutes, 30° C) for extraction of tannin. Then, they were centrifuged (Hermle®, Z216MK model, Germany) at 2865 g for 20 minutes.

Aliquots of 1 mL of supernatant were added to 2.5 mL of 1% solution of vanillin in methanol and 2.5 mL of 8% solution of HCl in methanol. The absorbance reading was held in spectrophotometer (MultiskanGo, Thermoscientific, USA) at 500 nm. The results were expressed as milligrams of catechin per gram of sample according to the analytical curve of catechin. For the construction of the curve, it was weighed 200 mg of catechin in a volumetric flask (200 mL) and completed the volume with methanol. Aliquots were withdrawn for 5, 10, 20, 25 and 50 mL of concentrated solution and the absorbance reading made at 500 nm.

2.11. Experimental design and statistical analysis

A completely randomized design was used. The data were analyzed using the t test (α=0.05) for independent samples to verify difference between chia seeds grown in different places. All statistical analyzes were conducted using SPSS software, version 20.

3. RESULTS

High concentrations of dietary fiber (35.3%, in means), lipids (31.2%, in means) and proteins (18.9%, in means) were found in chia seeds. Chia grown in the state of Rio Grande do Sul (RS) showed higher ($p<0.05$) content of moisture, lipids (saturated, monounsaturated and polyunsaturated) and carbohydrates compared to the chia grown in Mato Grosso (MT) (Table 1). Both chia seeds showed high concentration of polyunsaturated fatty acids, standing out the n-3.

Table 1. Nutritional composition of chia seeds grown in different places, Brazil, 2015 ($g.100g^{-1}$).

Variables*	Mean** ± SD***	Mean** ± SD***
	RS Chia flour	MT Chia flour
Moisture	7.14 ± 0.26^{a}	5.62 ± 0.03^{b}
Ash	4.56 ± 0.04^{b}	5.07 ± 0.07^{a}
Lipids	32.16 ± 0.29^{a}	30.17 ± 0.22^{b}
16:0	1.82 ± 0.12^{a}	1.85 ± 0.10^{a}
18:0	0.90 ± 0.07^{a}	1.03 ± 0.10^{b}
18:1 (n-9)	1.43 ± 0.10^{a}	1.67 ± 0.09^{b}
18:1 (n-7)	0.26 ± 0.02^{a}	0.23 ± 0.03^{a}
18:2 (n-6)	5.69 ± 0.42^{a}	5.09 ± 0.05^{b}
18:3 (n-3)	20.37 ± 1.38^{a}	18.74 ± 1.29^{b}
Saturated	2.88 ± 0.18^{a}	2.73 ± 0.03^{b}
Monounsaturated	1.89 ± 0.12^{a}	1.69 ± 0.06^{b}
Polyunsaturated	27.75 ± 1.80^{a}	25.73 ± 1.32^{b}
Protein	18.18 ± 1.20^{a}	19.72 ± 3.09^{a}
Food Total fiber	33.37 ± 0.26^{b}	37.18 ± 0.21^{a}
Soluble fiber	2.89 ± 0.09^{b}	3.88 ± 0.68^{a}
Insoluble fiber	30.47 ± 0.35^{b}	33.30 ± 0.46^{a}
Carbohydrates	4.59 ± 0.34^{a}	2.23 ± 0.56^{b}
Total energy value ($kcal \cdot 100\ g^{-1}$)	380.52 ± 1.83^{a}	359.33 ± 3.87^{b}

*values expressed in fresh matter; **mean of three replicates; ***standard deviation; same letters on the line do not differ by t test at 5% probability, RS: Rio Grande do Sul, MT: Mato Grosso.

Among the minerals presented chia seeds, iron, calcium, phosphorus and potassium highlight. Chia seed grown in RS presented higher ($p<0.05$) content of iron, manganese, boron, lead, aluminum, nitrogen, phosphorus, potassium, calcium, magnesium, sulfur and copper. The concentrations of zinc, cadmium and sodium were similar ($p>0.05$) in both seeds (Table 2).

Table 2. Mineral content presented in chia seeds grown in different places, Brazil, 2015.

Minerals* **Mean**** **± SD****	Fe (mg $100g^{-1}$)	Zn (mg $100g^{-1}$)	Mn (mg $100g^{-1}$)	Na (mg $100g^{-1}$)	B (mg $100g^{-1}$)	Pb (mg $100g^{-1}$)	Cd (mg $100g^{-1}$)	Al (mg $100g^{-1}$)
RS Chia Flour	9.39 ±052 [a]	3.65±0.09 [a]	4.05±0.12[a]	150±00.00[a]	1.12±0.02[a]	0.06±0.02[a]	0.08±0.03[a]	1.32±0.13[a]
MT Chia Flour	7.69±0.33 [b]	3.76±0.10 [a]	2.48±0.05[b]	140±13.42[a]	0.93±0.04[b]	0.00±0.00[b]	0.11±0.04[a]	0.99±0.25[b]

Minerals* **Mean**** **± SD****	N (mg $100g^{-1}$)	P (mg $100g^{-1}$)	K (mg $100g^{-1}$)	Ca (mg $100g^{-1}$)	Mg (mg $100g^{-1}$)	S (mg $100g^{-1}$)	Cu (mg $100g^{-1}$)	Cr (mg $100g^{-1}$)
RS Chia Flour	3607.00±22.68[a]	640.00±4.85[a]	620.00±17.45[a]	480.00±21.00[a]	350.00±4.09[a]	200.00±10.25[a]	1.32 ±0.03[a]	0.00 ±0.00[a]
MT Chia Flour	3620.00±20.36[b]	530.00±6.52[b]	550.00±6.71[b]	430.00±19.88[b]	330.00±13.437[b]	150.00±5.81[b]	0.63 ±0.01[b]	0.00 ±0.00[a]

*values expressed in fresh matter; **mean of three replicates; ***standard deviation; same letters on the line do not differ by t test at 5% probability Same letters in the column do not differ by t test at 5% probability. Fe: iron; Zn: zinc; Mn: manganese; Na: sodium; B: boron; Pb: lead; Cd: cadmium; Al: aluminum; N: nitrogen; P: phosphorus; K: potassium; Ca: calcium; Mg: magnesium; S: sulfur; Cu: copper; Cr: chrome, RS: Rio Grande do Sul, MT: Mato Grosso.

The analysis methods employed allowed good resolution of the peaks which ensured adequate identification and quantification of the carotenoids, vitamins and bioactive compounds in chia (Figure 1).

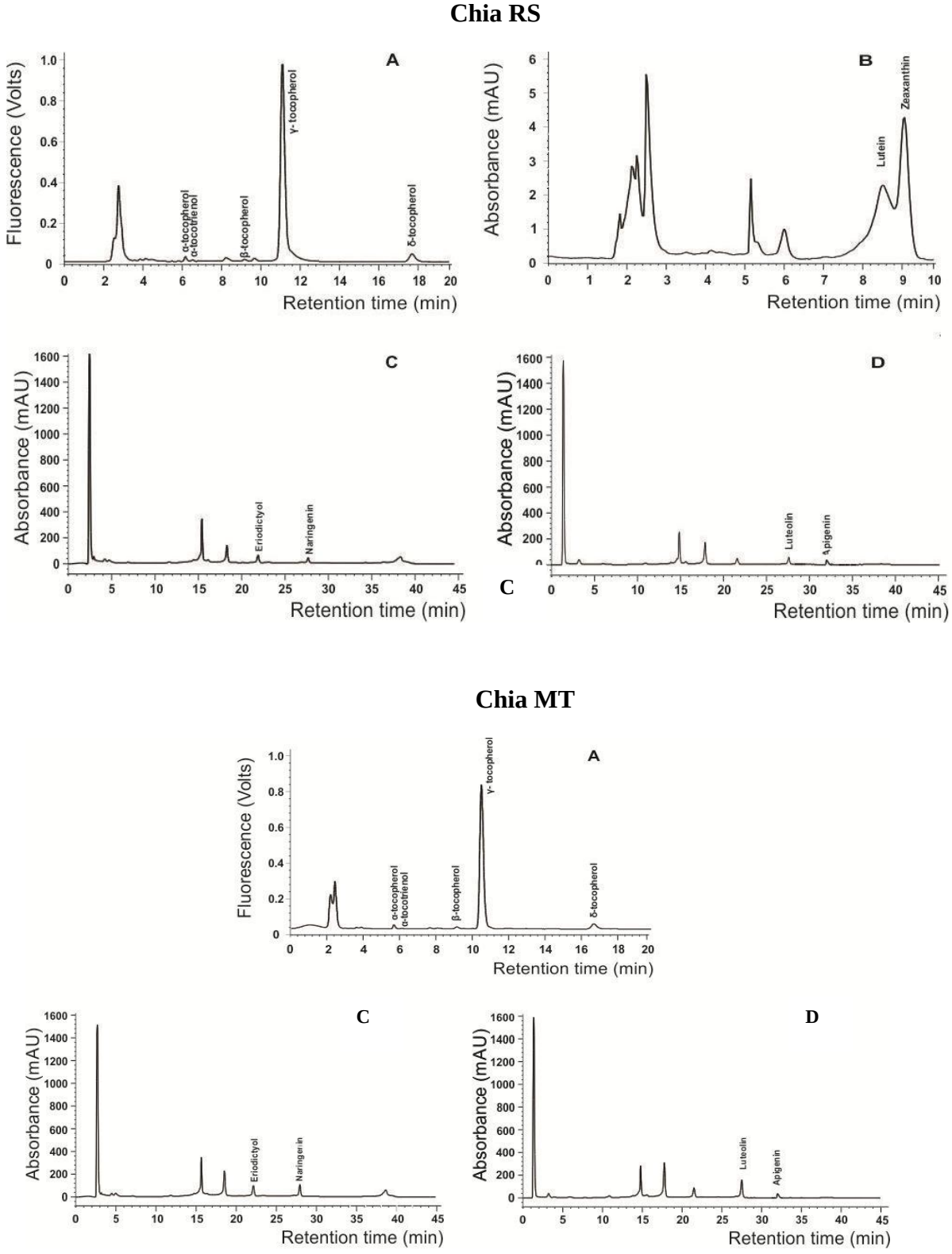

Figure 1. HPLC analyses of vitamin E (A), carotenoids (B) and flavonoids (C and D) in chia seeds grown in the Rio Grande do Sul and Mato Grosso

Chia seeds grown in RS ad MT showed high ($p>0.05$) vitamin E concentration. The γ-tocopherol was the main component found (on average, 90.9% and 91.5%, for chia seed grown in RS and MT, respectively). The occurrence of carotenoids in chia grown in MT was not observed as well as vitamin C and 3-deoxyanthocianidins in both seeds. The concentration of flavones and flavanones in the seed grown in MT was higher ($p<0.05$) than that grown in RS (Table 3).

Table 3. Occurrence and concentration of carotenoids, vitamins and bioactive compounds in in chia seeds grown in different places, Brazil, 2015.

Compounds*	Mean** ±SD*** RS Chia	Mean** ±SD*** MT Chia
Total Vitamin E (µg.100 g^{-1})	7743.38±161.92^{a}	7679.33 ±155.20^{a}
α-tocopherol	179.22±12.24^{a}	190.26±12.70^{a}
α-tocotrienol	43.71±3.22^{a}	16.79±1.61^{b}
β-tocopherol	88.78±2.10^{a}	77.77±2.80^{b}
β-tocotrienol	nd	nd
γ-tocopherol	7038.43±121.30^{a}	7024.59±138.20^{a}
γ-tocotrienol	nd	nd
δ-tocopherol	393.24±27.70^{a}	369.92±23.40^{a}
δ-tocotrienol	nd	nd
Sum of carotenoids (µg.100 g^{-1})	53.58±1.68^{a}	nd^{b}
Lutein	10.33±0.61^{a}	nd^{b}
Zeaxantin	43.25±1.33^{a}	nd^{b}
Vitamin C (µg.100 g^{-1})	nd	nd
Ascorbic acid	nd	nd
3-Deoxyanthocianidins (µg.100 g^{-1})	nd	nd
Luteolinidin	nd	nd
Apigeninidin	nd	nd
7-methoxy-apigeninidin	nd	nd
5-methoxy-luteliolinidin	nd	nd
Sum of flavones (µg.100 g^{-1})	5.71±0.03^{b}	15.07±0.06^{a}
Luteolin	5.56±0.05^{b}	14.84±0.08^{a}
Apigenin	0.15±0.01^{b}	0.33±0.03^{a}
Sum of flavanones (µg.100 g^{-1})	4.13±0.15^{b}	8.78±0.41^{a}
Naringenin	0.21±0.04^{a}	0.37±0.01^{a}
Eriodictyol	3.92±0.43^{b}	8.41±0.60^{a}

*values expressed in fresh matter; **mean of five replicates; ***standard deviation; nd: not detected; same letters on the line do not differ by t test at 5% probability; nd: not detected, RS: Rio Grande do Sul, MT: Mato Grosso

Similar concentrations ($p>0.05$) of phenolic compounds were observed in chia seeds (0.97 ± 0.01 mg GAE/g sample and 0.99 ± 0.02 mg GAE/g sample for chia grown in RS and MT, respectively). The chia seed grown in RS presented higher ($p\leq0.05$) antioxidant activity (478.2 ± 0.02 µmol TEAC/g sample) when compared to grown in MT (466.3 ± 0.06 µmol TEAC/g sample) (Figure 2). The concentration of phytic acid in the seed grown in RS and MT were similar ($p>0.05$) (0.96 and 1.16 g.100 g^{-1}, respectively) and the concentration of tannins were higher ($p<0.05$) in chia seed grown in Mato Grosso (19.08 ± 1.08 eq.catequina/g sample) than chia seed grown in Rio Grande do Sul (14.93 ± 0.24 eq.catequina/g sample).

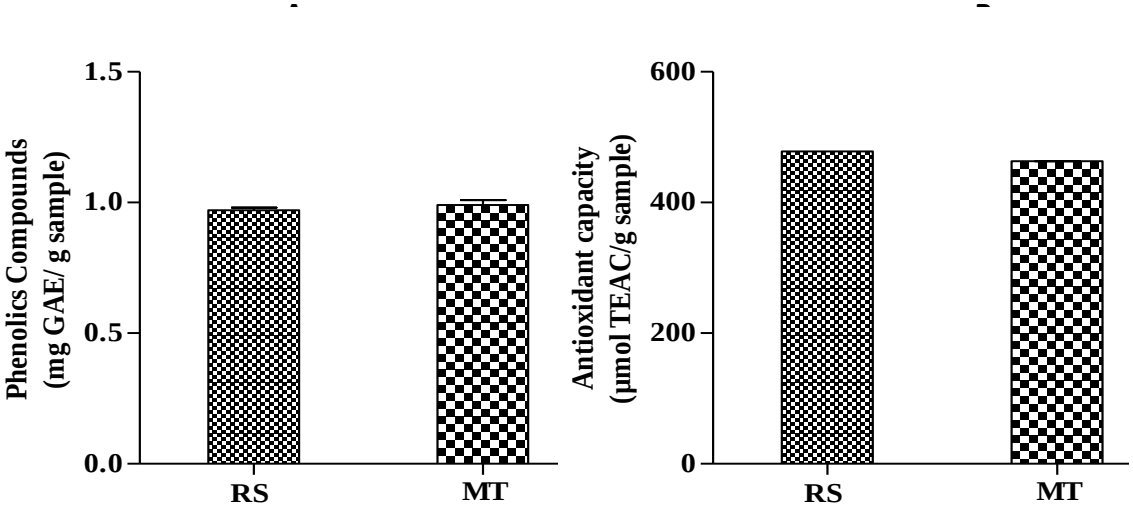

Figure 2. Concentration of total phenolic and antioxidant activity of Brazilian chia seeds, Brazil, 2015.

GAE: Gallic acid equivalent; same letters on the line do not differ by t test at 5% probability, RS: Rio Grande do Sul, MT: Mato Grosso.

4. DISCUSSION

The present study focused on the characterization of macronutrients and micronutrients of Brazilian chia seed grown in two distinct places. Brazilian chia seeds presented high concentrations of dietary fiber, lipids and proteins. The concentration of dietary fiber ranged from 30 to 38%, and about 6% of this amount comprises soluble fiber. Values similar to those observed in our study are reported in the literature for seeds grown in other countries (Capitani et al., 2012; Olivos-Lugo, Valdivia-López, & Tecante, 2010; Reyes-Caudillo et al., 2008; Vázquez-Ovando et al., 2009). The concentration of fiber in chia was higher than in other cereals and grains such as corn

(13.4%), soy (15%), wheat (12.6%), linseed (22.3%) and sesame (7.8%) (Dhingra, Michael, Rajput, & Patil, 2012). The American Dietetic Association (ADA, 2014) recommends a fiber intake of 14 g of fiber/1000 kcal for adults, and the intake of one portion of chia (20 g) satisfies approximately 20% of the recommended daily requirement.

The protein concentration observed in the chia seed was high (approximately 19%, in means), being this value similar to those verified by other authors for chia grown in other countries (Sandoval-Oliveros & Paredes-López, 2013). The protein content of the seed tends to decrease with increasing temperature at the place of cultivation (Ayerza & Coates, 2011). However, in our study, we found that chia grown in the state of Mato Grosso, which presents a high temperature, showed the same protein concentration of when compared to that grown in Rio Grande do Sul, with a lower temperature.

Chia seeds analyzed in our study were composed, in means, of 31% lipids, which may be noted in other studies for seeds grown in other countries (Ayerza & Coates, 2009; Monroy-Torres, Mancilla-Escobar, Gallaga-Solórzano, & Santiago-García, 2008). The weather influenced the concentration of lipids present in chia, since the chia seeds grown at high temperatures (Mato Grosso) had lower ($p < 0.05$) lipid content in relation to that grown at lower temperatures (Rio Grande do Sul). Our data corroborate with other studies that show that low temperatures generally increased the level of unsaturation of chia fatty acids (Ayerza & Coates, 2001).

The main type of fatty acid found in chia is polyunsaturated, mainly n-3 fatty acids. Chia essential oil has significantly higher content of α-linolenic and linoleic acids (Álvarez-Chávez, Valdivia-López, Aburto-Juarez, & Tecante, 2008) than linseed, canola and soybean oils (Gunstone & Padley, 1997). Polyunsaturated fatty acids has been associated with improved lipid profile, attenuating cardiometabolic risk and lowering the inflammation (Lesna, Suchanek, & Brabcova, 2013). The ratio n-6/n-3 observed in our study in chia seed was 1:3.6. The high concentration of n-3 is associated with reduction in the risk of coronary artery disease, hypertension, type 2 diabetes, rheumatoid arthritis, autoimmune disorders, and cancer (Connor, 2000).

The Brazilian chia seed highlighted in relation to the concentration of iron, zinc, calcium, manganese, potassium and phosphorus. The differences between the Brazilian chia seeds can be attributed to the geographic location and the type of soil in which they

are grown. Values similar to the present study are noted wherein the minerals calcium (631 mg.100 g^{-1}), potassium (407 mg.100 g^{-1}), magnesium (335 mg.100 g^{-1}), iron (7.72 mg.100 g^{-1}) and zinc (4.58 mg.100 g^{-1}) are the most significant in chia (USDA, 2015). The concentration of calcium in chia seed is six times higher than that of milk, whereas the iron concentration is 2.4 to 6 times higher than the other sources of this mineral, as meat (Beltrán & Romero, 2003).

The average content of vitamin E in chia (7711.35 μg.100 g^{-1}) was higher than that observed in other cereals like wheat, oats, barley, rye and sorghum (Cardoso et al., 2015; Okarter, Liu, Sorrells, & Liu, 2010; Tiwari & Cummins, 2009). The γ-tocopherol isomer was found in higher quantities in chia flour, followed by δ-tocopherol. A similar result was observed by Capitani et al. (2012) in chia grown in Argentina, wherein the component present in larger amounts in chia oil was γ-tocopherol. Controversial results were observed in other studies that used chia grown in Argentina and Guatemala (Capitani et al., 2012; Ixtaina et al., 2011) since the authors did not detect the presence of β-tocopherol in chia. In addition, α-tocopherol concentration of this study (179.2 to 190.3 μg.100 g^{-1}, for chia grown in Rio Grande do Sul and Mato Grosso, respectively) was superior to that observed by Ixtaina et al. (2011) (40-99 μg.100 g^{-1}) in chia oil cultivated in Argentina .

The total carotenoids were similar to that observed by Ixtaina et al. (2011) in chia oil (53-121 μg.100 g^{-1}). However, these authors found only the presence of β-carotene. In our study, lutein and zeaxanthin were verified only in the chia seed grown in the Rio Grande do Sul. Zeaxanthin was the main carotenoid (80.7%) observed in the seed. The zeaxanthin content was higher than in other cereals such as sorghum (15.48 μg.100 g^{-1}) (Cardoso et al., 2015).

The antioxidant activity in the samples was quite similar to that reported by Vázquez-Ovando et al. (2009) (488.8 TEAC, μmol/g) and Capitani et al. (2012) (446.4 TEAC, μmol/g) for a Mexican chia fibrous fraction. The high antioxidant activity of chia can be attributed to its high content of phenolic compounds, tocopherols and tocotrienols. Therefore, the consumption of chia seeds Brazilian can promote benefits to human health.

Although the content of phenolic compounds did not differ between Brazilian chia seeds (0.97 and 0.99 mg GAE/g of chia seeds grown in the Rio Grande do Sul and Mato Grosso, respectively), the average value was high to that reported in Mexican

(Porras-Loaiza, Jiménez-Munguía, Sosa-Morales, María Elena Palou, & López-Malo, 2014; Reyes-Caudillo et al., 2008) and Chilean (Marineli et al., 2014) chia seeds. The changes in total phenolic content between different studies can be attributed to factors such as cultivation techniques, weather conditions, as well as the methods used for the determination of phenolic compounds. The concentration of phytic acid present in both chia seeds was similar to that observed in another study (Ferreira, 2013).

5. CONCLUSION

Brazilian chia seeds showed high concentrations of lipids, proteins, total dietary fiber, minerals and vitamin E. The chia grown in RS showed a higher concentration of lipids, minerals like iron, manganese, boron, lead, aluminum, nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, copper and antioxidant capacity than chia grown in MT. The use of chia must be stimulated since this food presents a high nutritional value and bioactive compounds that are related to benefits to the human health.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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5.3. ARTIGO 2

CHIA SEED SHOWS GOOD PROTEIN QUALITY, HYPOGLYCEMIC EFFECT AND IMPROVES THE LIPID PROFILE AND LIVER AND INTESTINAL MORPHOLOGY OF *WISTAR* RATS

ABSTRACT

Chia has been consumed by the world population due to its high fiber, lipids and proteins content. The objective was to evaluate the protein quality of chia untreated (seed and flour) and heat treated (90°C/20min), their influence on glucose and lipid homeostasis and integrity of liver and intestinal morphology of *Wistar* rats. 36 male rats, weanling, divided into six groups which received control diet (casein), free protein diet (aproteic) and four diet tests (chia seed; chia seed with heat treatment; chia flour and chia flour with heat treatment) for 14 days were used. The protein efficiency ratio (PER), net protein ratio (NPR) and true digestibility (TD) were evaluated. The biochemical variables and liver and intestinal morphologies of animals were determined. The values of PER, NPR and TD did not differ among the animals that were fed with chia and were lower than the control group. The animals that were fed with chia showed lower concentrations of glucose; triacylglycerides, low-density lipoprotein cholesterol and very low-density lipoprotein and higher high-density lipoprotein cholesterol than the control group. The liver weight of animals that have been fed with chia was lower than the control group. Crypt depth and thickness of intestinal muscle layers were higher in groups that have been fed with chia. The consumption of chia has shown good digestibility, hypoglycemic effect, improved lipid and glycemic profiles and reduced fat deposition in liver of animals, and also promoted changes in intestinal tissue that enhanced its functionality.

Keywords: Protein digestibility; Heat treatment; Chia flour; Chia seed; Lipid; Glucose.

Accepted by *Plant Foods for Human Nutrition*

Abbreviations

ALT	alanine aminotransferase
ANOVA	analysis of variance
AST	aspartate aminotransferase
FER	food efficiency ratio
HDL	high-density lipoprotein cholesterol
LDL	low-density lipoprotein cholesterol
NPR	net protein ratio
PER	protein efficiency ratio
TC	total cholesterol
TD	true digestibility
TGL	triacylglycerides
VLDL	very-low density lipoprotein

Introduction

Chia (*Salvia hispanica L.*) is an oilseed that stands out because of its high nutritional value [1, 2]. The chemical composition and nutritional value of chia seed vary according to species, climate conditions, place of cultivation and soil [3] and its main components are lipids, dietary fibers and proteins [4]. Chia contains high concentration of proteins [3, 5, 6] which are complex organic compounds essential to the human body. The quality of chia protein refers to its ability to meet the nutritional needs of the organism by means of essential amino acids and nonessential nitrogen, for protein synthesis [7]. The heat treatment and grinding applied to chia seeds for flour production can raise the nutritional quality of food, due to denaturation of its proteins and therefore increasing digestibility [8, 9]. The difference in protein digestibility of chia may be due to the structural modification process which involves heat treating and grinding [9–11].

The presence of lipids, fiber, phenolic compounds and peptides confer to chia an antioxidant activity [12, 13]. In addition, these compounds are related to hypoglycemic effect in humans [14]. However, so far, studies that evaluated the effect of chia in intestinal and liver morphologies have not been found. There are *in vitro* studies evaluating the protein quality of chia with or without heat treatment [2, 15, 16]. Nevertheless, these studies evaluate apparent digestibility, being necessary to evaluate

the true digestibility, which is made in *in vivo* studies. However, *in vivo* studies are nonexistent. Since chia has a high protein concentration, it is of great interest to determine the utilization of this *in vivo* nutrient. The objective of this study was to evaluate the protein quality of chia untreated (seed and flour) and heat treated; the influence of the chia intake on the homeostasis of lipids and glucose, as well as the food action in the liver and intestinal integrities of *Wistar* rats for a short period of time.

Materials and Methods

Raw materials and preparation of flours

Chia seeds (*Salvia hispanica* L.) were purchased and came from the state of Rio Grande do Sul, Brazil, and were used along with four treatments, namely: chia seed untreated, chia seed with heat treatment, chia flour untreated and chia flour with heat treatment. To obtain flour, the seeds were grounded in three repetitions, using a knife mill with a particle size of 850 micrometers. For the heat treatment, the binomial time-temperature was based on *in vitro* study [16] with modifications in the heat exposure time. Chia seed and flours (522 g each) were exposed to 90°C in an oven with air circulation (New Ética®, model 400/6ND, Brazil) for 20 minutes. Subsequently, seeds and flours were packed in polyethylene bags covered with foil and stored in a freezer (-18 ± 1°C) until the time of analysis.

Chemical composition of chia flour

For the determination of chemical composition, 15 grams of chia flour were used. The determination of ash, protein, lipids, moisture and total dietary fiber was performed according to the methodology proposed by AOAC [17]. The concentration of carbohydrates was calculated by difference.

Determination of phenolic compounds and phytates

For the determination of phenolic compounds and phytates, 3 grams of chia flour were used. The total phenolic compounds in the flours were determined using the Folin-Ciocalteu method [18]. The concentration of phytate was determined by spectrophotometry according to Latta and Eskin [19] method with modifications [20].

Evaluation of protein quality seeds of chia

Determination of the True Digestibility (TD), Food Efficiency Ratio (FER), Protein Efficiency Ratio (PER) and Net Protein Ratio (NPR)

The true digestibility was calculated according to Bender and Doell [21]. The FER was calculated from the ratio between the weight gain and the total dietary intake by animals. The PER was determined by using the equation proposed by Hegsted [22]. The NPR was determined by using the equation proposed by Bender and Doell [21].

Biological assay

Preparation and composition of diets

The composition of the experimental diets were based on the AIN-93G diet [23], taking into account the chemical composition of chia flour (Table 1). The chia flour was used as a protein source to replace the casein in the diet tests. The amount of chia used to supply 100% of the protein recommendation for rodents, also provided 100% of the recommendation of lipids and cellulose, not being added in the AIN-93G diet tests. All diets were formulated to be isocaloric and isoproteic with protein percentage of 9.48% (Table 2).

Experimental animals

Thirty-six male rats (*Rattus norvegicus*, *Wistar*, *albinus* variation), newly weaned, and with 21 days of life have been systematically divided into 6 groups with 6 animals each. The animals were distributed in individual metabolic stainless steel cages in controlled temperature environment (22°C) and automatically controlled light and dark cycles of 12 hours. The animals received distilled water and their respective experimental diets ad libitum. The experimental groups received the following diets: positive control (casein); negative control (aproteic); chia seed untreated; chia seed heat treated (90°C for 20 minutes); chia flour untreated and chia flour with heat treatment (90°C for 20 minutes). After 28 days and after 12 hours fasting, the animals were anesthetized with isoflurane (Isoforine®, Cristália, Itapira, Brazil) and then were euthanized by cardiac puncture.

The study was approved by the Ethics Committee on Animal Research of the Federal University of Viçosa, Brazil (Protocol 97/2014).

Biochemical analysis

For the determination of biochemical analysis, 0.5 mL of plasma was used. Plasma glucose concentrations, total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL) and 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 following the manufacturer's instructions (Bioclin®, Belo Horizonte, Brazil). Analyses were performed on a Cobas Mira Plus device.

Histological analysis

Semi-serial histological sections of fragments of the liver and cecum with 3 µm thickness were obtained in automatic microtome (Reichert-Jung®, Germany) and were stained by Toluidine Blue technique. The slides were examined under an Olympus CX31 light microscope. It was used ImageJ software (Java) through grid containing 336 points. Points about nucleus, cytoplasm, fat vesicles and hepatocytes were counted. To measure crypt depth and thickness of the circular and longitudinal muscle layers, twenty random fields per animal were selected [24] and were obtained by using the ImagePro-Plus® software version 4.5 (Media Cybernetics, Rockville USA).

Statistical analysis

The treatments were conducted in a completely randomized design, with six replications. The results were analyzed by analysis of variance. For "F-value" significant, the Duncan test was used to compare means among the experimental groups. Statistical analyzes were performed using the Statistical Analysis System software, version 9.1. P-value <0.05 was considered statistically significant.

Results and Discussion

High concentrations of dietary fiber (33.4%), lipids (32.2%) and proteins (18.2%) were found in chia in the present study. Chia showed a concentration of phenolic compounds equals to 0.97 ± 0.01 mg GAE / g sample and phytates corresponding to 0.96 g ± 0.11 / 100 g (Table 1). Animals fed with chia showed weight gain, FER, PER, NPR and TD lower ($p<0.05$) than the control group (casein). The PER values observed in the groups

fed with chia ranged from 1.73 to 1.92. However, there was no difference ($p>0.05$) in the TD among the groups fed with chia. The weight of feces and fecal nitrogen were higher in the group who ate chia compared to the control group ($p<0.05$) (Table 3). The lower digestibility observed in the groups fed with chia may be associated with a higher concentration of soluble fiber present in the food matrix (2.89 g/100g) compared to casein (control) and the presence of phenolic compound (0.97g/100g) and phytic acid (0.96 g/100g) that can act as anti-nutritional factors. The phenolic compound, phytic acid and dietary fiber can complex with the intestinal contents, preventing access of digestive enzymes and absorption of nutrients; and reducing protein digestibility [25, 26]. Chia presented good protein digestibility regardless of the heat treatment and grinding. The conversion efficiency of chia protein to promote weight gain was about 50% in relation to casein, as it can be observed by means of PER values. Authors [16] observed increased protein digestibility *in vitro* isolated chia when subjected to heat treatment; however, our *in vivo* study contradicts this finding because the protein digestibility, PER and NPR did not differ among the groups fed with chia seed untreated, chia seed with heat treatment, chia flour untreated and chia flour with treatment heat. The digestibility values similar to our study were observed in heat treated soybean (approximately 79%) [27] and in raw and heat treated flaxseed flour (approximately 78%) [28]. The PER values observed in the studies above were higher than the ones in the present study. Moraes et al. [29] evaluated the protein quality of sorghum flour and they reported greater digestibility (86% on average) and lower PER values than in our investigation which suggests that the amino acid composition of chia is better than sorghum, because a lower digestibility of chia protein was effective to promote weight gain of the animals. The animals fed with different diets containing chia showed blood glucose levels lower ($p<0.05$) than animals fed with casein (Table 4). This fact may be associated with the increased presence of soluble dietary fiber fraction of chia compared to control diet (casein), which received only cellulose. The soluble dietary fiber increases the viscosity of the intestinal lumen, reducing the contact of glucose with the enterocyte, thus decreasing its absorption [30]. The effect of chia intake in our study was beneficial, because the food promoted greater control of plasma glucose levels in a short period of time (28 days). The groups fed with chia seed or flour, with or without heat treatment, decreased ($p<0.05$) TGL, LDL, VLDL and increased HDL (Table 4). This can be justified by the supply of chia that satisfied 100%

of the need for fiber and fat of animals. The reduction of HDL was observed in normal rats consuming chia seed oil for four weeks [1]. It is known that high concentrations of fatty acids present in the n-3 chia [31] are related to the reduction of VLDL and TGL. Total cholesterol was lower ($p<0.05$) in the group fed with chia seed with heat treatment compared than in the control one. Thus, chia intake for a short period of time was able to promote improvement in the homeostasis of lipids in animals. Urea and creatinine concentrations did not differ among the experimental groups ($p>0.05$) and the concentrations of ALT and AST were higher ($p<0.05$) in all groups that ingested chia (approximately 1.4 and 1.6 times for AST and ALT, respectively) compared to the control group (casein); however, the concentrations of liver enzymes are within normal range for rodents [32]. The weight of the liver, volume of the cytoplasm and the percentage of hepatic fat were lower ($p<0.05$) in the groups fed with chia than in the control group (casein). The reduction of the volume of hepatocytes in animals fed with different ways of processing chia was directly associated with the decrease in concentration of fat globules in the liver (Online Resource 1). Chia was able to decrease the percentage of liver fat and liver weights due to lower accumulation of lipids in the body, which may have led to increased excretion of fat in the feces, since animals fed with chia showed an increase in fecal weight compared to the control one. Poudyal et al. [33] offered diets containing chia seed for obese mice for eight weeks and observed a reduction of hepatic steatosis in animals. This result may be due to the ability of chia to induce redistribution of lipids in the body, reducing its accumulation in the liver and visceral tissue, thereby exerting a hepatoprotective effect [33]. In our study, normal rats fed with chia for 28 days showed cardio protective effect that may have had a positive effect on insulin sensitivity, reducing plasma glucose levels of animals fed with chia. Animals receiving the chia flour untreated showed a cecum weight lower than the others ($p<0.05$). The depth of the crypts and the longitudinal muscle layer thickness, circular and total, were higher in groups fed with chia than in the control group (Online Resource 1). This result is probably due to increased motility of the digestive tract by the intact form that the fiber is found in chia, besides the formation of gel promoted by the fiber soluble fraction, leading to hyperplasia and/or hypertrophy of muscle cells. The same can be explained for increased crypt depth, although cell size, cell turnover and the number of mitoses of intestinal cells had increased [34]. This same effect was observed in studies using soy flour [35] in rats. Thus, chia consumption was able to

stimulate the acceleration of intestinal transit, and be beneficial in the prevention of diseases.

The results of our experiment demonstrated that animals fed with chia showed PER, NPR, TD, blood glucose levels, TGL, VLDL, LDL, weight of the liver and percentage of hepatic fat lower than the control group. The thickness of the intestinal muscular layer and HDL were higher in animals fed with chia. The consumption of chia in seed or flour form, with or without heat treatment, for a short period of time, showed good protein digestibility, hypoglycemic effect, improved lipid profile and reduced the deposition of hepatic fat, and promoted changes in the intestinal tissue which favored its functionality.

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Compliance with Ethical Standards All procedures performed in this study involving animals were in accordance with the ethical standards of the Federal University of Viçosa.

Conflict of Interest Statement The authors declare that they have no conflict of interest.

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Tables

Table 1 Nutritional composition of chia flour, dry basis (g $100g^{-1}$)

Compounds	**Concentration (g $100g^{-1}$)**
Moisture	7.14±0.26
Ash	4.56±0.04
Lipids	32.16±0.29
Protein	18.18±1.20
Total dietary fiber	33.37±0.26
Soluble Fiber	2.89±0.09
Insoluble Fiber	30.47±0.35
Carbohydrates	4.59±0.34
Phenolic Compounds	0.97±0.07
Phytate	0.96±0.11

Table 2 Composition of diet AIN 93-G (g Kg^{-1})

	Experimental Groups					
Ingredients (g Kg^{-1})	Casein	Aproteic	Chia seed untreated	Chia seed with HT	Chia flour untreated	Chia flour with HT
Casein	117.2	-	-	-	-	-
Chia seed untreated	-	-	522	-	-	-
Chia seed with HT	-	-	-	522	-	-
Chia flour untreated	-	-	-	-	522	-
Chia flour with HT	-	-	-	-	-	522
Dextrinized Starch	132	132	132	132	132	132
Sucrose	100	100	100	100	100	100
Soybean oil	168.08	168.08	-	-	-	-
Cellulose	154.5	154.5	-	-	-	-
Mineral Mix	35	35	35	35	35	35
Vitamin Mix	10	10	10	10	10	10
L-cystine	3	3	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
Starch	277.72	394.92	195.5	195.5	195.5	195.5
Protein (%)	9.38*	-	9.48	9.48	9.48	9.48
Caloric density (kcal g^{-1})	4.08	4.17	3.93	3.93	3.93	3.93

HT: Heat treatment; * Considering that casein has 80% purity

Table 3 Effect of chia ingestion in protein quality index in *Wistar* rats (n = 6) for a period of 14 days

Groups	Casein	Chia seed untreated	Chia seed with HT	Chia flour Untreated	Chia flour with HT
Weight Gain (g)	68.66 ± 4.76[a]	19.83 ± 5.49[b]	24.00 ± 5.96[b]	27.00 ± 8.43[b]	25.33 ± 4.13[b]
FER	0.34 ± 0.04[a]	0.16 ± 0.02[b]	0.20 ± 0.05[b]	0.18 ± 0.03[b]	0.19 ± 0.03[b]
PER	3.45 ± 0.49[a]	1.73 ± 0.22[b]	1.84 ± 0.47[b]	1.82 ± 0.32[b]	1.92 ± 0.33[b]
NPR	4.51 ± 0.60[a]	3.22 ± 0.64[b]	3.49 ± 0.65[b]	3.32 ± 0.32[b]	3.52 ± 0.42[b]
Fecal dry weight (g)	5.16 ± 0.49[b]	6.98 ± 0.41[a]	7.55 ± 0.69[a]	7.59 ± 1.28[a]	7.45 ± 1.11[a]
Fecal nitrogen (%)	0.94 ± 0.15[b]	2.14 ± 0.12[a]	2.20 ± 0.14[a]	2.29 ± 0.18[a]	2.21 ± 0.14[a]
TD (%)	89.42 ± 4.10[a]	69.43 ± 6.99[b]	67.03 ± 5.34[b]	72.23 ± 5.87[b]	69.22 ± 4.55[b]

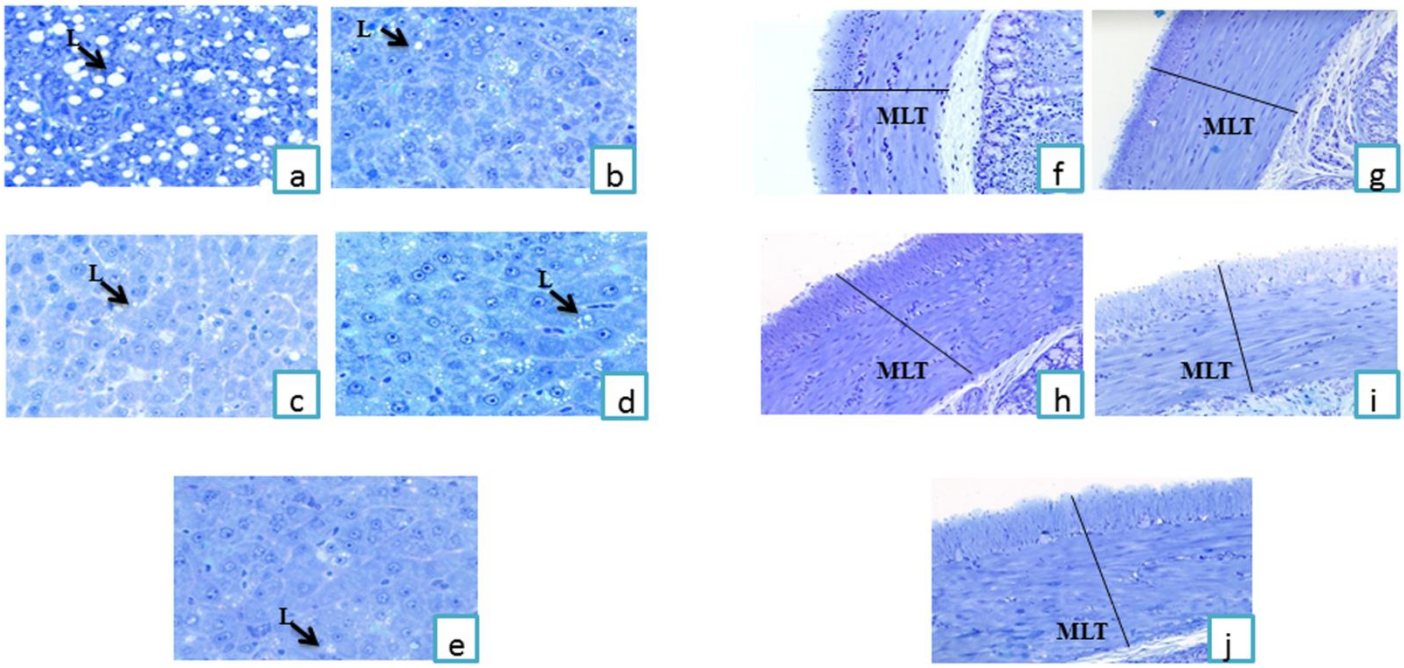
FER: food efficiency ratio; PER = protein efficiency ratio; NPR = net protein ratio; TD: true digestibility; HT = heat treatment. Average scores on the lines followed by different letters differ by Duncan test ($p<0.05$).

Table 4 Effect of chia intake on the biochemical variables in *Wistar* rats (n = 6) for a period of 28 days

Groups	Casein	Chia seed untreated	Chia seed with HT	Chia flour untreated	Chia flour with HT
Glucose (mg dL^{-1})	172.33 ± 14.22[a]	118.83 ± 7.31[b]	128.33 ± 20.66[b]	127.17 ± 20.72[b]	127.67 ± 16.77[b]
HDL (mg dL^{-1})	28.00 ± 4.38[b]	35.50 ± 3.23[a]	32.20 ± 3.63[a]	33.83 ± 3.50[a]	37.50 ± 4.36[a]
TC (mg dL^{-1})	63.83 ± 10.34[a]	58.17 ± 4.79[ab]	53.60 ± 10.06[b]	58.67 ± 11.91[ab]	61.00 ± 10.77[a]
TGL (mg dL^{-1})	47.50 ± 17.75[a]	27.33 ± 4.97[b]	27.33 ± 3.83[b]	26.34 ± 5.05[b]	32.50 ± 5.61[b]
AST (U L^{-1})	98.33 ± 15.96[b]	155.33± 32.21[a]	116. 50 ± 3.83[a]	139.33 ± 13.75[a]	126.33 ± 18.90[a]
ALT (U L^{-1})	26.50 ± 5.01[b]	46.17 ± 6.37[a]	38.33 ± 10.88[a]	44.00 ± 8.20[a]	45.50 ± 8.73[a]
VLDL (mg dL^{-1})	9.50 ± 3.55[a]	5.47 ± 0.99[b]	5.47 ± 0.77[b]	5.27 ± 1.01[b]	6.50 ± 1.12[b]
LDL (mg dL^{-1})	26.33 ± 11.13[a]	17.20 ± 4.79[b]	13.70± 8.47[b]	19.57 ± 4.59[b]	17.00 ± 5.93[b]
Urea (mg dL^{-1})	1.12 ± 0.19[a]	1.20 ± 0.23[a]	1.27 ± 0.31[a]	1.12 ± 0.19[a]	0.92 ± 0.43[a]
Creatinin (mg dL^{-1})	0.20 ± 0.04[a]	0.17 ± 0.06[a]	0.15 ± 0.02[a]	0.20 ± 0.04[a]	0.21 ± 0.09[a]

HT: heat treatment; HDL: high-density lipoprotein; TC: total cholesterol; TGL: triacylglyceride; AST: alanine aminotaminotransferase; ALT: aspartate aminotransferase; VLDL: lipoprotein very-low density; LDL: low- densit lipoprotein. Average scores on the lines followed by different letters differ by Duncan test ($p<0.05$)

ESM_1: Electronic Supplementary Material



Online Resource 3. Liver photo of animals (a, b, c, d, and e) and cecum photo of animals (f, g, h, i, and j). Groups treated with casein (a and f); Groups treated with seed chia untreated (b and g); Groups treated with seed chia with HT (c and h); Groups treated with chia flour untreated (d and i); Groups treated with chia flour with HT (e and j); HT: heat treatment; L: lipids; MLT: muscular layer total.

5.4. ARTIGO 3

HIGH FAT DIET DOES NOT AFFECT THE IRON BIOAVAILABILITY IN *WISTAR* RATS FEED WITH CHIA AND INCREASES GENE EXPRESSION OF IRON METABOLISM PROTEINS

ABSTRACT

This study evaluated the effect of chia on the iron bioavailability and gene expression of proteins involved in iron metabolism in animals fed with high fat diet and standard diet. Four experimental groups were tested (n=8): standard diet + ferrous sulfate (SD+FS); standard diet + chia (SD+C); high fat diet + ferrous sulfate (HFD+FS); high fat diet + chia (HFD+C). The hemoglobin gain, hemoglobin regeneration efficiency, biological relative value of HRE, serum ferritin and transferrin, liver iron concentration and gene expression of proteins were evaluated. The SD+C group showed lower transferrin expression when compared to the control group. The control group showed serum transferrin concentration higher than the others groups. Serum ferritin and liver iron concentration did not differ among the animals that received chia Ferritin and hephaestin expression was lower in experimental groups when compared with the control group. The peroxisome proliferator activated receptor expression was higher in animals fed with SD+C than in the control group. The expression of duodenal cytochrome B and divalent metal transporter 1 in the HFD+C group was higher and ferroportin was lower in the groups containing chia. Animals fed with chia showed similar iron bioavailability compared to animals fed with ferrous sulfate.

Keywords: gene expression; *Salvia hispanica* L.; lipids; ferritin; transferrin; PPAR-α; DMT-1; DcytB; ferroportin; hephaestin.

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1. Introduction

Iron is the fourth most abundant element in nature, comprising about 4.7% of the Earth's surface. Despite this abundance, iron deficiency is the most prevalent nutritional deficiency in the world, reaching more than 2 billion people. Iron deficiency anemia is highly prevalent in developing countries, but it also remains a problem in developed countries where other forms of malnutrition have been virtually eliminated [1,2].

Chia (S*alvia hispanica* L.) is a food that has been consumed by the world population due to its protective, functional and antioxidant effects [3,4], attributed to the presence of phenolic compounds, vitamins and minerals, including iron [5,6], which appears in a high concentration in this seed [7]. However, one should take into account the bioavailability of this mineral for use in metabolic processes or for storing and incorporation into heme iron [8]

Mechanisms to regulate iron homeostasis at the systemic and cellular levels are known. These involve transcriptional mechanism that regulates proteins involved in the expression of genes related with iron metabolism [9,10]. The uptake of ferric iron is mediated by duodenal cytochrome **b** (DcytB), which reduces iron to its ferrous (Fe^{2+}) form, and divalent metal transporter 1 (DMT-1), which transports the iron across the cell membrane of the enterocyte [11]. The main exporter of iron from the cell to the plasma is ferroportin and the hefaestin is responsible for converting Fe^{+2} into Fe^{+3} for its incorporation in transferrin. Ferritin, in turn, is responsible for the iron storage [12].

Intake of high fat diet is able to reduce the action of peroxisome proliferator activated receptors (PPARs) and increases iron transport genes, interfering therefore in iron metabolism [13]. The PPARs are ligand-activated transcription factors that activate the transcription of genes involved in many different processes, including lipid metabolism and inflammation [14]. Among the types of PPAR stands out PPAR-α, which is found in high concentration in the liver and stimulates lipid metabolism and regulates numerous genes involved in fatty acid uptake and activation [15–17]

There are no *in vivo* studies evaluating the iron bioavailability of chia as well as the influence of high fat diet intake in the expression of proteins involved in iron metabolism. High fat diet intake was used to verify if a diet containing high concentrations of lipids can reduce the bioavailability of iron. Since this food shows a high iron concentration, it is of great interest to determine the bioavailability of this nutrient *in vivo*. Thus, the objective of this study was to evaluate the effect of chia on

the bioavailability of iron and gene expression of proteins involved in iron metabolism in animals fed with high fat diet and standard diet.

2. Materials and Methods

2.1 Sample and staple food flours preparation

Chia seeds (*Salvia hispanica* L.) grown in the state of Rio Grande do Sul (Brazil) were used. To obtain the flour, the seeds were ground in three repetitions, using knife mill (sieve of 600 μm, size 30 mesh; Grinder Vertical Rotor MA 090 CFT, Marconi Equipment, Brazil) with a particle size of 850 micrometers. Subsequently, chia flour was packed in polyethylene bags covered with foil and stored in a freezer (-18 ± 1°C) until the time of analysis.

2.2 Determination of iron and zinc

The determination of iron and zinc content of food and iron content of the diets were performed according to Gomes [18]. Briefly, 1.0 g of the samples was oxidized with 10 mL of nitric acid for 8 hours at room temperature. After, the samples were heated in the digester block with ventilation at approximately 120°C for 16 hours. The iron and zinc concentrations were determined by coupled plasma atomic emission spectrometry (model Optima 3300 DV, Perkin Elmer, Massachusetts, USA) with an inducible plasma argon source. Analysis was performed under the following conditions: power of 1,300 W, plasma argon flow rate of 15 L min^{-1}, auxiliary argon flow rate of 0.7 L min^{-1}, nebulizer argon flow rate of 0.5 L min^{-1}, rate of sample introduction of 1.5 mL min^{-1}. The analyses were performed in triplicate (Table 1).

2.3 Chemical composition, phytate and phenolic compounds

The chemical composition was determined according to AOAC [19]. Phytate content was determined by ion exchange and spectrophotometry according to Latta; Eskin [20], with modifications [21]. The determination of the concentration of phenolic compounds in foods was performed using the Folin-Ciocalteu reagent [22]. The analyses were performed in triplicate (Table 1).

Table 1. Chemical composition and phytate/iron and zinc/iron molar ratio of chia, on a dry basis

Compounds	**Chia**
Moisture ($g.100g^{-1}$)	7.14±0.26
Ash ($g.100g^{-1}$)	4.56±0.04
Lipids ($g.100g^{-1}$)	32.16±0.29
Protein ($g.100g^{-1}$)	18.18±1.20
Total dietary fiber ($g.100g^{-1}$)	33.37±0.26
Soluble fiber	2.89±0.09
Insoluble fiber	30.47±0.35
Carbohydrates ($g.100g^{-1}$)	4.59±0.34
Total phenolic (mg de EqAG/g)	0.97±0.07
Minerals	
Iron (mg.$100g^{-1}$*)*	9.39±5.20
Zinc (mg.$100g^{-1}$*)*	3.65±0.95
Phytic acid (*mg.*$100g^{-1}$)	0.96±0.11
Molar Ratio	
Phytate/iron	8.56±1.67
Zinc/iron	0.33±0.02

Data presented as mean ± standard deviation.

2.4 Animals and diets

Controlled experimental test was used and the bioavailability of iron was evaluated by the hemoglobin depletion/repletion method modified [19]. At 21 days of age, 32 male rats (*Ratus norvegicus albinus* Wistar) from the Central Animal Facility of the Center for Life Sciences and Health at Federal University of Viçosa, Minas Gerais, Brazil, were placed in individual temperature-controlled (22 ± 2ºC) cages, with a photoperiod of 12 h. The experimental diets were based on the standard AIN-93G [23] and high fat diet (Research Diets, New Brunswick, NJ). The standard diet was

comprised by 22% protein, 15% fat and 63% carbohydrate. The high fat diet was prepared in the proportion 61% fat, 21% protein and 18% carbohydrate (Table 2).

Animals initially received a depletion diet containing Fe-free mineral mixture to reduce hemoglobin (Hb) concentrations and deionized water *ad libitum*, for 21 days [24]. Animals were then divided into four groups (n=8) so that the Hb concentration was not statistically different among groups: 1) standard diet + ferrous sulfate (SD+FS); 2) standard diet + chia (SD+C); 3) high fat diet + ferrous sulfate (HFD+FS); 4) high fat diet + chia (HFD+C). The repletion diet was pair fed to control food and Fe intake, and deionized water was offered *ad libitum*, for 14 days.

Table 2. Nutritional composition of experimental diets.

	Depletion Phase		Repletion Phase			
Ingredients (1kg of diet)	**Standard diet without iron**	**High fat diet without iron**	**SD + FS**	**SD + C**	**HFD + SF**	**HFD + C**
Ferrous sulfate (mg)	-	-	59.73	-	59.73	-
Chia (g)	-	-	-	127.80	-	127.80
Albumin (g)	218.22	280.00	218.22	188,36	280.00	250.18
Dextrinized starch (g)	132.00	132.00	132.00	132.00	132.00	132.00
Sucrose (g)	100.00	100.00	100.00	100.00	100.00	100.00
Soybean oil (mL)	70.00	70.00	70.00	28.91	70.00	28.91
Lard (g)	0.00	300.00	00.00	0.00	300.00	300.00
Microcrystalline cellulose (g)	50.00	50.00	50.00	7.36	50.00	7.36
Mineral mix without iron (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)	3.00	3.00	3.00	3.00	3.00	3.00
Choline bitartrate (g)	2.50	2.50	2.50	2.50	2.50	2.50
BHT (g)	0.008	0.008	0.008	0.008	0.008	0.008
Corn starch (g)	379.27	17.49	379.09	365.09	17.43	3.23
Nutritional composition						
Total calories (Kcal)	3847.88	5447.96	4017.80	3864.89	5447.72	5364.67
Caloric density (Kcal/g)	4.15	5.65	4.20	4.17	5.65	5.58
Iron (mg/Kg)*	0.30 ± 0.02^{b}	0.28 ± 0.04^{b}	18.37 ± 0.25^{a}	19.17 ± 0.49^{a}	18.63 ± 0.55^{a}	18.30 ± 0.53^{a}

* Analyzed according to the methodology proposed by Gomes (1996). SD+FS: standard diet + ferrous sulfate; SD+C: standard diet + chia; HFD+FS: high fat diet + ferrous sulfate; HFD+C: high fat diet + chia. Means with different letters in the same line show significant difference ($p<0.05$) by Newman-Keuls test

In the repletion phase chia was used as a source of iron, and the ferrous sulfate in AIN-93G diet was used as a positive control, and 12 mg of iron per kg of diet were used for each treatment. At the end of both depletion and repletion phases, blood samples were collected from the rat tails to determine Hb concentrations. On the 36th day, after fasting for 12 hours, the animals were anesthetized with isoflurane (Isoforine, Cristália®) and were euthanized by cardiac puncture [24]. Blood was stored in a test-tube and centrifuged under 4°C at 3000 rpm for 10 min (Fanem-204, São Paulo, Brazil) to obtain serum. The liver and duodenum were removed and were immediately frozen in liquid nitrogen and stored at - 80°C before analyses.

All experimental procedures with animals were performed in accordance with the ethical principles for animal experimentation and the study was approved by the Ethics Committee of the Federal University of Viçosa (Protocol 97/2014; date of approval: March 13th 2015).

2.5 Blood tests

Serum hemoglobin was measured by cyanide methemoglobin method [19], using a colorimetric kit (Bioclin®, Brazil) for *in vitro* diagnosis. A volume of 20 µL of blood was pipetted and mixed with 5 mL of Drabkin's solution color regent (containing potassium cyanide, and hydrogen cyanide). The reading of absorbance was done in UV-Visible Multiskan (Thermo Scientific, Massachusetts, MA, USA) at a wavelength of 540 nm. The analyses of ferritin and transferrin serum were performed using a specific kit, according to manufacturer's recommendation (Bioclin®, Brazil).

2.6 Liver iron concentration

The liver iron concentration was carried out by atomic absorption spectrophotometry, according to Gomes [18].

2.7 Iron bioavailability

The iron bioavailability was calculated according to Hernandez et al. [25]. The hemoglobin regeneration efficiency (HRE%) was calculated by the formula: HRE% = [(mg Fe final Hb - mg Fe initial Hb) / 100] / mg Fe consumed. The iron in hemoglobin content was estimated by: [Body weight (g)*Hb (g/L)*0.335*6.7]/1000. This variable was calculated assuming the total blood volume equals to 6.7% of the rats' body weight,

and the body iron in hemoglobin content as being 0.335. The use of iron was calculated as: [HRE% *% dietary iron]/100, and the absorption of iron was calculated as: [Fe intake - excretion Fe].

2.8 Extraction of mRNA in liver and duodenal tissue and cDNA synthesis

The tissues were macerated in liquid nitrogen in RNAse free conditions and samples were aliquoted for total RNA extraction. Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) using the manufacturer's recommendations. 2 µL of extracted mRNA were used to synthesize the cDNA using M-MLV reverse transcription kit (Invitrogen Corp., Grand Island, USA) according to the manufacturer's protocol.

2.9 Determination of gene expression of proteins involved in iron metabolism by reverse transcriptase polymerase chain reaction (RT-qPCR)

Expression of mRNA levels in the duodenal mucosa and the liver of proteins involved in iron metabolism were analyzed by RT-qPCR. The SYBR green PCR master mix from Applied Biosystems (Foster City, CA, USA) was used and analyses were performed on the StepOne™ Real-Time PCR System (Thermo Fisher Scientific) using the measurement system by SYBR-Green Fluorescence and Primer Express software (Applied Biosystems, Foster City, CA, USA). The PCR involved an initial denaturation cycle of 95°C (10 min) and then 40 cycles with 1 min denaturation (94°C), 1 min annealing (56°C) and 2 min elongation (72°C), followed by a standard dissociation curve. Sense and antisense primer sequences (GenOne Biotechnologies, Rio de Janeiro, Brazil) were used to amplify protein divalent metal carrier (DMT-1), duodenal cytochrome b (DcytB), ferroportin and hephaestin from duodenum, and proteins ferritin, transferrin and PPAR-α from liver. The relative expression levels of mRNA were normalized by the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 3). All steps were performed using open conditions with RNase.

Table 3. Sequence of primers used in the RT-PCR analysis.

Genes	Oligonucleotide (5'-3')	
	Forward	Reverse
GAPDH	AGGTTGTCTCCTGTCACTTC	CTGTTGCTGTAGCCATATTC
DMT-1	CTGATTTACAGTCTGGAGCAG	CACTTCAGCAAGGTGCAA
DcytB	TGCAGACGCAGAGTTAAGCA	CCGTGAAGTATACCGGCTCC
Ferroportin	TTCCGCACTTTTCGAGATGG	TACAGTCGAAGCCCAGGACCGT
Hephaestin	GGCACAGTTACAGGGCAGAT	AGTAACGTGGCAGTGCATCA
Ferritin	CAGCCGCCTTACAAGTCTCT	ATGGAGCTAACCGCGAAGAC
Transferrin	AGCTGCCACCTGAGAACATC	CGCACGCCCTTTATTCATGG
PPAR-α	CCTGCCTTCCCTGTGAACT	ATCTGCTTCAAGTGGGGAGA

GAPDH: glyceraldehyde 3-phosphate dehydrogenase; DMT-1: divalent metal transporter-1 Protein; DcytB: duodenal cytochrome B; PPAR-α: peroxisome proliferator-activated receptor alpha.

2.10 Statistical Analysis

Chia flour was analyzed in replicates. The results were analyzed by ANOVA. For significant "F-value", post hoc Student Newman-Keuls test was used to compare the averages of all experimental groups. The mean dispersion was expressed as standard deviation. Statistical analyses were carried out using SPSS statistics version 20.0 software considering a 5% significance level.

3. Results

3.1 Concentration of iron in the experimental diets

The experimental diets showed approximately 18.54 mg Fe/kg, with no difeerences from each other ($p>0.05$).

3.2 Effect of consumption of chia on the bioavailability of iron in rats fed with AIN-93G diet and high fat diet

It was observed that the total diet consumption and iron intake were lower in animals that received high fat diet ($p\leq0.05$). The depletion phase (three weeks) was sufficient to produce anemia in the animals, with hemoglobin means equals to 5.69 ± 1.02 g/dL. In the repletion period, all groups showed body weight gain similar to that of

the controls groups (SD+FS and HFD+FS). Body weight gain, hemoglobin concentrations, hemoglobin gain, HRE%, RBV-HRE did not differ ($p>0.05$) among the experimental groups (Table 4).

Table 4. Total consumption, body weight gain, total iron intake, hemoglobin levels and indices for assessing iron bioavailability in the repletion phase (n=8).

	SD + FS	**SD + C**	**HFD + FS**	**HFD + C**
Total consumption (g)	192.45±16.48[a]	193.24±15.28[a]	145.49±16.79[b]	158.93±20.44[b]
Body weight gain (g)	41.25±9.86[a]	41.00±3.16[a]	42.63±10.51[a]	41.50±9.48[a]
Fe intake (g)	2.31±0.19[a]	2.32±0.18[a]	1.75±0.20[b]	1.91±0.24[b]
Initial hemoglobina (g/dL)	5.58±0.99[a]	5.53±0.75[a]	5.82±1.15[a]	5.83±1.19[a]
Final hemoglobin (g/dL)	10.20±1.17[a]	9.15±0.99[a]	9.40±1.09[a]	9.82±0.99[a]
Hemoglobin gain (g/dL)	4.83±1.48[a]	3.81±1.22[a]	3.58±1.27[a]	4.00±1.82[a]
HRE%	105.35±27.52[a]	91.64±18.48[a]	102.35±24.49[a]	103.39±30.97[a]
RBV-HRE	1.01±0.28[a]	0.94±0.19[a]	0.93±0.22[a]	0.94±0.28[a]

Data presented as mean ± standard deviation. SD+FS: standard diet + ferrous sulfate; SD+C: standard diet + chia; HFD+FS: high fat diet + ferrous sulfate; HFD+C: high fat diet + chia; Fe: iron; HRE: hemoglobin maintenance efficiency; RBV-HRE: relative biological value of HRE. Means followed by different small letters in line differ by Newman-Keuls test ($p<0.05$).

3.3 Gene expression of proteins involved in iron metabolism, analysis of ferritin and transferrin serum and liver iron concentration

The mRNA expression of DMT-1 (Fig. 1A) and DcytB (Fig. 1B) was higher ($p\leq0.05$) in the group that received high fat diet containing chia (HFD + C) when compared to the control group SD + FS (6.92 and 2.18 fold, respectively). However, the hephaestin expression was lower in all experimental groups compared with the control group (SD + FS) (Fig. 1C). The gene expression of ferroportin (Fig. 1D) was lower in the groups containing chia (0.16 and 0.01 fold for SD + C and HDF + C, respectively) when compared to the control group (SD+FS; $p\leq0.05$), while HFD + SF showed similar results to the control group ($p>0.05$).

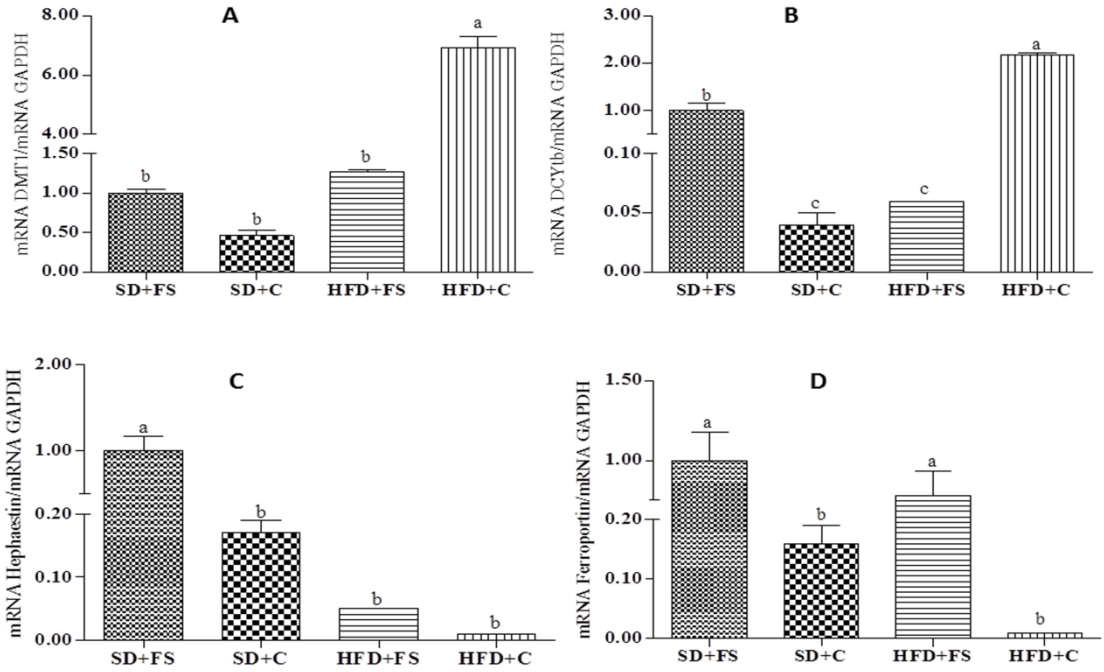

Figure 1. Effect of chia intake (standard diet and high fat diet) on the gene expression of proteins in duodenal tissue. RT-qPCR Analysis. (A) DMT-1, (B) Dcytb (apical membrane of the enterocyte), (C) Hephaestin (D) Ferroportin (the enterocyte basolateral membrane). SD+FS: standard diet + ferrous sulfate; SD+C: standard diet + chia; HFD+FS: high fat diet + ferrous sulfate; HFD+C: high fat diet + chia. Different letters indicate statistical differences at 5% probability by Newman-Keuls test.

The SD + C group showed 0.51 times less expression of transferrin when compared to the control group (SD + FS), while the groups treated with high fat diet showed a higher expression (2.01 fold for HFD + FS and 3.93 fold for HFD + C; $p \leq 0.05$; Fig 2B). However, analyzing the serum transferrin concentration, it is possible to verify that the control group (SD + FS) showed higher ($p \leq 0.05$) concentration of this protein compared to the other experimental groups. The ferritin expression was lower in all experimental groups compared to the control (FS + SD; $p \leq 0.05$; Fig 2D). The analysis of liver iron concentration and serum ferritin showed that the group fed with high fat diet with ferrous sulfate (HFD + FS) showed a lower ($p \leq 0.05$) concentration of iron stores markers, which can be observed by the lower iron concentration in tissue and serum ferritin in relation to the control group (SD + FS). The increased PPAR-α gene expression (Fig.2F) in animals fed with standard diet with chia (SD + C) was 3.32 fold higher ($p \leq 0.05$) than that observed in the control group (SD + FS), while the groups

treated with high fat diet showed reduced 0.28 and 0.39 fold (HFD + FS and HFD + C, respectively).

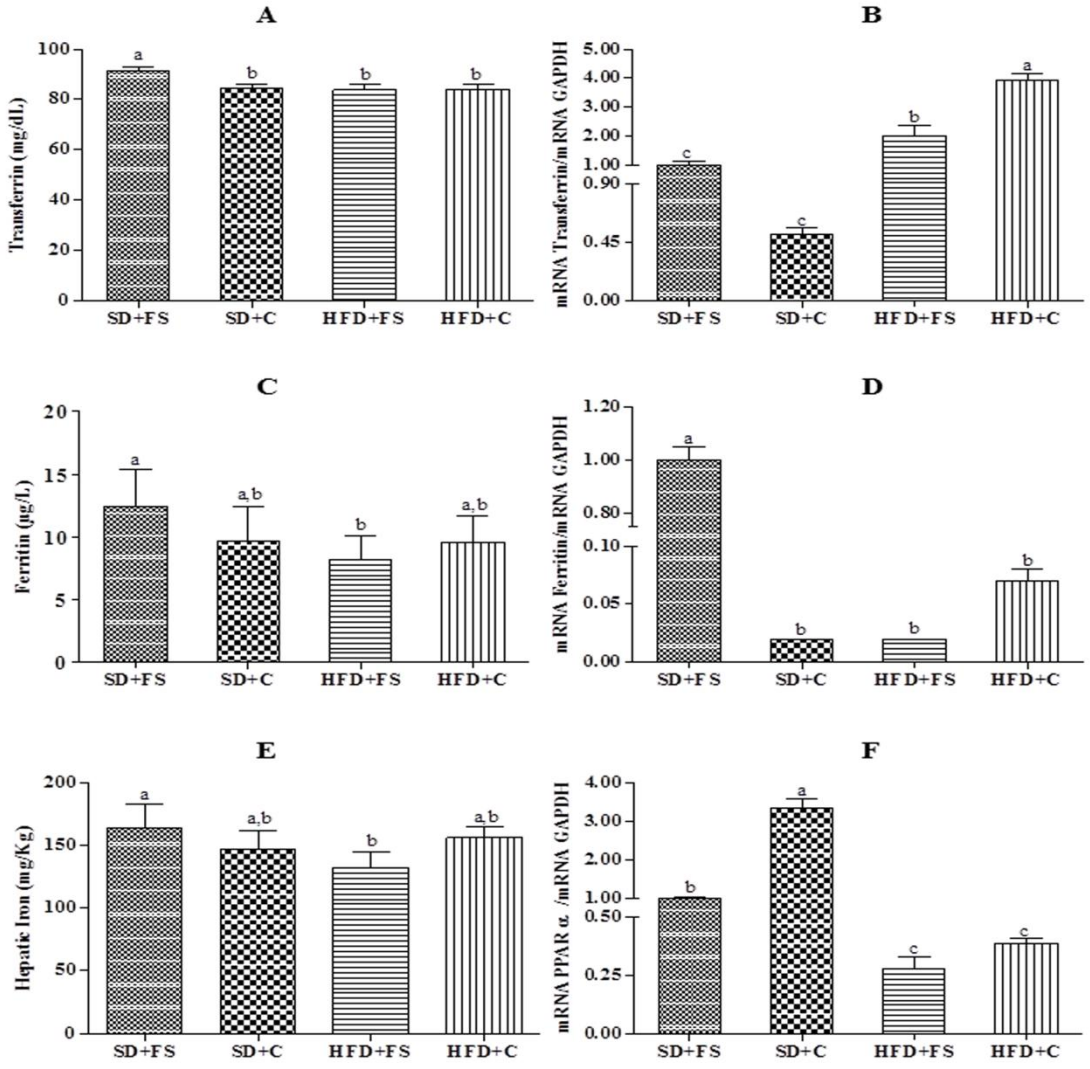

Figure 2. Effect of chia intake on the gene expression of proteins and on serum and tissue markers of iron stores in liver tissue. RT-qPCR analysis. (A and B) Transferrin; (C, D and E) Ferritin; (F): PPAR-α. SD+FS: standard diet + ferrous sulfate; SD+C: standard diet + chia; HFD+FS: high fat diet + ferrous sulfate; HFD+C: high fat diet + chia. Different letters indicate statistical differences at 5% probability by Newman-Keuls test.

4. Discussion

Studies that evaluated the iron bioavailability of chia in animals fed with standard diet and high fat diet are not found. Thus, the present study focused on the

bioavailability of iron from chia in animals fed with standard diet and high fat diet to verify if a diet containing high concentrations of lipids, such as that consumed by western diets, can reduce the bioavailability of iron in the body.

The influence of phytate on the bioavailability of iron depends not only on the phytate contents in the diet but also on the interaction between phytate and iron. The phytate: minerals molar ratios can be used to predict the inhibitory effect of phytate on the bioavailability of minerals, such as iron [26]. The molar ratio phytate: iron in the present study was 8.56. Phytate: iron molar ratio lower than 1 can decrease the iron absorption in humans [27]. However, molar ratio phytate: iron of 27 did not affect the iron bioavailability from staple crops enriched with iron or carotenoids [24] in agreement with the present study, which used the same experimental model.

The molar ratio zinc: iron was 0.33, without effect on iron biovaliability, since researches that measured the effect of zinc intake on iron absorption in human subjects conclude that when zinc is present in a meal, a ratio of 5:1 (zinc to iron) did not interfere with iron absorption [28]. Also, the concentration of phenolic compounds offered by chia did not affect the iron bioavailability. The same was observed in crops combinations of beans and rice, targets for iron and carotenoids biofortification [24].

The lower consumption of high fat diet found in this study can be attributed to higher energy density and greater satiety by animals. The same was observed in a study which evaluated the iron status of rats fed with high fat diet, and the authors concluded that the reduction of consumption occurs due to higher energy concentration of the diet [29]. The gain weight of the animals fed with different diets did not differ. This result was also observed in other studies that evaluated the intake of high fat diet in rats for 16 and 12 weeks [29,30]. The consumption of chia, regardless of being available on the standard diet or high fat diet resulted in a gain of hemoglobin, hemoglobin regeneration efficiency and relative biological value of hemoglobin regeneration efficiency similar to that of standard group supplemented with ferrous sulfate, suggesting that the iron in chia showed good bioavailability.

DcytB is responsible for converting Fe^{+3} to Fe^{+2} for absorption in enterocytes [31]. We found that a DcytB mRNA expression was higher in animals that received high fat diet with chia. This fact can be explained by the absorptive efficiency which increases the front lower intake of iron. However, a study that evaluated rats fed with

standard diet and high fat diet found decreased DcytB mRNA expression in animals fed with high fat diet compared with animals feed with standard diet [32].

DMT-1 plays a role to transport Fe^{+2} into the enterocyte [33]. Animals that received high fat diet with chia showed duodenal higher DMT-1 mRNA expressions when compared to animals fed with standard diets (sulfate ferrous and chia) and high fat diet with sulfate ferrous. This result is expected since it is known that mRNA levels of DMT-1 significantly increased in iron deficiency in experimental models [34], increasing the captation. The same result was observed in others studies [32,35–37]. Thus, when the ferrous sulfate was associated with the high fat diet, animals exhibited decreased DMT-1 expression, suggesting that supplementation (ferrous sulfate), as expected, was more effective than chia intake.

Hephaestin is a ferroxidase that converts Fe^{+2} to Fe^{+3} for incorporation into transferrin [12]. The hephaestin mRNA expression was lower in animals that received high fat diet and standard diet with chia. This result is expected, leading to lower oxidation of iron and consequently less absorption and iron accumulation in enterocytes and macrophages [12]. A study that evaluated animals fed with standard diet and high fat diet found decreased hephaestin mRNA expression in rats fed with high fat diet when compared with standards animals [32].

Deficiency of iron intake causes increased in absorption of this mineral in the body. To meet this increased demand, there is a greater expression of proteins involved in this process, as ferroportin [38], that carries Fe^{+2} the enterocyte into the bloodstream [39]. However, this result was not found in our study providing further evidence that intestinal iron absorption was affected by chia intake. Chia intake was able to reduce ferroportin expression, since the animals fed with standard diet and high fat diet containing chia showed less expression of the gene.

Transferrin has the function of iron transporting in the bloodstream [40]. The animals fed with standard diet with ferrous sulfate showed higher serum transferrin concentration in relation to the others experimental groups, showing that, in this group, there was a higher translation of transferrin, increasing its concentration in serum. However, the animals fed with high fat diet showed increased transferrin expression compared to standard group. This reinforces the idea that the front lower consumption of the mineral, as observed by intake of iron values in animals fed with high fat diet, there is an increased gene expression since the body has a compensating action in order

to maintain the homeostasis [41]. In addition, in our study, the animals fed with standard diet containing chia and high fat diet with chia or ferrous sulfate had lower ferritin expression than other diets, thereby suggesting lower iron storage. Similar results were observed in another study that evaluated the expression of genes involved in iron metabolism after intake of high fat diet [42]. However, when the analyses of liver iron concentration and serum ferritin were performed, we observed that the animals fed with high fat diet with ferrous sulfate presented lower liver iron concentration and serum ferritin concentration in relation to the control group. Thus, the ferritin translation in animals that ingested chia in standard diet or in hight fat diet was similiar to the animals that received control diet, thus showing the efficiency of chia in iron storage.

The intake of high fat diet for 5 weeks causes a reduction in the PPAR-α mRNA expression. This may be related to the installation of inflammation present in the diet-induced obesity, since a short period of time was able to affect the inflammatory marker [43]. It is known that PPAR-α ligands have anti-inflammatory effects in various cells for causing apoptosis in cytokine-activated macrophages and inhibiting of NF-kB signaling [44,45]. In addition, the increased inflammation may increase the hepicidin production and affect the ferroportin production, impairing the bioavailability of iron [46]. Thus, we conclude that chia, in a standard diet, was able to reduce the anti-inflammatory effects since the mRNA expression was higher in animals fed with standard diet with chia than those who received standard diet with sulfate ferrous. Furthermore, the lower PPAR-α expression observed in the groups that receive high fat diet confirm the presence of inflammation arising out diet intake containing high concentrations of lipids. Different outcome of our study was observed in animals fed with high fat diet for 26 weeks reported an increase in hepatic expression of PPAR-α [15].

5. Conclusions

The high fat diet intake during 5 weeks was not able to interfere with the gain of hemoglobin, hemoglobin maintenance efficiency and relative biological value of hemoglobin maintenance efficiency and increased some gene expression of proteins involved in iron metabolism. Animals fed with high fat diet showed similar iron bioavailability to animals fed with standard diet. The animals fed with standard diet

with ferrous sulfate showed serum transferrin concentration higher than the others experimentals groups. Serum ferritin concentration and liver iron concentration did not differ among the animals that received chia, in standard or high fat diet, in relation to the animals that received standard diet with ferrouse sulfate (control group).

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

Abbreviations

The following abbreviations are used in this manuscript:

BHT: Butylated hydroxytoluene
cDNA: Complementary DNA
DCYTb: Duodenal cytochrome B
dL: deciliter
DMT-1: Divalent metal transporter 1
DNAse: Deoxyribonuclease
EqAG: Gallic acid equivalente
Fe: Iron
g: gram
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
HFD+C: High fat diet + chia
HFD+FS: High fat diet + ferrous sulfate

HG: Hemoglobin gain

HRE%: Hemoglobin regeneration efficiency

IRP1: Iron - responsive element - binding protein 1

IRP2: Iron - responsive element - binding protein 2

Kg: Kilogram

mg: Milligram

mL: Milliliter

mRNA: Messenger RNA

PPAR-α: Peroxisome proliferator-activated receptors

RBV-HRE: Biological relative value of HRE

RT-PCR: Reverse transcription polymerase chain reaction

SD+C: Standard diet + chia

SD+FS: Standard diet + ferrous sulfate

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6. GENERAL CONCLUSIONS

The Brazilian seed chia evaluated in this study showed high nutritional value, especially for polyunsaturated fatty acids, protein and dietary fiber, and is a source of minerals (especially calcium, iron and manganese) and vitamin E. Chia grown in Rio Grande do Sul presented higher concentration of lipids, iron, manganese, boron, aluminum, nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, copper and antioxidant capacity than chia grown in Mato Grosso.

The consumption of chia seed or chia flour, with or without thermal treatment for a short period of time (28 days), showed good protein digestibility, hypoglycemic effect, improved lipid profile, reduced the deposition of fat in liver and promoted changes in intestinal tissue of *Wistar* rats, favoring its functionality. The heat treatment used in the study (90°C for 20 minutes) was not able to inactivate the tannins, phytic acid and total phenolic compounds that can link to the proteins presented in chia, since it was not observed better true digestibility in groups fed with chia seed or chia flour heat treated.

In relation to the iron bioavailability in chia, the animals fed with high fat diet showed iron bioavailability similar to animals fed with standard diet. The iron presented in chia showed bioavailability similar to the ferrous sulfate. A high intake of lipids during 5 weeks did not affect the gain of hemoglobin, hemoglobin maintenance efficiency, relative biological value of hemoglobin maintenance efficiency, and increased gene expression of proteins involved in iron metabolism, although it reduced the iron reserve in *Wistar* rats.

7. FINAL CONSIDERATIONS

Through the obtained results, the use of chia should be recommended in the amount of 20 g/day (1 heaped tablespoon). This amount will provide approximately 3.5 grams of protein, 6 g of lipids and 7 grams of dietary fiber, daily.

As a limitation of protein quality study, we can mention the fact that the animals showed preference for chia flour consumption in relation to chia seed consumption. In relation to the iron bioavailability study, it was not possible to perform the analysis of hepcidin gene expression, an important marker of iron metabolism. The analysis of this protein is very high cost, which prevented its realization in our study.

The chia showed high concentration of calcium, and there are no literature studies evaluating the bioavailability of this mineral *in vivo*. Thus, the realization of this analysis would be interesting in order to contribute with data about this food, which has been widely used by the world's population.

8. APPENDIX

APPENDIX 1. RECEIPT OF APPROVAL OF RESEARCH PROJECT BY THE ETHICS COMMITTEE ON RESEARCH WITH ANIMALS (UFV)

Viçosa, 13 de março de 2015

Ilma. Profª.
Helena Maria Pinheiro Sant'Ana
Coordenadora do projeto
DNS/UFV

Srª. Coordenadora,

Após avaliação da Metodologia utilizada no Projeto de Pesquisa intitulado "Chia *Salvia hispanica L.*: ocorrência e concentração de nutrientes e compostos bioativos, qualidade proteica e biodisponibilidade de ferro", aqui nomeado Processo 97/2014, a CEUA/UFV emite parecer favorável ao protocolo de utilização de animais proposto, tendo como base para análise a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTI, bem como 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.

Acresce a esse Parecer a exigência de Relatório Final de Atividades conforme itens a seguir:

RESUMO DOS RESULTADOS FINAIS OBTIDOS A PARTIR DOS EXPERIMENTOS ENVOLVENDO A ***UTILIZAÇÃO DE ANIMAIS*** *NO PROJETO DE PESQUISA*

1 Número do protocolo de submissão do projeto de pesquisa à CEUA/UFV:
2 Metodologia completa obrigatoriamente com:
- Local (is) Geral (is) e específico (s) oficial (is) onde ocorreu a experimentação;
- O nome científico do animal em questão;
- Número total de animais utilizados na pesquisa.
3 Resultados:
4 Nome do Coordenador do Projeto:
Assinatura:
5 Nome do Responsável Técnico:
Assinatura:
Inscrição em CRMV:

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

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 97/2014, intitulado "Chia *Salvia hispanica L.*: ocorrência e concentração de nutrientes e compostos bioativos, qualidade proteica e biodisponibilidade de ferro", coordenado pela professora Helena Maria Pinheiro Sant'Ana do Departamento de Nutrição e Saúde, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTI, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTI, portanto sendo aprovado por esta Comissão em 13/03/2015, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 97/2014, named " Chia Salvia hispanica L .: occurrence and concentration of nutrients and bioactive compounds, protein quality and bioavailability of iron", is in agreement with the a ctual Brazilian legislation (Lei Nº 11.794, 2008), Normative Resolutions edited by CONCEA/MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific Purposes and Teaching) and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI therefore being approved by the Committee on March 13, 2015 valid for 12 months.

Átima Clemente Alves Zuamon

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