OSCAR DAVID MEDINA MARTINEZ

EFFECT OF WHOLE SORGHUM BRS 305 HYBRID FLOUR ON INFLAMMATION, OXIDATIVE STRESS, GLUCOSE METABOLISM, ADIPOSITY, HEPATIC LIPOGENESIS, AND INTESTINAL HEALTH IN RATS FED WITH A HIGH-FAT HIGH-FRUCTOSE DIET

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

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BIOGRAPHY

OSCAR DAVID MEDINA MARTINEZ, filho de Roger Antonio Medina Guerra e de Ruth Marlene Martinez Villeroz, nasceu em Sincelejo, Sucre, Colômbia, no dia 10 de novembro de 1990.

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Em março do 2018, iniciou o doutorado no Programa de Pós-Graduação em Ciência da Nutrição da Universidade Federal de Viçosa, na área de Valor Nutricional e Funcional de Alimentos e de Dietas, com foco no efeito do sorgo na inflamação, estresse oxidativo, metabolismo da glicose, adiposidade, lipogêneses e esteatose hepática e saúde intestinal de ratos alimentados com dietas ricas em gordura saturada e frutose.

ABSTRACT

MEDINA MARTINEZ, Oscar David, D.Sc., Universidade Federal de Viçosa, September 2022. **Effect of whole sorghum flour BRS 305 on inflammation, oxidative stress, glucose metabolism, adiposity, hepatic lipogenesis and intestinal health in Wistar rats fed with a high-fat high-fructose diet.** Advisor**:** Hércia Stampini Duarte Martino**.** Co-advisors: Frederico Augusto Ribeiro de Barros, Valéria Aparecida Vieira Queiroz, Mariana Grancieri and Bárbara Pereira da Silva.

Introduction: The consumption of high fat and high fructose (HFHF) diet promotes an increase in free fatty acids (FFA) circulating in adipose, muscle and liver tissues, causing an increase in adipogenesis that results in the accumulation of visceral fat, insulin resistance, inflammation and impairment of lipid metabolism, as well as an increase in fatty deposits in the liver that results in liver steatosis. In this sense, sorghum BRS 305 stands out for being a cereal with bioactive compounds that can help prevent obesity and hepatic steatosis with the ability to modulate the composition of the intestinal microbiota, reducing the problems generated by the diet in addition to being a low-cost, easy-to-produce, gluten-free. **Objective**: The aim of the study was evaluate the effect of sorghum flour on insulin resistance, glucose tolerance, adiposity, hepatic steatosis, microbiota modulation, inflammation and oxidative stress in Wistar rats fed a high-fat high-fructose diet. **Methodology**: The study was divided into two phases. In the first phase male *Wistar* rats (n=30) at 40 days of age, were allocated into two groups: control AIN93-M (normal control: n=10) and high-fat and high-fructose (HFHF) (30% pork fat and 20% of fructose) (n=20) for 7 weeks to induce metabolic changes. In the second phase, the control AIN93-M manteined with the same diet, and the HFHF group were divided into two groups: HFHF (HFHF control: n=10) and HFHF + sorghum flour (replacing 50% dietary fiber, 100% starch, 19.8% protein and 22.5% lipids in the experimental diet), (n=10), for 10 weeks. At the end of the period, the animals were euthanized. Blood, liver, feces, colon and adipose tissues were collected to evaluate biochemical and inflammatory markers, intestinal microbiota and antioxidant capacity. Data will be submitted to ANOVA followed by the Newman-Keuls test (α=0.05) performed in the GraphPadPrism software, version 7.0. Microbiota data were corrected for multiple comparisons using false discovery rate (FDR) in the STAMP software. **Results: Article 1**: The consumption of sorghum flour reduced

adiposity, as well as the levels of triglycerides, uric acid, alanine aminotransferase, hepatic steatosis and lipogenesis. In addition, sorghum improves glucose metabolism by act directly on insulin sensitivity and glucose tolerance and increased the concentration of PPARα protein in the liver. **Article 2:** Sorghum flour decreased the concentration of nitric oxide, phosphoprotein kinase B (Akt), nuclear factor-kappa B (p65-NFκB), toll-like receptor 4 (TLR4) and lipid peroxidation in the liver. In addition, sorghum flour improved superoxide dismutase and catalase activities and total plasma antioxidant capacity. **Article 3:** The intake of the whole sorghum flour showed higher relative abundance of Firmicutes and higher Firmicutes/Bacteroidetes ratio in relation to the other experimental groups, and lower abundance of Bacteroidetes when compared to the HFHF group. Despite this, sorghum increased the production of short-chain fatty acid (SCFA) as propionate and the circular muscle layer, as well as the abundance of the genera *Roseburia* and *Lachnospiraceae_NK4A136_group* in relation to the HFHF group. **Conclusion**: Consumption of sorghum BRS 305 with high content of tannin and resistant starch improved inflammation and oxidative stress by inhibiting the activation of NFκB-p65; further modulated adiposity and glucose metabolism, reducing liver steatosis and lipogenesis. Finally, the consumption of the HFHF diet associated with sorghum flour modulated the composition of the intestinal microbiota and the abundance of SCFA-producing bacteria.

Keywords: Adiposity. Metabolic change. Resistant starch. Tannins. Glucose tolerance. Intestinal microbiome. Free fatty acids. Intestinal homeostasis. Short-chain fatty acids.

RESUMO

MEDINA MARTINEZ, Oscar David, D.Sc., Universidade Federal de Viçosa, setembro de 2022. **Efeito da farinha integral de sorgo BRS 305 na inflamação, no estresse oxidativo, no metabolismo de glicose, na adiposidade, na lipogênese hepática e na saúde intestinal de ratos alimentados com dieta rica em gordura saturada e frutose.** Orientadora: Hércia Stampini Duarte Martino. Coorientadores: Frederico Augusto Ribeiro de Barros, Valéria Aparecida Vieira Queiroz, Mariana Grancieri E Bárbara Pereira da Silva.

Introdução: O consumo de dietas rica em gordura saturada e frutose (HFHF) promove o incremento nos ácidos graxos livres circulantes nos tecidos adiposo, muscular e hepático, proporcionando aumento na adipogênese que resulta no acúmulo de gordura visceral, resistência à insulina, inflamação e comprometimento do metabolismo lipídico, bem como aumento nos depósitos de gordura no fígado que resulta em esteatose hepática. Nesse sentido o sorgo BRS 305 se destaca por ser um cereal com compostos bioativos que podem auxiliar na prevenção da obesidade e da esteatose hepática pela capacidade de modular a composição da microbiota intestinal reduzindo os agravos gerados pela dieta, além de ser de baixo custo, fácil produção e sem glúten. **Objetivo**: O objetivo do estudo foi avaliar o efeito da farinha de sorgo do híbrido BRS 305 na resistência à insulina, na tolerância a glicose, na adiposidade, na esteatose hepática, na modulação da microbiota intestinal, na inflamação e no estresse oxidativo de ratos Wistar alimentados com dieta rica em gordura saturada e frutose. **Metodologia**: O estudo foi dividido em duas fases. Na primeira, ratos machos *Wistar* (n=30), com 40 dias de idade, foram alocados em dois grupos: controle com dieta AIN93-M (controle normal: n=10) e com dieta rica em gordura satura e frutose (HFHF) (30% de gordura de porco e 20% de frutose) (n=20) durante 7 semanas para indução das alterações metabólicas. Na segunda fase, os animais do grupo 1 (controle) continuaram com a mesma dieta, e os animais do grupo HFHF foram divididos em dois grupos: HFHF (controle HFHF: n=10) e HFHF + farinha de sorgo, (n=10), por 10 semanas. Ao final do período os animais foram eutanasiados. Foi coletado sangue, fígado, fezes, colón e tecido adiposo para avaliação das alterações bioquímicas, inflamatórias, microbiota intestinal e ação antioxidante. **Resultados: Artigo 1:** O consumo da farinha integral de sorgo reduziu a adiposidade, assim como os níveis de triglicerídeos, ácido úrico, alanina aminotransferase, esteatose hepática

e lipogênese. Além disso, o sorgo melhorou o metabolismo da glicose, atuando diretamente na sensibilidade à insulina e tolerância à glicose e aumentou a concentração da proteína PPARα no fígado. **Artigo 2:** A farinha integral de sorgo diminuiu a concentração de oxido nítrico, fosfoproteína quinase B (Akt), fator nuclear-kappa B (p65-NFκB), receptor toll-like 4 (TLR4) e a peroxidação lipídica no fígado. Além disso, a farinha integral sorgo melhorou as atividades de superóxido dismutase e catalase e a capacidade antioxidante total do plasma, demostrando seu efeito na melhora do estresse oxidativo e redução da inflamação. **Artigo 3:** A ingestão da farinha integral de sorgo apresentou maior abundância relativa de Firmicutes e maior razão Firmicutes/Bacteroidetes em relação aos demais grupos experimentais, e menor abundância de Bacteroidetes em relação ao grupo HFHF. Apesar disso, o sorgo aumentou a produção de ácido graxo de cadeia curta (AGCC) como propionato e a camada muscular circular, assim como a abundância dos gêneros *Roseburia* e *Lachnospiraceae_NK4A136_group* em relação ao grupo HFHF. **Conclusão:** O consumo de sorgo BRS 305 com alto teor de tanino e amido resistente melhorou a inflamação e o estresse oxidativo pela inibição da ativação do NFκB-p65, também modulou a adiposidade e o metabolismo da glicose reduzindo a esteatose e a lipogênese hepática, além disso, o consumo da dieta HFHF associada à farinha de sorgo modulou a composição da microbiota intestinal e a abundância de bactérias produtoras de AGCC.

Palavras-chave: Adiposidade. Alterações metabólicas. Amido resistente. Taninos. Tolerância à glicose. Microbioma intestinal. Ácidos graxos livres. Homeostase intestinal. Ácidos graxos cadeia curta.

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LIST OF ACRONYMS AND ABBREVIATIONS

AIN-93M: normal control diet
AOAC: Association of official analytical chemistry
ALT - alanine aminotransferase
AST - aspartate aminotransferase
AKT: protein kinase B
AMPK: Adenosine monophosphate-activated protein kinase
AUC: Area under the curve
CAT: catalase
CNA: comprimento nasoanal
DMT2 - diabetes mellitus tipo 2
DNS: Departamento de Nutrição e Saúde
EROs - espécies reativas de oxigênio
EER: energy efficiency ratio
FFA: free fatty acids
FA: Fatty acids
FER: Food efficiency ratio
HDL- high-density lipoprotein
HOMA-IR: homeostasis model assessment of insulin resistance
HFCS: high fructose corn syrup
HFHF: High Fat and High Fructose diet
HPLC: High Performance Liquid Cromatography
iAUC: incremental area under the curve
iGTT: intraperitoneal glucose tolerance test
IL-1β: interleukin 1β
IL-6: interleukin 6
IL-10: interleukin 10
IgA: imunoglobulin A
KEGG: Kyoto Encyclopedia of Genes and Genomes
BMI: body mass index
IR: insulin resistance
ITT: intraperitoneal insulin tolerance test

LDL: low-density lipoprotein

LPS: lipopolissacarídeo

LEfSe: linear discriminant analysis effect size method

MDA – malondialdeído

NAFLD: nonalcoholic fatty liver disease

NAFL: nonalcoholic fatty liver

NASH: nonalcoholic fatty liver

NFκB: nuclear factor-kappa B

NCD: chronic non-communicable diseases

NO: nitric oxide

OTU: operational taxonomic units

PCR: C-reactive protein

PPAR-α: Peroxisome proliferator-activated α receptor

PFK: phosphofructokinase

RNS: reactive nitrogen species

RPL: platelet/lymphocyte ratio

RNL: neutro- phil/lymphocyte ratio

SOD - superóxido dismutase

SCFAs: short-chain fatty acids

TA: tecido adiposo

TAC: total antioxidant capacity

TCA - trichloroacetic acid

TG: triglycerides

T2DM: type 2 diabetes mellitus

TNF-α: tumor necrosis factor alpha

TLR4: toll-like receptor 4

TyG: triglyceride/glucose index

UFV: Universidade Federal de Viçosa

SUMMARY

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1. GENERAL INTRODUCTION

High intake of energy-dense ultra-processed foods are characteristic of a western-style diet, rich in saturated fats and, or sugar, which are the main ingredients in this diet. The high-fat high-fructose (HFHF) diet have been widely used in animal studies to simulate the intake of Western-style foods, and thus understand the negative effects and mechanisms involved in chronic non-communicable diseases (NCDs) (Moreira et al., 2022; Zaki, Fattah e Hassan, 2019). The intake of this type of diets causes an increase in the excessive accumulation of free fatty acids (FFA) circulating in adipose, muscular and hepatic tissues (Martinez, Theodoro, Grancieri, Toledo, Queiroz, et al., 2021), as well as increased fat deposits, providing hypertrophy and hyperplasia of adipocytes (Martinez, Theodoro, Grancieri, Toledo, Barros, de, et al., 2021; Theodoro, Martinez, Grancieri, Toledo, Dias Martins, et al., 2021). This diet also negatively affects glucose metabolism due to high levels of glucose and insulin in plasma, which results in insulin resistance (IR) (Nemati *et al.*, 2017). Thus, in the liver, insulin resistance leads to an increase in gluconeogenesis and liver lipogenesis due to excessive accumulation of FFA resulting in in hepatic steatosis (Enes *et al.*, 2020; Theodoro, Martinez, Grancieri, Toledo, Binoti, *et al.*, 2021). This type of diets affect inflammatory properties, which can stimulate the formation of reactive oxygen species (ROS), induce greater oxidative stress and activate NFκB, further increasing the inflammatory process (Martinez, Theodoro, Grancieri, Toledo, Barros, de, *et al.*, 2021). In addition, the HFHF diet can damage the intestinal barrier function and induce intestinal dysbiosis by altering the modulation of the microbiota by increasing the phylum Firmicutes and reducing the phylum Bacteroidetes (Gomes *et al.*, 2022; Rinninella *et al.*, 2019). This type of food contributes to visceral fat accumulation and lipotoxicity in tissues, including the liver, leading to metabolic changes and affecting the inflammatory properties and intestinal microbiota (Martinez, Theodoro, Grancieri, Toledo, Barros, de, *et al.*, 2021; Sousa, de *et al.*, 2018, 2019).

Considering the high risk of HFHF diet consumption, nutritional strategies should be investigated to prevent/treat these negative effects. It is thus, that with the accelerated increase of NCDs, nutritional strategies in order to prevent and treat the damage generated by these diets and their injuries have been the focus of many studies (Arbex *et al.*, 2018; Enes *et al.*, 2020; Sousa, de *et al.*, 2018, 2019; Theodoro,

Martinez, Grancieri, Toledo, Binoti, *et al.*, 2021; Theodoro, Martinez, Grancieri, Toledo, Dias Martins, *et al.*, 2021), demonstrating that regular consumption of whole grains rich in dietary fiber and complex carbohydrates such as resistant starch (AR) and dietary fiber that can be fermented by the intestinal microbiota, thus stimulating the growth of certain bacteria in the colon, producing short-chain fats acid (SCFAs), such as acetate, propionate and butyrate that can reduce the risk of cardiovascular disease and diabetes, improve blood glucose regulation (Flight e Clifton, 2006; Stefoska-Needham *et al.*, 2015) and decrease intestinal inflammation and permeability, preserving mucosal integrity and colonic homeostasis (Gomes *et al.*, 2022; Moreira *et al.*, 2022; Theodoro, Martinez, Grancieri, Toledo, Dias Martins, *et al.*, 2021).

In this context, sorghum (*Sorghum bicolor L. Moench*) has been highlighted as a food with functional claims and potential for the prevention and modulation of chronic diseases, which is associated with its content of dietary fiber, resistant starch, phenolic compounds, tannins and flavonoids, such as anthocyanins, flavones and flavanones (Moraes *et al.*, 2012; Queiroz *et al.*, 2014; Stefoska-Needham *et al.*, 2015; Teixeira *et al.*, 2016). The effect of sorghum has been investigated in the form of extruded flour (Arbex *et al.*, 2018; Sousa, de *et al.*, 2019) and whole grain flour (Moraes *et al.*, 2012), demonstrating its antioxidant and anti-inflammatory activity. Sousa, de *et al.* (2019) observed that the consumption of extruded sorghum flour (SC 319 genotype) improves intestinal dysbiosis, inflammation and oxidative stress by reduction of the concentrations of p65 NF-κB in the liver, lipids peroxidation and increasing the total antioxidant capacity of plasma. Furthemore, the sorghum decreased hepatic lipogenesis and grade of hepatic steatosis by increasing protein expressions of peroxisome proliferator-activated receptor α (PPARα) and reduced metabolic risk of hepatic steatosis associated with lipogenesis and obesity in rats fed a high-fat diet. Arbex *et al.* (2018) showed that the consumption of extruded sorghum flour (genotype SC 319) reduced the blood levels of glucose, percentage of adiposity, adipocyte hypertrophy and the metabolic risk of obesity associated with adiposity and inflammation in rats fed a high-fat diet.

Among the sorghum genotypes, BRS 305 has a high content of fiber, resistant starch and tannins. Its extruded form has demonstrated a protective effect in changed metabolic conditions. Moreover, the probiotic milk with the extruded sorghum (BRS 305) alleviated the inflammation and oxidative stress in patients with chronic kidney

disease as observed in Lopes *et al.* (2018). Further, the consumption of dairy drinks with extruded sorghum flour (BRS 305) showed a decreased urea concentration, higher fecal butyric acid and lower pH during seven weeks in hemodialysis subjects (Lopes *et al.*, 2019). Silva *et al.* (2020) observed an improvement in oxidative stress and lipid profile by the intake of toasted sorghum flour (BRS 305) on oxidative stress induced by paracetamol in *Wistar* rats for 29 days. Further, BRS 305 sorghum flour may reduce inflammation and oxidative stress without altering the jejunum morphology in rats fed a high-fat diet for 35 days (Moraes *et al.*, 2012). And finally, Martinez et al. (2020) highlighted the effect of BRS 305 sorghum flour on enhancing the total antioxidant capacity in Wistar rats submitted to oxidative stress with sodium fluoride. However, there are few studies relating the effect of sorghum flour BRS 305 with a high content of tannins and resistant starch in the form of whole meal flour on a diet with high fat and high fructose and the consequences on adiposity, oxidative stress, inflammation and gut health. Thus, we hypothesize that BRS 305 hybrid sorghum flour rich source of resistant starch, condensed tannins, and phenolic compounds can improve sensitivity to insulin and glucose tolerance, promote the control of liver lipogenesis, increasing β-oxidation and improve inflammation, oxidative stress, gut microbiome composition and colon histomorphometry in animals fed a HFHF diet.

2. OBJECTIVES

2.1. General objective

To evaluate the bioactive properties of hybrid BRS 305 rich in tannins and resistant starch sorghum flour (*Sorghum bicolor L.*) on weight gain, adiposity, insulin resistance, glucose tolerance, oxidative stress, inflammation, hepatic steatosis, and intestinal microbiota in Wistar rats fed a high-fat high-fructose diet.

2.2 Specific objective

- To characterize the chemical composition of sorghum flour, profile of phenolic compounds and resistant starch;
- To evaluate the effect of sorghum flour on food consumption, weight gain and biometric measurements in Wistar rats fed a high-fat, high-fructose diet;

- To verify the effect of sorghum flour on the biochemical markers of renal and hepatic function;
- To investigate the effect of sorghum flour on glucose metabolism, insulin resistance, lipogenesis, β-oxidation, and histomorphometry of liver tissue;
- To evaluate the effect of sorghum on biomarkers of oxidative stress and inflammation in Wistar rats fed a high-fat, high-fructose diet;
- To evaluate the effect of sorghum on gut microbiota modulation, intestinal health and histomorphometry of colon tissue in Wistar rats fed high-fat high-fructose diet.

3. THEORETICAL REFERENCE

3.1. HIGH-FAT HIGH-FRUCTOSE (HFHF) DIETS

Diet and nutrition are essential factors in promoting and maintaining health. Research shows a strong relationship between diets and some chronic diseases (Harrison, Couture e Lamarche, 2020; Lozano *et al.*, 2016). The Western-style diet is characterized by the high intake of energy-dense ultra-processed foods rich in saturated fats and, or sugar. The consumption of this type of food, combined with genetic predisposition, physical inactivity and the environment can trigger chronic non-communicable diseases (NCDs) such as obesity, dyslipidemias and type 2 diabetes mellitus (T2DM) (Harrison, Couture e Lamarche, 2020; Nemati *et al.*, 2017; Renzo, Di, Gualtieri e Lorenzo, De, 2021).

Studies have shown that consumption of diets high in saturated fat can result in the development of metabolic syndrome that involves: weight gain, higher levels of circulating triglycerides and insulin insensitivity (Harrison, Couture e Lamarche, 2020; Woodie e Blythe, 2017). A diet containing a high amount of saturated fatty acids and a low amount of unsaturated fatty acids negatively affects glucose metabolism, such as increased plasma glucose and insulin levels as a consequence of insulin resistance (IR) (Clegg *et al.*, 2011; Nemati *et al.*, 2017). This type of diet can cause these changes

by increasing fat deposits, especially intra-abdominal fat (Lozano *et al.*, 2016; Siri-tarino *et al.*, 2010) and also increasing plasma levels of free fatty acids (McArdle *et al.*, 2013; RD *et al.*, 2013; Sears e Perry, 2015). According to the Brazilian Society of Cardiology (Guideline on Fat Consumption and Cardiovascular Health), the recommended intake of saturated fatty acids is 10% of the total daily calories, and the consumption of this in addition to the recommended can cause a change in lipid profile, such as increased low-density lipoprotein (LDL) and decreased high-density lipoprotein (HDL), increased blood pressure, development and, or progression of type 2 diabetes mellitus and obesity and increased inflammation (RD *et al.*, 2013).

Fructose consumption increased, because of an increase in the consumption of fructose-enriched foods as a sugar substitute, in the preparation of condiments and carbonated beverages (soft drinks), breakfast cereals, roasts, and desserts prepared and sweetened with sucrose and fructose-rich corn syrup (HFCS). HFCS is produced by enzymatic isomerization of fructose dextrose. Commercial use of HFCS (addition of fructose to food) began to increase in the 1970 and in 1985 HFCS accounted for 35% of the total amount of dry weight sweeteners in the food supply (Elliott *et al.*, 2002; Lozano *et al.*, 2016; Mai e Yan, 2019). Fructose absorbed by the intestine is almost completely metabolized in the liver through metabolic pathways distinct from those of glucose; In addition, the initial steps of its metabolism are independent of insulin and therefore, fructose is widely metabolized without the need for insulin secretion and without increasing plasma glucose (Elliott *et al.*, 2002; Tappy *et al.*, 2010). As fructose metabolism is not dependent on insulin secretion (at least in its initial steps) its ingestion cause only a limited increase in blood glucose, fructose was initially proposed as a natural substitute for sucrose for diabetic patients (Le *et al.*, 2006). However, the increased dietary intake of this sugar, is associated with increased plasma triglyceride concentrations, hepatic steatosis, obesity, decreased glucose tolerance, IR and increased blood pressure (Jensen *et al.*, 2018; Jeong e Kim, 2019; Tappy e Lê, 2010; Zaki, Fattah e Hassan, 2019).

High-fat high-fructose diet (HFHF) has been shown to be efficient in inducing chronic non-communicable diseases, efficiently simulating a Western-style diet. In a study conducted by Zaki; Fattah; Hassan, (2019) a diet rich in saturated fat and fructose (HFHF) was used compared to the diet rich in saturated fat (HFD) and the high fructose diet (HF). All diets promoted a significant increase in weight gain in male

Sprague-Dawley rats in 6 weeks. The groups also developed liver disorders, steatosis up to necrosis and inflammation, and metabolic changes including elevated serum albumin, glucose, triglyceride (TG) and LDL cholesterol and reduced HDL cholesterol. In the groups fed a HFD and HFHF diets, lipid droplets hepatocytes were observed, demonstrating severe form of liver injury, indicating cell necrosis. Further, oxidative stress was developed in the three diet groups, with highest effect in the HFHF group.

In other study with rats fed three types of diet (HFD, HF, HFHF) for 8 months, it was observed that the HFHF diet was able to induce type 2 diabetes associated with long-term metabolic disorders such as fasting hyperglycemia, hyperinsulinemia pre and postprandial, insulin resistance, glucose intolerance and dyslipidemia (hypertriglyceridemia and hypercholesterolemia), Furthermore, the HFHF diet-induced complications associated with T2DM, such as hepatic steatosis with fibrosis and inflammation (Lozano *et al.*, 2016).

3.1.1. FRUCTOSE METABOLISM

Fructose is highly available in almost all foods, with the possible exception of some nuts, which contain negligible amounts. As a monosaccharide, fructose is absorbed directly into the bloodstream from the small intestine, as well as glucose and galactose (Le *et al.*, 2006). This sugar has attracted much attention from researchers in recent decades because it has unique metabolic characteristics, as it is essentially metabolized by splanchnic organs (intestinal and liver cells) by insulin-independent mechanisms (Le *et al.*, 2006; Tappy e Lê, 2010).

Due to its independent insulin metabolism and low glycemic index, fructose was initially investigated as a potential beneficial sweetener in patients with type 2 diabetes mellitus (Mai e Yan, 2019; Tappy e Lê, 2010). But when administered acutely, diets with high fructose content, demonstrated metabolic and cardiovascular adverse effects, with development of almost all characteristics of metabolic syndrome, i.e., dyslipidemia, IR, impaired glucose homeostasis, increased body fat and increased blood pressure (Dekker *et al.*, 2010; Tappy *et al.*, 2010; Tappy e Lê, 2010; Zaki, Fattah e Hassan, 2019).

The hepatic metabolism of fructose has effects on glucose and lipid metabolism, is absorbed and delivered to the liver via the portal vein. This is distinct from glucose

metabolism in that glucose is mainly metabolized by glucokinase or hexokinase, while fructose is mainly metabolized by fructokinase. Fructose is phosphorylated in the liver by adenosine triphosphate (ATP) to form fructose-1-phosphate. The enzyme fructokinase catalyzes this reaction. Fructose-1-phosphate is split by aldolase B into glyceraldehyde and dihydroxyacetone phosphate. From this stage, fructose metabolism is similar to glucose metabolism and results in the generation of glucose, glycogen and triglycerides. Therefore, the unique aspect of fructose metabolism is in its first two enzymatic steps (Elliott *et al.*, 2002; Jensen *et al.*, 2018). The products of fructose metabolism in the glycolytic pathway of the liver are glucose, glycogen, lactate and pyruvate. Fructose absorption by the liver is not inhibited at the level of phosphofructokinase, so fructose intake results in higher increases in circulating lactate comparable to glucose (Elliott *et al.*, 2002; Renzo, Di, Gualtieri e Lorenzo, De, 2021).

The main isoform of fructokinase in the liver is fructokinase C, which phosphorylates fructose rapidly without any negative feedback control, resulting in a drop in intracellular ATP and phosphate. The drop in intracellular phosphate activates the enzyme adenosine monophosphate (AMP) deaminase, which converts AMP to inosine monophosphate (IMP), resulting in a turnover of purine nucleotides that culminates in the formation of uric acid (Jensen *et al.*, 2018). This drop in ATP level induces a series of reactions, including a transient blockage in protein synthesis, an induction of oxidative stress and mitochondrial dysfunction that turns out to play a key role in fructose-mediated effects. (Jensen *et al.*, 2018; Lanaspa *et al.*, 2012). Thus, fructose induces uric acid production by increasing the breakdown of ATP to AMP (a precursor of uric acid), so the increase in uric acid can directly stimulate hepatic fat accumulation (Lanaspa *et al.*, 2012).

3.2. NON-ALCOHOLIC FATTY LIVER DISEASE

Non-alcoholic fatty liver disease (NAFLD) describes a condition of fatty infiltration of the liver in the absence of the other common cause of steatosis, as alcohol consumption. It is characterized by excessive fat accumulation in the liver (hepatic steatosis). NAFLD spans the spectrum from simple steatosis or non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH). NAFL describes hepatic steatosis without significant inflammation leading to hepatocellular injury or fibrosis, is generally

considered benign and reversible, with minimal risk of progression to cirrhosis or liver failure. In contrast, NASH refers to hepatic steatosis with inflammation and hepatic balloon-shaped liver damage and resultant cell necrosis. Hepatic steatosis and steatohepatitis can be distinguished only by liver biopsy and histology (Carr, Oranu e Khungar, 2017; Sweet, Khoo e Nguyen, 2017; Wilkins *et al.*, 2013).

The prevalence of NAFLD increased as more patients developed a sedentary lifestyle, metabolic syndrome, and obesity. NAFLD is the most common form of hepatic steatosis and affects 30% to 40% of men and 15% to 20% of women in the general population. It is accepted as the hepatic manifestation of metabolic syndrome and has a strong relationship with insulin resistance, atherosclerosis, obesity, dyslipidemia and hypertension (Idilman, Ozdeniz e Karcaaltincaba, 2016; Targher, 2007). Estimates are that NAFLD affects 30% of the population of the United States; 32% of the population of the Middle East; 30% of the South American population; 27% of Asian populations (the highest of East Asians); 24% of the European population; and 13% of the African population (Carr, Oranu e Khungar, 2017). The accumulation of lipids in hepatocytes causes oxidative stress and inflammatory response that leads to NASH, which can progress to cirrhosis. It is estimated that NAFLD would become the most common indication for liver transplantation by 2030 (Idilman, Ozdeniz e Karcaaltincaba, 2016).

Both hyperinsulinemia and insulin resistance are central to the pathophysiology of NAFLD. Insulin acts on various metabolic tissues, including adipose tissue to promote fatty acid esterification and storage in lipid droplets, while inhibiting the opposite process of lipolysis. In hepatocytes, insulin has three main actions: promoting glycogen storage, inhibiting gluconeogenesis, and activating key regulators of lipogenesis *de novo* (Carr, Oranu e Khungar, 2017). NAFLD patients have reduced insulin sensitivity not only in muscle, but also in liver and adipose tissue, which play an important role in this pathogenesis. Due to IR, there is an increase in adipocyte lipolysis and high availability of circulating free fatty acids for later hepatic uptake, a reduction in hepatic glycogen storage and an increase in gluconeogenesis (Carr, Oranu e Khungar, 2017; Than e Newsome, 2015). In addition, in response to IR, hyperinsulinemia develops increasing hepatic lipogenesis *de novo* pathways (Carr, Oranu e Khungar, 2017).

The increase of circulanting lipids load result from reduced capacity of adipocytes to store these lipids in lipid droplets. In hepatocytes, the inability to storage the lipids expose the cells to lipotoxic lipids. This lipotoxicity further impairs insulin signaling, causing oxidative damage and promoting inflammation and fibrosis, and these effects are responsible for the progression from NAFL to NASH and development of fibrosis and hepatocellular carcinoma in NAFLD patients (Carr, Oranu e Khungar, 2017).

3.3. β-OXIDATION

Fats are stored in our body as TG, which are hydrolyzed into free fatty acids and glycerol by lipases, as the first step of lipid catabolism. β-oxidation is an important process by which fatty acids are oxidized, and this oxidation occurs in both mitochondria and peroxisomes (Reddy e Hashimoto, 2001). The β-oxidation of fatty acids represents an important source of energy in periods of catabolic stress in various tissues, including liver, heart and skeletal muscle. This metabolic pathway plays a central role in maintaining energy homeostasis in situations such as fasting or prolonged exercise that require glucose storage and increased energy supply (Rinaldo, Matern e Bennett, 2002). The fatty acid β-oxidation pathway consists of multi-step reactions that oxidize fatty acids by degrading them two carbons at a time, producing acetyl-CoA, which provides energy to other tissues when glycogen stores are depleted. Medium and short fatty acids are transported directly to the cytosol and mitochondria, and long-chain fatty acids are conjugated with carnitine and transported across the mitochondrial membrane and released as acyl-CoA for use in β-oxidation pathways (Rinaldo, Matern e Bennett, 2002; Rupasinghe *et al.*, 2016).

Mitochondrial β-oxidation of linear-chain saturated fatty acids is a catabolic pathway that involves the participation of several enzymes and generates acetyl-CoA, NADH and FADH2 from acyl-CoA esters. Very long-chain fatty acyl-CoA esters (> 20 carbon atoms) are initially shortened by peroxisomal enzymes and then proceed to the mitochondrial network for β-oxidation (Adeva-Andany *et al.*, 2019). Therefore, a system is needed that allows the transport of long- and medium-chain fatty acyl-CoA esters across the mitochondrial membrane. Thus, the so-called carnitine transport system, catalyzed by carnitine acyltransferase-1 or carnitine palmitoyltransferase-1 (CPT-1), converts fatty acyl-CoA into acyl-carnitine (by carnitine acyltransferase I)

which enters the mitochondrial matrix and the acyl -CoA fatty is regenerated by a reaction catalyzed by carnitine acyltransferase II (Adeva-Andany *et al.*, 2019; Rupasinghe *et al.*, 2016), that is, CPT-1 catalyzes the transfer of acyl groups from coenzyme A to L-carnitine, generating acyl-carnitine esters, and is involved in the passage of long and medium-chain fatty acyl-CoA esters across the mitochondrial membrane (Adeva-Andany *et al.*, 2019).

Fatty acid oxidation occurs in mitochondria, peroxisomes, and smooth endoplasmic reticulum, and some of the critical enzymes in these oxidation systems are transcriptionally controlled by the peroxisome proliferator-activated receptor α (PPARα) a member of the nuclear hormone receptor superfamily. Eicosanoids, fatty acids and fibrates are well-known activators of PPARα (Reddy e Hashimoto, 2001). PPARα is a key regulator of hepatocyte intermediary metabolism, especially fatty acid β-oxidation, when plasma triglycerides levels are high. In a fasting situation, fatty acids mobilized from adipose tissue are transferred to the liver and enter the process of β-oxidation. Stimulation of PPARα increases hepatic fatty acid catabolism and decreases plasma TG levels, upregulating the expression of the enzymes carnitine palmitoyltransferase and acyl-CoA oxidase (Navidshad e Royan, 2016). CPT I is markedly induced by peroxisome proliferators and fatty acids / fatty acyl-CoAs, which activate PPARα, and in this sense, this transcription factor plays a critical role in the regulation of mitochondrial β-oxidation (Reddy e Hashimoto, 2001).

In β-oxidation, adiponectin helps to increase the oxidation of fatty acids, reducing the accumulation of TG, and its receptors are preferentially expressed in adipose (AdipoR1) and hepatic (AdipoR2) tissues. AdipoR1/R2 are the main physiological adiponectin receptors, with the function of measuring parts of adiponectin binding actions and adiponectin actions *in vivo*, these receptors activate AMP activated protein kinase (AMPK) (AdipoR1) pathways and PPARα (AdipoR2) pathways, which in turn lead to a lower triglyceride content. Thus, both pathways can increase insulin sensitivity through different signaling mechanisms (Yamauchi *et al.*, 2007, 2014). In contrast, simultaneous discontinuation of AdipoR1 and R2 almost completely cancels the binding of adiponectin to the liver and its effect on glucose reduction, resulting in marked glucose intolerance and insulin resistance (Yamauchi *et al.*, 2007).

3.4. LIPOGENESE

The lipogenese is the synthesis of fatty acids and triglycerides, which will subsequently be stored in the liver and adipose tissue. For TG biosynthesis, the adipocyte needs glycerol-3-phosphate and free fatty acids (FFA) complexed with coenzyme A, producing AcylCoA molecule. The main source of FFA are circulating lipoproteins (chylomicrons and very low density lipoproteins (VLDL)), which are hydrolyzed by the action of the lipoprotein lipase (LPL) enzyme (present in the endothelial cells of the capillaries of adipose tissue) for storage in adipocytes or oxidation in peripheral tissues (Wang e Eckel, 2009). Hepatic lipid accumulation may be a product of *de novo* hepatic lipogenesis, esterification of plasma-free fatty acids, or increased dietary fat intake. Thus, a high-carbohydrate diet can stimulate hepatic fatty acid synthesis, channeling excess acetyl units from glucose catabolism to fat biosynthesis in the liver through *de novo* lipogenesis.

After a high-carbohydrate diet, there is an increase in glucose oxidation and a decrease in fatty acid (FA) oxidation, this increase in oxidation induces an increase in the synthesis of fatty acids, which are finally esterified and stored as TG in the liver (the called de novo lipogenesis). For *de novo* lipogenesis, glucose is converted to acetyl-CoA through glycolysis and pyruvate oxidation. The mitochondrial acetyl-CoA resulting from catabolism is initially condensed with oxaloacetate, thus forming citrate, which is transported out of the mitochondria by the citrate transporter (an integral protein of the inner mitochondrial membrane) and, once in the cytosol, is cleaved in the presence of ATP in oxaloacetate and acetyl-CoA by the enzyme citrate lyase. This acetyl-CoA is the substrate for fatty acid synthesis. Acetyl-CoA is then converted to malonyl-CoA by acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) catalyzes the formation of palmitic acid from malonyl-CoA and acetyl-CoA. (Ferramosca *et al.*, 2013; Kawano e Cohen, 2013). ACC plays a crucial role in *de novo* lipogenesis because its product, malonyl-CoA, is not only an intermediate of fatty acid synthesis, but also an inhibitor of carnitine palmitoyltransferase (CPT-1) and therefore an inhibitor of oxidation of fatty acids (Brownsey e Denton, 1997; Ferramosca *et al.*, 2013).

Lipogenesis is mainly controlled at the transcriptional level. Postprandially, plasma concentrations of glucose and insulin increase, both of which are required for

full activation of lipogenesis. Glucose and insulin promote lipogenesis by activating carbohydrate response element binding protein (ChREBP) and sterol regulatory element binding protein 1c (SREBP1c), respectively. These are major transcription factors, which in turn promote transcription of lipogenic genes. SREBP1c is a transcription factor that regulates lipid homeostasis by controlling the expression of lipogenic genes, including FAS, ACC, SCD1 and ACL. The expression of SREBP1c is transcriptionally controlled by several nutritional and hormonal factors, which insulin being one of the most potent activators (Kawano e Cohen, 2013; Mao e Zhang, 2018). Insulin promotes transcriptional upregulation of SREBP1c by a phosphoinositide 3-kinase (PI3K)-dependent mechanism and the participation of the liver X receptor (LXR). A potent activator of SREBP-1c transcription is LXRα. LXRα is a nuclear hormone receptor with high hepatic expression that plays a key role in the regulation of cellular cholesterol homeostasis and is activated by oxysterols (cholesterol metabolites) (Ferré e Foufelle, 2010; Kawano e Cohen, 2013). Single LXR activation of SREBP-1c transcription is not sufficient to generate activity from SREBP-1c. In fact, insulin is absolutely necessary to release the active form of SREBP-1c from endoplasmic reticulum membranes (Ferré e Foufelle, 2010).

During the energy abundance, lipogenesis can also be activated by mTOR, a pathway known to control cell growth and metabolism in response to nutrients, growth factors, or energy status (Wang *et al.*, 2015). mTOR is the conserved serine/threonine kinase that interacts with various proteins to form two distinct complexes with different protein functions and components: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Caron, Richard e Laplante, 2015; Mao e Zhang, 2018). mTORC1 controls lipid synthesis and promotes anabolism, regulating the expression of many lipogenic genes and controlling hepatic lipogenesis through regulation in the SREBP1c-dependent pathway by phosphorylation of ribosomal protein S6 kinase 1 (S6K1) (Düvel *et al.*, 2010). Thus, activation of mTORC1 leads to increased production of SREBP-1c, and this facilitates the storage of excess nutrients such as triglycerides. It can be activated under nutrient-abundant conditions to enhance protein translation, and when active, promotes protein synthesis, primarily through ribosomal protein S6 kinase 1 (S6K1) phosphorylation (Caron, Richard e Laplante, 2015). mTORC1 can also be activated by Akt through phosphorylation and inhibition of the TSC2 protein within the TSC1-TSC2 complex (Yecies *et al.*, 2011). Hyperactivation of mTORC1

during overfeeding can trigger an S6K-dependent negative feedback loop; when activated, mTORC1 and S6K1 directly phosphorylate insulin receptor substrate 1 (IRS1), which promotes IRS1 degradation and contributes to insulin resistance (Caron, Richard e Laplante, 2015; Zoncu, Sabatini e Efeyan, 2011).

3.5. GLICONEOGENESE

Gluconeogenesis is the synthesis of glucose from compounds that are not carbohydrates, the main substrates being lactate, glycerol, alanine and glutamine. Together they represent 90% of gluconeogenic substrates; however, other amino acids and citrus cycle intermediates can also serve as substrates for gluconeogenesis. (Hatting *et al.*, 2019). Prolonged fasting induces *de novo* liver glucose synthesis (termed gluconeogenesis) by acute activation of key regulatory enzymes including glucose-6-phosphatase (G6Pase), fructose 1,6-bisphosphatase (Fbpase), pyruvate carboxylase (PC)) and phosphoenolpyruvate carboxykinase (PEPCK) (Oh *et al.*, 2013). From lactate or an α-keto acid derived from amino acid breakdown, pyruvate can be generated for gluconeogenesis. Pyruvate is converted by carboxylation to oxaloacetate in mitochondria. After an intermediate step that allows oxaloacetate to leave the mitochondria via malate, oxaloacetate is decarboxylated and then phosphorylated to form phosphoenolpyruvate by the enzyme PEPCK (this enzyme is a regulator of the rate of gluconeogenesis, and its transcription is targeted by multiple factors, including glucagon and insulin). After several steps of reverse glycolysis, Fbpase converts fructose 1,6-bisphosphate to fructose 6-phosphate. And finally glucose-6-phosphate (formed from fructose 6-phosphate by phosphoglucoisomerase) can be used in other metabolic pathways or dephosphorylated by G6Pase to form free glucose (Hatting *et al.*, 2019).

In gluconeogenesis, two elements are known to play significant physiological roles: serine/threonine kinase Akt (also known as protein kinase B, PKB) and forkhead box protein O1 (FoxO1). FoxO1 is a member of the forkhead transcription factor family and its transcriptional activity is regulated by phosphorylation and intracellular localization. It can directly bind to the target DNA sequence of PEPCK and G6Pase to regulate their expression levels in the liver (Ren *et al.*, 2018), and when it is phosphorylated directly by Akt, it prevents FoxO1 from entering the nucleus, reducing the expression of its target genes necessary for gluconeogenesis (Ren *et al.*, 2018;

Sajan *et al.*, 2018). Thus, activation of Akt promotes the nuclear exclusion of FoxO1 by phosphorylation, blocking transcriptional regulation of gluconeogenic genes.

In the liver, mTORC2 directly activates Akt by phosphorylating its hydrophobic motif (Ser473) (a site necessary for its maximal activation) (Hagiwara *et al.*, 2012; Laplante e Sabatini, 2013), and regulates it to control the phosphorylated state of FoxO1, decreasing expression of gluconeogenic genes. Thus, hepatic mTORC2 controls glucose homeostasis via activation of glycolysis (through activation of glucokinase and transcription factor ChREBP) and inhibition of gluconeogenesis (by inhibiting nuclear accumulation of FoxO1 via phosphorylation of Akt Ser473) (Cornu, Albert e Hall, 2013; Li *et al.*, 2017).

3.6. INTESTINAL MICROBIOTA

The small and large intestines are home to more than a trillion microorganisms, made up of hundreds of species. Growing evidence links changes in gut bacteria to the development of obesity-related risk factors and associated pathologies such as dyslipidemia, systemic inflammation, and insulin resistance (Burcelin, 2016). Studies based on metagenomic analysis and 16S ribosomal RNA gene sequencing have shown that the human gut microbiota comprises more than 1,000 phylotypes. In the intestines of mouse and humans, Bacteroidetes (Gram-negative) e Firmicutes (Gram-positive) phyla constitute about 90% of all intestinal microbial cells, while Actinobacteria (Gram-positive),Proteobacteria (Gram-negative) and Verrucomicrobia (Gram-negative), constitute relatively minor proportions (Singh *et al.*, 2019; Yang, Hur e Lee, 2017). Microbial diversity is characterized by α and β diversity metrics, where α diversity explains the richness of the community or diversity of species in different sites or habitats within a local scale. β diversity explains the difference in the community and the diversity between habitats, measured by the heterogeneity of the community structure (Martinez, Leone e Chang, 2017). Studies show that a low abundance of Bacteroidetes, a high abundance of Firmicutes, and reduced bacterial diversity are associated with individuals and rats with metabolic disturbances (Festi *et al.*, 2014; Moszal, Szulinska e Bgdanski, 2020; Verdam *et al.*, 2013).

Metabolic syndrome is associated with an altered gut microbiota, known as dysbiosis (Clemente *et al.*, 2012; Winer *et al.*, 2016). The main consequences of the changed microbial composition is the increase in intestinal permeability, which

increases the leakage of bacteria or bacterial products, such as lipopolysaccharide (LPS) (the main component of the outer membrane of Gram-negative bacteria, such as Escherichia coli), through the intestinal barrier to the systemic circulation characterizing endotoxemia (Winer *et al.*, 2016). Elevated plasma levels of LPS are associated with obesity, and bacterial products trigger the innate immune system, such as macrophages, in different tissues resulting in chronic inflammation and metabolic disease (Shen, Obin e Zhao, 2013).

Interactions between diet and microbiota also affect epithelial integrity and intestinal homeostasis, for example, non-digestible carbohydrates are fermented in the intestine to produce short-chain fatty acids (SCFAs) (acetate, propionate, and butyrate), which bind to G protein-coupled receptors (GPRs) that suppress inflammation and improve barrier function (Tremaroli e Bäckhed, 2012). Furthermore, the gut microbiota increases the host's energy harvest through hydrolysis and fermentation of dietary polysaccharides that the host cannot digest, this generates monosaccharides and SCFAs, which can be absorbed and used as energy by the host (Shen, Obin e Zhao, 2013). The consumption of saturated fats and sugars in the diet can generate a high proportion of Firmicutes in relation to Bacterioidetes (Bibbò *et al.*, 2016; HILDEBRANDT *et al.*, 2009). In the same way that the type of diet and the state of health influence the intestinal microbiota, it also affects the organism's homeostasis.

3.7. DIET ON GUT MICROBIOTA COMPOSITION

Diet affects several aspects of host homeostasis through modulations of gut microbiota composition. Protein, fat, and carbohydrates are the most components in diets. Thus, the type and amount of food present in diets affect the microbiota composition. The protein degradation occurs in the distal end of the colon by major microbial phyla (Firmicutes, Bacteroidetes, and Proteobacteria) (Rinninella *et al.*, 2019; Shi *et al.*, 2017). Products of protein degradation are amino acids, ammonia, amines and SCFAs. The effects of protein consumption positively correlate with overall microbial diversity and vary according to the protein type (animal-derived proteins versus plant-derived proteins) (Moszal, Szulinska e Bgdanski, 2020; Rinninella *et al.*, 2019). The carbohydrates play an essential role in modulating the gut microbiota composition, and their effects on the bacterial community have been evidenced. The simple carbohydrates (e.g., sucrose, fructose, glucose) are enzymatically degraded in

the small intestine and rapidly released as glucose in the bloodstream, causing rapid deregulation in the microbiota composition and hence metabolic dysfunction in the host (Moszal, Szulinska e Bgdanski, 2020).

Evidence indicates that high-fructose diets may increase the Firmicutes-to-Bacteroidetes ratio and Proteobacteria (one of the best sources of lipopolysaccharide (LPS)) (Rinninella *et al.*, 2019). High levels of glucose or fructose in the diet modulate the gut microbiota and increase intestinal permeability, which leads to the development of metabolic endotoxemia, inflammation, lipid accumulation, and finally, hepatic steatosis (Do *et al.*, 2018). Complex and indigestible carbohydrates such as dietary fiber showed the elevated fecal butyrate production (Moszal, Szulinska e Bgdanski, 2020). Dietary fiber resist to digestion in the small intestine (because they are resistant to pancreatic amylase), and reach the large intestine (they are fermented by intestinal microbiota, producing short-chain fatty acids), include non-starch polysaccharides (cellulose and hemicellulose), lignin, resistant starches, and non-digestible oligosaccharides) (Rinninella *et al.*, 2019). Dietary fiber and resistant starch are non-digestible by the host's metabolism and made available for gut microbiota as prebiotics, which stimulates the proliferation or activity of desirable bacterial populations in the intestine (colon), benefiting the host health, as well as the production of SCFAs (butyrate, propionate and acetate) (Paiva, de *et al.*, 2020; Rinninella *et al.*, 2019). Firmicutes mainly produce butyrate, propionate is produced by Bacteroidetes, and most gut anaerobes make an acetate. They have multiple beneficial effects on the host, reducing intestinal permeability and decreasing the risk of inflammation (Moszal, Szulinska e Bgdanski, 2020; Paiva, de *et al.*, 2020). The SCFAs may modulate the colonic homeostasis, reduce intestinal permeability, risk of inflammation, stimulate the proliferation and differentiation of epithelial cells, absorption of salts and water, maintenance of mucosal integrity and recovery of intestinal symbiosis (Paiva, de *et al.*, 2020; Rinninella *et al.*, 2019).

On the other hand, the intake of high-fat can also increase intestinal permeability and Gram-negative bacteria, reduce Gram-positive bacteria (Bibbò *et al.*, 2016), Bacteroidetes, and increase Firmicutes and Proteobacteria in mice (Kolodziejczyk, Zheng e Elinav, 2019), thus increasing the passage of bacterial products, such as LPS, through the intestinal barrier to the systemic circulation, which characterizes endotoxemia (Bibbò *et al.*, 2016; Winer *et al.*, 2016). A high-fat diet generates

dysbiosis, leading to a significant reduction in the abundance of *Roseburia* species (Bibbò *et al.*, 2016).

Dietary polyphenols are natural compounds identified in various plants and foods such as fruits, vegetables, tea, medical plants, microalgae, herbs, seeds, and cereals, and in beverages such as coffee, tea, cocoa, and wine (Cardona *et al.*, 2013; Rinninella *et al.*, 2019). Due to their degree of structural complexity, high molecular weights, and polymerization, up to 95% of the consumed dietary polyphenols cannot be absorbed in the small intestines and reach the colon almost unchanged (oligomeric and polymeric polyphenols such as condensed or hydrolyzable tannins) where colon bacteria enzymatically act on their structure (transform the polyphenols into bioactive compounds), sequentially producing metabolites with different physiological meanings (Cardona *et al.*, 2013; Wan, Co e El-Nezami, 2020). Gut microbiota can modulate the transformation of phenolic compounds and breakdown of the original polyphenolic structures into a series of low-molecular-weight phenolic metabolites that are absorbable, influencing their bioavailability and modifying their properties (Cardona *et al.*, 2013; Singh *et al.*, 2019). Thus, dietary polyphenols reaching the gut microbiota may modulate the microflora community by exhibiting prebiotic effects and antimicrobial action against pathogenic intestinal microflora (Singh *et al.*, 2019), showing that phenolic compounds can alter the gut microbiota, resulting in a greater abundance of beneficial microbes.

3.8. GUT MICROBIOTA AND METABOLIC SYNDROME

The metabolic syndrome describes a cluster of risk factors such as obesity, weight gain, hyperglycemia, dyslipidemia, higher levels of circulating triglycerides, insulin insensitivity and others. when uncontrolled, it will eventually lead to non-alcoholic fatty liver (NAFLD) (Harrison, Couture e Lamarche, 2020; Wang *et al.*, 2020). The metabolic syndrome is related to a variety of factors, such as glucose intolerance, insulin resistance, increased inflammation, oxidative stress, and liver steatosis (Harrison, Couture e Lamarche, 2020; Martinez, Theodoro, Grancieri, Toledo, Barros, de, *et al.*, 2021; Martinez, Theodoro, Grancieri, Toledo, Queiroz, *et al.*, 2021). Furthemore, it found that gut microbiota disorder is a risk factor for the development of the metabolic syndrome (Wang *et al.*, 2020). The gut microbiota plays a significant role in maintaining the homeostasis of human health and the function of the host immune

system, and dysbiosis caused by various factors leads to extensive physiological changes and increases the plasma concentration of the inflammatory bacterial lipopolysaccharide, inducing inflammation and abnormal lipid accumulation in several tissues (due to increased levels of inflammatory cytokines), resulting in the insulin resistance and glucose intolerance (Do *et al.*, 2018). Thus, there is a close association between gut microbiota dysbiosis and numerous non-communicable diseases such as cardiovascular diseases, obesity, diabetes, cancer, and gastrointestinal diseases (Rinninella *et al.*, 2019; Shi *et al.*, 2017; Wang *et al.*, 2020).

Studies have confirmed that metabolic syndrome can lead to gut microbiota imbalance, and dysbiosis of the gut microbiota may alter host metabolism, aggravating metabolic disorders. Liu *et al.* (2017) observed that the microbiome was altered in high-fat diet mice, such as the increased abundance of Firmicutes (families *Streptococcaceae*, *Enterococcaceae*, and *Carnobacteriaceae*) and the decreased abundance of Bacteroidetes (*Porphyromonadaceae* and *Bacteroidaceae*) associated with the obese phenotype. Song *et al.* (2017) found in rats with hyperlipidemia higher levels of gut LPS-producing bacteria (*Bilophila*, *Sutterella*, *Oscillibacter* and *Proteus*) and mucosa-damaging bacteria (*Bilophila* and *Akkermansiamuciniphila*). The gut microbiome of obese rats fed a high-fat diet increases the abundance of firmicutes phylum, intestinal dysbiosis and permeability, allowing the LPS translocation to blood and tissue, such as the liver (Sousa, de *et al.*, 2019). In another study, patients with metabolic syndrome and T2DM have a gut microbiota enriched in *Bacteroides, Clostridiales, Erysipelotrichaceae,* and low in *Lachnospiraceae,* and metabolic syndrome subjects decrease of abundance, *F. prausnitzii A. muciniphila.* and *F. prausnitzii* strains (considered major butyrate producers in the human intestine) (Pircalabioru *et al.*, 2022). So, gut microbiota can promote the development of insulin resistance and diabetes by inducing metabolic endotoxemia, and the presence of bacteria producing butyrate (such as *Bifidobacteria* or *Lactobacillus)* may improve glucose tolerance with a decrease in endotoxemia (Festi *et al.*, 2014)

3.9. SORGHUM

Sorghum, a plant of the Poaceae family, sorghum species [Sorghum bicolor (L.) Moench], is among the five most cultivated cereals in the world, behind rice, wheat, corn and barley, which are the main grains in the world. It is an essential source of

carbohydrates, calories, and protein in many countries in Africa, Asia, and Central America (Althwab *et al.*, 2015). Sorghum consumption is likely the result of its ability to grow in different agricultural conditions, including dry and arid regions or in severe temperature fluctuations areas (INTSORMIL, 2010; Rosa, 2012). Thus, sorghum has advantages to be efficient in extracting water and nutrients from the soil, allowing it to be cultivated in dry and, or hot areas where the productivity of other cereals would be uneconomical. So, the consumption of sorghum as a source of human food is growing slowly but steadily due to the different research on the application of sorghum in human food that have been showing its positive effects on health (Bernardo *et al.*, 2019; Prado, do *et al.*, 2019) and, increasing demand for gluten-free foods and beverages and other intolerances that make it impossible to consume wheat, barley, or rye products (Queiroz *et al.*, 2014).

Sorghum consists of three distinct parts: pericarp, endosperm and germ, and some varieties have a fourth structure called testa pigmented, located between the pericarp and endosperm, which is responsible for the presence of condensed tannins (Awika e Rooney, 2004). The proportion and chemical composition of each of these parts will depend on the genotype and growing conditions (Antunes *et al.*, 2007; Conceição *et al.*, 2009). Thus, due to its versatility and production capacity, sorghum has been increasingly researched and used in the elaboration of a wide variety of food products, such as in the manufacture of bakery products, including gluten-free products, snacks, flours, porridges, couscous, tortillas, beer and more (Queiroz *et al.*, 2009, 2014; Taylor, Schober e Bean, 2006).

High concentrations of bioactive compounds, with high antioxidant capacity, which can benefit human health, has aroused the interest of researchers, resulting in scientific studies that demonstrate that sorghum phenolic compounds modulate parameters related to chronic non-communicable diseases such as obesity, diabetes, dyslipidemias, cardiovascular diseases, cancer and hypertension (Awika e Rooney, 2004; Moraes *et al.*, 2012; Morais Cardoso, de *et al.*, 2017; Sousa, de *et al.*, 2018; Stefoska-Needham *et al.*, 2015). However, the efficiency of this grain largely depends on the genotype used. Therefore, research developed by Embrapa Milho and Sorgo evaluated hundreds of sorghum genotypes and identified that the hybrid BRS 305 has a high content of condensed tannins (Moraes *et al.*, 2012) and resistant starch (Teixeira *et al.*, 2016).

3.10. BIOACTIVE COMPOUNDS FROM SORGHUM

The chemical composition of sorghum is similar to that of corn, having as main components: starch, proteins, lipids, non-starch polysaccharides and phytochemicals such as phenolic compounds, phytosterols and policosanol, it also contains dietary fiber, resistant starch and micronutrients, including vitamins and minerals (Althwab *et al.*, 2015; Stefoska-Needham *et al.*, 2015).

Sorghum is a source of phenolic compounds that provide its high antioxidant capacity. (Dykes *et al.*, 2006; Morais Cardoso, de *et al.*, 2017). The levels of sorghum phenolic compounds, although determined by genetic factors and growing conditions, can also vary according to processing methods, mainly due to changing levels of measurable phenolic compounds (Cardoso, L. de M. *et al.*, 2015; Dlamini, Taylor e Rooney, 2007), like the use of the dry heat treatment processing that has the advantage of maintaining and preserving of phenolic compounds, higher antioxidant activity and resistant starch content in the whole sorghum grains, compared to wet heat treatment, as has been shown in previous studies (Cardoso, L. de M. *et al.*, 2015; Silva *et al.*, 2020; Teixeira *et al.*, 2016).

The bioactive compounds responsible for these antioxidant effects include phenolic acids (FA), tannins, flavonoids (anthocyanins, flavones and flavanones), and also, resistant starch, phytosterols and policosanols (Awika e Rooney, 2004; Stefoska-Needham *et al.*, 2015). Sorghum phenolic acids are derived from benzoic or cinnamic acids and are found in the pericarp (bran) of the grains. These include gallic, p-hydroxybenzoic, vanillic, syringic, protocatechuic, coumaric, caffeic, ferulic, sinapic acid, among others (Althwab *et al.*, 2015; Awika e Rooney, 2004; Dykes e Rooney, 2006). Among the anthocyanins most commonly found in sorghum cultivars is 3-deoxyanthocyanin, comprising luteolinidines and apigeninidines. Pigmented testa sorghum grain varieties also contain condensed tannins (known as proanthocyanidins) which are composed of polymers of flavan-3-ols, flavan-3,4-diols and flavan-4-ol (glycosylated and non-glycosylated) (Cardoso, L. D. M. *et al.*, 2015).

In sorghum, tannins are located in the testa (thick layer located just below the pericarp), the presence of tannins in the grain can impair its nutritional value and decrease its metabolizable energy, as they bind to proteins, carbohydrates and other

nutrients, limiting its nutritional value and decreasing its digestibility (Barros, Awika e Rooney, 2012; Dunn *et al.*, 2015). Although tannins have historically been considered undesirable from a nutritional point of view, evidences have shown the antioxidant potential of tannins, as well as anticarcinogenic, antimutagenic and antimicrobial properties, important in protecting cells against oxidative damage, reducing the risk of NCDs (Arbex *et al.*, 2018; Lopes *et al.*, 2018; Morais Cardoso, de *et al.*, 2017; Queiroz *et al.*, 2009; Sousa, de *et al.*, 2018).

In vivo studies by our research group showed that extruded sorghum SC319 can improve intestinal dysbiosis, inflammatory markers and reduce oxidative stress by inhibiting the production of reactive species, contributing to a greater total antioxidant capacity (Sousa, de *et al.*, 2019). Furthermore, a reduction in the accumulation of fat in the liver of rats fed a high-fat diet was also observed, helping to prevent obesity and hepatic steatosis. (Sousa, de *et al.*, 2018), in addition to positively altering the expression of antioxidant and adipogenic genes (PPAR-γ, LPL e FAS), reducing the metabolic risk of obesity associated with adiposity and inflammation (Arbex *et al.*, 2018) in rats fed a high-fat diet for 8 weeks. In a another study, the intake of extruded sorghum (BRS 305) consumed with non-fermented probiotic dairy drink was evaluated in patients with chronic kidney disease during 7 weeks, the results showed that sorghum had positive effects by improving oxidative stress and inflammation (Lopes *et al.*, 2018). It has also been determined that the ingestion of extruded sorghum (SC319, SC391 and BDLO357) in overweight individuals has the potential to decrease body fat, in addition to reducing oxidative stress by increasing antioxidant enzymes such as glutathione peroxidase (Anunciação *et al.*, 2019). MORAES *et al.*, (2012) evaluated the effect of a high-fat diet containing sorghum flour (BRS 305) which reduced oxidative stress and inflammation markers.

However, there are currently no studies relating the impact of BRS 305 hybrid whole sorghum flour rich source of resistant starch, condensed tannins, and fiber (Martinez *et al.*, 2020; Teixeira *et al.*, 2016) on rats fed with a high-fat high-fructose (HFHF) diet (which mimics the current classic Western diet) and its effects in inflammation, oxidative stress, insulin resistance, glucose tolerance, adiposity, liver steatosis and liver lipogenesis, on gut microbiome composition and colon histomorphometry.

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5. Papers

Article	Article title
Original 1 – published - Journal of Cereal Science, impact factor 3.616, Doi: 10.1016/j.jcs.2021.103201	Dry heated whole sorghum flour (BRS 305) with high tannin and resistant starch improves glucose metabolism, modulates adiposity, and reduces liver steatosis and lipogenesis in Wistar rats fed with a high-fat high-fructose diet
Original 2 – published - Food & Function, impact factor 5.396, Doi: 10.1039/d1fo00802a	Dry heated sorghum BRS 305 hybrid flour as a source of resistant starch and tannins improves inflammation and oxidative stress in Wistar rats fed with a high-fat high-fructose diet
Original 3 – published to European Journal of Nutrition, impact factor 5.291; Doi:10.1016/j.jcs.2021.103201	Sorghum flour BRS 305 hybrid source of resistant starch and tannins has the potential to modulate the intestinal microbiota of rats fed with a high-fat high-fructose diet

Original 1 – published - Journal of Cereal Science, impact factor 3.616, Doi: 10.1016/j.jcs.2021.103201

Dry heated whole sorghum flour (BRS 305) with high tannin and resistant starch improves glucose metabolism, modulates adiposity, and reduces liver steatosis and lipogenesis in *Wistar* rats fed with a high-fat high-fructose diet.

ABSTRACT

This study aimed to evaluate the effect of dry heated whole sorghum flour (BRS 305 hybrid) on insulin resistance, glucose tolerance, adiposity, steatosis, and liver lipogenesis in rats fed with a high-fat high-fructose diet (HFHF). Male Wistar rats (n = 10/group), 45–50 days old, were fed with AIN93-M diet, HFHF (31% saturated fat and 20% fructose) and HFHF + sorghum flour (replacing 50% of dietary fiber, 100% starch, 19.8% protein and 22.5% lipids in the experimental diet), for 10 weeks. The sorghum flour restored the effect of the HFHF diet, decreasing triglycerides, uric acid, alanine aminotransferase (ALT), liver steatosis, and lipogenesis. Further, sorghum improved insulin sensitivity and glucose tolerance and increasing the concentration of PPARα protein in the liver. Thus, the BRS 305 whole sorghum flour with high tannin and resistant starch showed potential as a functional food, since it improves glucose metabolism, modulates adiposity, and reduces liver steatosis and lipogenesis in Wistar rats fed with a high-fat high-fructose diet

Keywords: glucose tolerance, insulin resistance, PPARα, uric acid, metabolic changes.

1. INTRODUCTION

Ultra-processed foods and beverages dense in energy, fat, and sugar are present in a Western diet, which combined with a genetic predisposition can trigger metabolic syndrome with weight gain, high plasmatic triglycerides, and lower insulin sensibility. These effects may increase the deposits of fat into adipose tissue and serum levels of free fatty acids, besides negatively affect glucose metabolism, raising plasma glucose, and insulin levels, generating insulin resistance (IR) (Nemati *et al.*, 2017).

The consumption of high-fat high-fructose diet (HFHF) promotes an increase in free fatty acids (FFA) circulating in adipose, muscle, and liver tissues, as well as an

increase in fat deposits, providing hypertrophy and hyperplasia of adipocytes (McArdle *et al.*, 2013). Further, the HFHF diet negatively affects glucose metabolism due to high plasma glucose and insulin levels, which results in IR. Thus, in the liver, insulin resistance leads to an increase in gluconeogenesis and hepatic lipogenesis, due to the excessive accumulation of FFA resulting in liver steatosis (McArdle *et al.*, 2013).

Due to the growing number of individuals with metabolic syndrome, nutritional strategies are essential to combat this condition. Sorghum (*Sorghum bicolor* L.), a cereal native to Africa, has high nutritional and functional value, with potential benefit for human health, able of improving the antioxidant and inflammatory conditions (Morais Cardoso, de *et al.*, 2017). There are sorghum hybrids, such as BRS 305, which has brown pericarp and pigmented test with a high content of condensed tannins and resistant starch (RS) (Teixeira *et al.*, 2016). Resistant starch can improve end points related to acute and long-term glucose and insulin responses, including the down-regulation of post- prandial peak glucose, postprandial glucose and insulin at specific points in time, and the incremental area under the curve (iAUC) for postprandial glucose and insulin (Harris, 2019). On the other hand, condensed tannins, represented especially by catechin, epicatechin and procyanidin B2, are a class of flavonoids related to the reduction of oxidative stress, which is related to less inflammation and better homeostasis of glucose metabolism (Laddha and Kulkarni, 2019). Further, sorghum tannins can reduce the activity of glycolytic enzymes, reducing the absorption rate and consequently improving the glycemic response (Mkandawire et al., 2013). In this way, the BRS 305 sorghum as a source of both these compounds can be a viable alternative to improve glucose and insulin metabolism.

Recent studies, by our research group, showed that SC 319 sorghum genotype (with a high content of 3-deoxyanthocyanidins, flavones, proanthocyanidins, and dietary fiber), in extruded form, promoted a reduction of fat accumulation in the liver, increasing β-oxidation, besides an anti-inflammatory action, helping to reduce obesity and liver steatosis in *Wistar* rats fed with a high-fat diet (Sousa, de *et al.*, 2018). ANUNCIAÇÃO et al. (2019) observed that the daily consumption of a breakfast cereal made with SC 319 sorghum genotype reduced the percentage of body fat and oxidative stress, increasing antioxidant enzymes in overweight men compared to wheat consumption.

However, there are currently no studies relating the intake of BRS 305 sorghum flour, which has high content of condensed tannins (Martinez et al., 2020) and resistant starch (Teixeira et al., 2016) on rats fed with a high-fat high fructose diet (HFHF). Thus, we hypothesize that BRS 305 hybrid sorghum flour can improve sensitivity to insulin and glucose tolerance, and promote the control of liver lipogenesis, increasing β-oxidation in animals fed a HFHF diet. The objective of this study was to evaluate the effect of dry heated whole sorghum flour (BRS 305 hybrid) on insulin resistance, glucose tolerance, adiposity, steatosis, and liver lipogenesis in rats fed with a high-fat high-fructose diet (HFHF).

2. MATERIAL AND METHODS

2.1 Material

The sorghum BRS 305 hybrid grains, with brown pericarp and high tannin (Martinez et al., 2020) and resistant starch (Teixeira et al., 2016) developed by Embrapa Milho and Sorgo Sete Lagoas, MG, harvest August 2015, were used. The seeds were manually selected, sieved, to eliminate impurities. Then, the grains were submitted to heat treatments at 105 ◦C in an air circulation oven, for 30 min, as proposed by Martinez et al. (2020). Then, the sorghum was ground in a knife mill (Bra- bender®, Dusburg, Germany) with a 1.0 mm stainless steel sieve to obtain the flour.

The chemical analysis of protein, lipids, moisture, ash, fiber (soluble, insoluble, and total), carbohydrates, and condensed tannins in BRS 305 dry heating sorghum were previously carried out and already published in Martinez et al. (2020). The chemical composition of this sorghum was moisture: 9.09 ± 0.14%; protein: 12.97 ± 0.19%; lipids: 5.01 ± 0.12%; ash: 1.13 ± 0.04%; total dietary fiber: 14.28 ± 0.69%; insoluble fiber: 13.95 ± 0.77%; soluble fiber: 0.33 ± 0.08%; carbohydrates: 66.61 ± 0.69%; and condensed tannins 59.53 ± 1.80 mgEC/g (milligrams of catechin per gramme of sample).

2.2 Chemical analysis of resistant starch

The resistant starch content (RS) was determined through simulated digestion with pancreatic α-amylase and amyloglucosidase, using a commercial kit (Resistant star assay kit AACC 32–40, Megazyme), following the instructions of manufacturer. These analyzes were per- formed after dry heating.

2.3 Identification of phenolic compounds

The 1g of sorghum dry heating flour was added in 5 mL of a methanol/water solution (50:50 v/v), mixed in the vortex for 1 min, and placed in a water bath with sonication at 24 ◦C for 20 min. Then it was mixed again for 1 min and centrifuged (4000g,15 min). The supernatant was filtered with a 0.20 μm Teflon syringe and stored at - 20 ◦C until use. The extracts and standards were analyzed by an Agilent 1220 Infinity liquid chromatography (LC) (Agilent Technologies, Santa Clara, CA, USA), coupled to an Advion expression LC compact mass spectrometer (Advion, Ithaca, NY, USA). Then,10 μL samples were injected and passed through a 3.5 μm 2.1 × 100 mm X Bridge Shield RP18 column (Waters, Milford, MA, USA) at 0.6 mL/min with the controlled temperature at 40 ◦C. The mobile phase was ultrapure water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The polyphenols were eluted using linear gradients of 84.4% A in 1.50 min, 81.5% A in 2.25 min, 77.0% A in 6.25 min, 55.0% A in 1.25 min, 46.0% A in 2.25 min, 94.0% A in 2.25 min and maintaining 94.0% A for 2.25 min, for a total run time of 18 min. From the column, the flow was directed to an ultraviolet (UV) detector of variable wavelength (265 and 278 nm), and later to an Advion LCMS mass spectrometer, and ESI mass spectrometry was performed in negative ionization mode using selected ion monitoring with a scan time of 200 ms. The temperature and voltage of the capillaries were 250 ◦C and 180 V, respectively, the voltage of the ESI source and the temperature of the gas were 2.5 kV and 250 ◦C, with a flow of desolvation gas at 240 L/h. Advion Mass Express™ software (Advidon, Ithaca, USA) was used to control LC, CMS instrumentation, and data acquisition. Individual polyphenols were identified and confirmed by comparing the m/z and LC retention times with authentic standards. Analysis of MS and UV data was performed using the Advion Data Express™ software.

2.4 Animals and experimental diets

Thirty Wistar rats (Rattus norvegicus) from 45 to 50 days of age were obtained from the Central Bioterium of Biological and Health Sciences at Universidade Federal de Viçosa. The animals were distributed in indi- vidual stainless-steel cages, kept at a temperature of 21 ± 3 ◦C with a 12- h photoperiod automatically controlled, and received distilled water and their respective experimental diets ad libitum. The animals were randomized using the animals' body weight to maintain homogeneity among experimental groups and the experiment was divided into two phases. In phase I, to induced metabolic changes, the animals were divided into two groups: normal control

group (155.98 ± 17.05g; n = 10), which received the AIN93-M diet (Reeves et al., 1993) and the HFHF group (162.67 ± 19.07g; n = 20), that received a high-fat and high-fructose diet, with 4% soy oil, 31% lard and 20% fructose, for 8 weeks. At the end of this period, the animals have fasted for 12 h a sample of blood was collected by a caudal puncture to analyze the concentration of glucose and triglyceride. Further, biometric parameters waist circumference and naso-anal length were measure to calculate the Lee index ($\sqrt[3]{P(g)}/CNA(cm)$). In phase II, the animals in the control

group were kept with the same diet AIN-93M diet (349.9 ± 30.71g; n = 10) and the animals from group HFHF were redistributed into two groups: a group that received an HFHF diet (HFHF control) (366.88 ± 36.90g; n = 10) and HFHF added with sorghum flour (368.44 ± 34.11g; n = 10) for 10 weeks. The addition of sorghum flour to the diet allowed the replacement of 50% fiber, 100% corn starch, 22.5% soybean oil, and 19.8% protein from sorghum flour (Table 1).

At the end of phase II, the animals were anesthetized with isoflurane (Isoforine, Crist´alia®) and euthanized by cardiac puncture. Blood was collected in tubes with EDTA heparin anticoagulant and centrifuged (1006g; 10 min) to obtain plasma. The liver of animal and adipose tissue were collected and washed in saline (PBS), weighed and immediately immersed in liquid nitrogen and subsequently stored at −80 ◦C. A fragment of the liver tissue was fixed in 10% formaldehyde for histological analysis.

The current project was approved by the Animal Use Ethics Committee of the Federal University of Viçosa (CEUA/UFV), protocol No. 38/2019. All the experimental procedures with animals were performed in accordance with Directive 86/609/EEC of November 24, 1986, in compliance with the ethical principles for animal experimentation.

Table 1. Composition of experimental diets

Ingredients (g/kg diet)	AIN93-M	HFHF	HFHF Sorgo**
Albumin*	136.6	136.6	108.8
Dextrinized starch	155.0	45.0	46.5
Corn starch	463.5	135	-
Sucrose	100.0	28.6	31
Soybean oil	40.0	40.0	31.1
Sorghum	-	-	195.4
Lard	-	310.0	310.0
Fructose	-	200.0	200.0
Microcrystalline Cellulose	55.8	55.8	27.9
Mineral mix	35.0	35.0	35.0

Vitamin mix	10.0	10.0	10.0
L-cystine	1.8	1.8	1.8
Choline bitartrate	2.5	2.5	2.5
Macronutrients			
Protein (%)	12.6	12.6	12.4
Carbohydrate (%)	82.8	47.1	46.2
Lipids (%)	4.6	40.3	42.4
Energetic density (Kcal/g)	3.8	5.3	5.2

*Albumin with 88% of protein content. ** Chemical composition of sorghum in g/100g is carbohydrates: 58.93g; total dietary fiber: 14.28g; lipids: 4.56g; protein: 12.11g; moisture: 9.09g; antioxidant capacity (µmol Trolox Equivalent/g): 296.41; condensed tannins (mgEC.g-1): 59.53 (Martinez et al., 2020). AIN-93M: standard diet for rodents; HFHF: high-fat high-fructose diet; HFHF Sorghum: HFHF diet plus sorghum flour.

2.5 Food intake and biometric measures

The food intake and body weight were measured weekly. The nasoanal length and waist circumference of the animals were measured using an inelastic measuring tape. The BMI was calculated as body weight/height^2. Total body adiposity (%) was calculated by the weight of total adipose tissues (epididymal, abdominal, and retroperitoneal), divided by the final body weight, multiplied by 100. The feed efficiency ratio (FER) was calculated by the ratio between body weight gain and total diet consumption (Sousa, de *et al.*, 2018).

2.6 Biochemical analyzes

The concentration of glucose and triglycerides was performed at the end of the induction of metabolic changes (phase I) and the end of the intervention (phase II). The animals have fasted for 12 hours and the blood was collected by a caudal puncture to analyze the concentration of glucose and triglycerides, using the commercial kits AccuChek Active and Accutrend devices, respectively, both from the Roche Company.

Plasma concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and uric acid were evaluated by colorimetric methods according to the manufacturer's instructions (Bioclin®). Insulin was analyzed by immunoassay according to the protocol (RayBio® Rat Insulin) in serum samples.

2.7 Assessment of glucose and insulin tolerance

The glucose intraperitoneal tolerance test (GTT) was conducted in the ninth week of the experiment (phase II). After fasting for 12 h, a 50% D-glucose solution (2g / kg body weight) was administered into intraperitoneal cavity of animals. The glucose concentration was determined in blood obtained from the caudal vein at baseline (zero) and after 30, 60, 90, and 120 minutes. Blood glucose was quantified using a glucometer with an appropriate test strip (Accu-Chek, Roche). The area under the glucose curve (AUC) was calculated using GraphPad Prism 6 (GraphPad Software, San Diego, CA).

In the tenth week (phase II), a week before the euthanasia of animals, the intraperitoneal insulin tolerance test (ITT) was performed with the animals fasted for 12 hours. Glycemia was measured at baseline (zero) and 5, 10, 15, 20, 25 and 30 minutes, after insulin injection intraperitoneally (Novolin®, Novo Nordisk), at the rate of 0.75 U/kg of body weight. The mean of the glycemia decay constant (KTTII) was calculated using the formula: [0.693 / (t1 / 2)], with t1/2 being the time needed to have baseline blood glucose. The glucose t1/2 was calculated from the slope of the glucose decay curve during the linear phase.

2.8 Quantification of PPARα

The extraction of the nuclear fraction from liver samples was performed using a specific kit (NE-PER ™ Nuclear and Cytoplasmic Extraction Reagents - Thermo Scientific), following the manufacturer's instructions. The quantification of the protein concentration of the peroxisome proliferator- activated receptor alpha (PPARα) was performed using the Rat PPAR-α ELISA kit (Elabscience, USA) by reading the absorbance of the samples in an ELISA reader at 450 nm (Multiskan Microplate Photometer, ThermoFisher Scientific, MA, USA).

2.9 Gene expression

Expression of mRNA levels was performed using the Quantitative Polymerase Chain Reaction technique in real-time (RT-qPCR). Briefly, 1 µL of cDNA (1:10), 3 mL H_2O, 5 µL of Applied FastStart Master SYBR Green Biosystems (Foster City, CA), and oligonucleotides at a final concentration of 100 nM were mixed to a final volume of 10 µL. Quantification was performed on the StepOnePlus ™ Real-Time PCR System, using the SYBR-Green fluorescence quantification system and Primer Express software (Applied Biosystems, Foster City, CA). Sense and antisense oligonucleotide sequences (Sigma-Aldrich Brasil Ltda) were used: AMPK: 5'-TGA AGC CAG AGA

AGG TGT TG-3' (forward) and 5'-ATA ATT TGG GGA TCC ACA-3' (reverse); AKT: 5'-GGG CCA CGG ATA CCA TGA AC-3' (forward) and 5'-AGC TGA CAT TGT GCC ACT GA-3' (reverse); PFK: 5'-CCA CCT GGA GGC CAT TGT AGA-3' (forward) and 5'-GGG ATG ACG CAC ATG ACGA-3' (reverse); PEPCK: 5'-GTT GCA GGC CCA GTT GTT GA-3' (forward) and 5'-CTC ACC TCT GGC CCA GAT TGG TA-3' (reverse). The β-actin was used as an endogenous control to normalize the relative expression of mRNA.

2.10 Histological analyzes of the liver tissue

The liver tissue samples were fixed in 10% formaldehyde and then embedded in resin. The sections were cut to 3 µm thick with 12 cuts of the distance between them and set up on glass slides and stained with gomori and picrosirius trichrome using the hematoxylin/eosin technique. The analyzes were performed and photographed under a bright field microscope (Olympus AX 70 TRF, Tokyo, Japan) in a 40X objective.

In the images of the histological sections, the cytoplasm, lipid vesicles, and collagen type I, III, and total were quantified using Image J® 1.48v software (National Institute of Health, USA). Ten liver fields were selected for each animal, with each histological field with a 266-point reticulum on the images until reaching the sum of 1064 points per animal in a 20x increase. The degree of liver steatosis was assessed semi-quantitatively according to the grade scale: grade 0, if the fat percentage was absent or <5%; Grade 1, if ≥5% and <25%; Grade 2, if ≥25% and <50%; Grade 3, if ≥50% and <75% (Turlin *et al.*, 2001).

2.9 Molecular Docking

The main phenolic compounds of sorghum (Catechin, Procyanidin B1, Procyanidin B2, Quercetin, Quercetin 3-glucoside, Quercetin, and 3-rutinoside) were used as potential ligands for PPARα. The Protein Data Bank (PBD) website (http://www.rcsb.org/pdb/home/home.do) was used to obtain the 3D crystal structures of PPARα. The binding sites of inhibitors or co-crystallized substrates were selected as the center of the anchorage area. The structures of ligants Catechin, Procyanidin B1, Procyanidin B2, Quercetin, Quercetin 3-glucoside, Quercetin, and 3-rutinoside recovered from the PubChem Compound database (https://pubchem.ncbi.nlm.nih.gov/).

The binder files were opened in AutoDock Tools to add partial loads of Gasteiger and detect the root of each rotating link in the set of structures. The dimensions of the survey space, the center point, and the flexible torsions were assigned with AutoDock Tools and the coupling calculations were performed using AutoDock Vina (Oleg e Arthur J., 2010). Several different runs per ligand were performed, and the pose with the highest binding affinity, with the least binding energy (BE) being saved. Protein-ligand interactions and binding modes were visualized in Discovery Studio Client (Dassault Systèmes Biovia Corp).

2.10 Statistical Analysis

The normality of the data was assessed by the Kolmogorov-Smirnov test. The data that did not present a normal distribution were transformed into Log_{10} before the parametric statistical analysis. The results were submitted to analysis of variance (ANOVA) and *post-hoc* of Newman-Keuls test, at 5% probability. The area under the curve (AUC) for insulin and blood glucose was calculated using the trapezoidal rule. A significance level of 5% was considered. All statistical analyzes were performed using the Graphpad Prism version 7.0 program.

3. RESULTS

3.1 The BRS 305 hybrid sorghum flour resistant starch and phenolic composition

The dry heated whole BRS 305 sorghum flour showed 37.2 ± 1.5 g/100g of resistant starch. The mains phenolics found were the condensed tannins catechin, procyanidin B1, procyanidin B2, quercetin, quercetin 3-glucoside, and quercetin 3-rutinoside, and they concentrations were 45.5 ± 2.2; 29.9 ± 7.8; 47.5 ± 5.5; 2.0 ± 0.5; 0.1 ± 0.0; and 0.1 ± 0.0 nmol/g, respectively.

3.2 Food intake and body composition

The food intake of the HFHF and HFHF sorghum group was lower ($p < 0.05$) than that of the AIN-93M group **(Supplementary Figure S1A).** The food efficiency ratio (FER), energy efficiency ratio (EER), and body weight were similar ($p > 0.05$) between the experimental groups (Supplementary Figure S1B, S1C, and S1D). The epididymal, retroperi- toneal, and visceral adipose tissues increased ($p < 0.05$), as well as, in the percentage of adiposity in the HFHF group compared with the AIN93-M group

(Supplementary Figures S1E, S1F, S1G, and S1H). Sorghum flour reduced ($p < 0.05$) the retroperitoneal and visceral adipose tissues and the percentage of adiposity relative to the AIN93-M group ($p > 0.05$) (Supplementary Figures S1E, S1F, S1G, and S1H). However, no difference was observed for epididymal adipose tissue between the HFHF groups and the sorghum flour ($p > 0.05$) (Supplementary Figure S1E). Further, BMI and Lee's index did not differ among the experimental groups ($p < 0.05$)

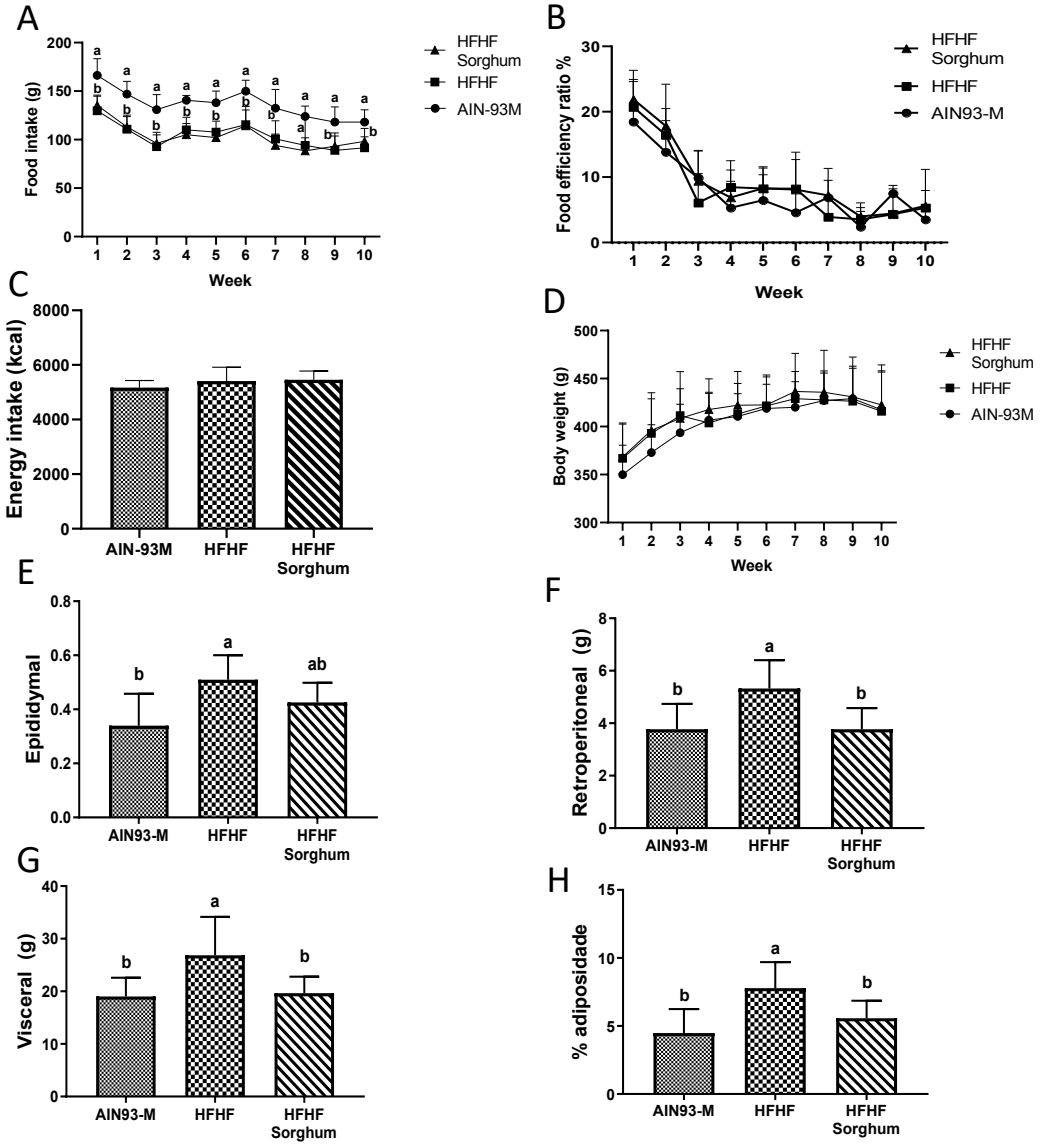


Figure S1. Food intake and body composition of animals fed with a HFHF diet and treated with BRS 305 sorghum flour after 10 weeks. Food consumption (A), food efficiency ratio (FER) (B), energy intake (C), body weight (D), epididymal adipose tissue (E), retroperitoneal adipose tissue (F), visceral adipose tissue (G), percentage of body adiposity (H). Values are represented by means and standard deviation (n = 10), means followed by the same letter do not differ by the Newman-Keuls test at 5% probability. AIN-93M: standard diet for rodents; HFHF: high-fat high-fructose diet; HFHF Sorghum: HFHF diet plus sorghum flour.

3.3 Biochemical analyses

The levels of AST did not differ among experimental groups ($p > 0.05$) (Supplementary Table S1). The HFHF group increased the levels of ALT, uric acid, TG, glycemia, and insulin, while the sorghum flour reduced these parameters become similar to the AIN93-M group (Sup- plementary Table S1). In the glucose tolerance test, the AUC of the HFHF group was higher than the group fed with sorghum flour ($p < 0.05$), demonstrating that the sorghum was able to recover glucose tolerance (Fig. 1A and B). Further, the HFHF group showed a lower insulin tolerance test than the AIN93-M group ($p < 0.05$) (Fig. 1C and D). The glycemia of the animals fed with sorghum flour was lower ($p < 0.05$) relative to the HFHF group, at 30 min of glucose tolerance test (Fig. 1B).

Table S1. Biochemical parameters of Wistar rats induced to metabolic changes with the HFHF diet and treated with sorghum flour for ten weeks

Parameters	AIN93-M	HFHF	HFHF Sorgo
AST (U/L)	122.0 ± 26.2 [a]	141.2 ± 32.5 [a]	131.9 ± 23.7 [a]
ALT (U/L)	31.1 ± 9.7 [b]	50.7 ± 13.3 [a]	36.6 ± 5.9 [b]
Uric acid (mg/dL)	1.1 ± 0.4 [b]	1.8 ± 0.5 [a]	0.9 ± 0.3 [b]
TG (mg/dL)	117.8 ± 20.3 [b]	147.8 ± 21.6 [a]	113.0 ± 12.9 [b]
Glycemia (mg/dL)	86.0 ± 7.2 [b]	100.8 ± 7.8 [a]	92.0 ± 4.6 [b]
Insulin (μUI/mL) **	13.7 ± 2.4 [b]	25.4 ± 13.2 [a]	11.8 ± 2.3 [b]

Values are represented by means and standard deviation (n = 10), means followed by the same letter do not differ by the Newman-Keuls test at 5% probability. AIN-93M: normal diet for rodents; HFHF: high-fat high-fructose diet; HFHF Sorghum: HFHF diet with added sorghum flour. AST: aspartate aminotransferase; ALT: alanine aminotransferase; TG: triglyceride. ** Postprandial insulin.

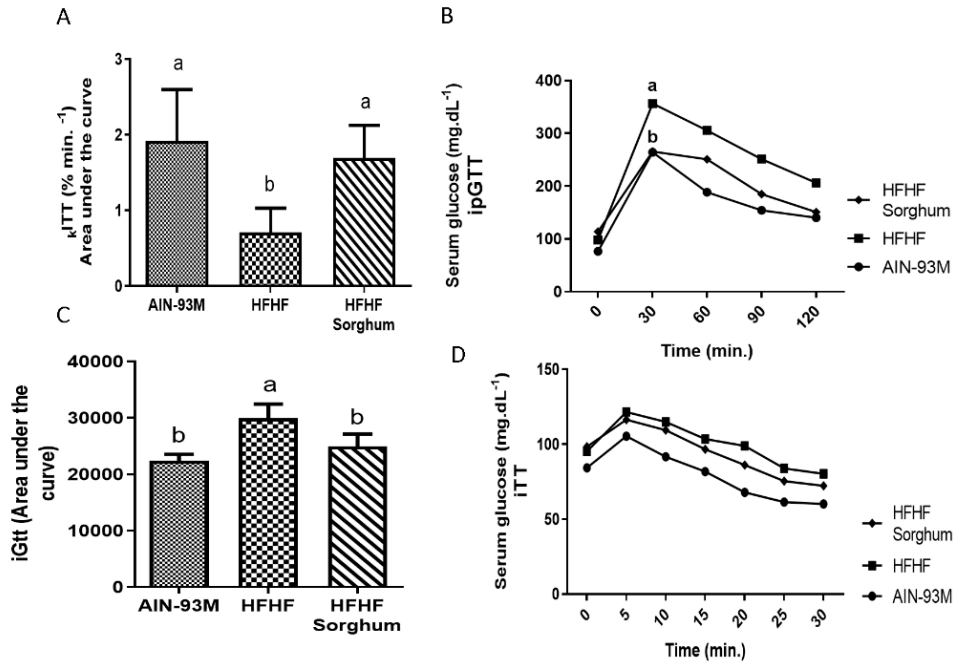


Fig. 1. Glycaemia and insulin resistance of ani- mals fed with a HFHF diet and treated with BRS 305 sorghum flour after 10 weeks. Glucose and insulin tolerance were accessed through intra- peritoneal glucose tolerance test (iGTT) and in- sulin tolerance test (ITT). Area under the curve of the glucose tolerance test (A), glucose tolerance test (B), area under the curve of the insulin tolerance test (C), insulin tolerance test (D). Values are represented by means and standard deviation (n = 10), means followed by the same letter do not differ by the Newman-Keuls test at 5% probability. AIN-93M: standard diet for ro- dents; HFHF: high-fat high-fructose diet; HFHF Sorghum: HFHF diet plus sorghum flour.

3.4 Gene expression and histomorphometry measurements in liver tissue

Sorghum flour up-regulated the mRNA levels ($p < 0.05$) of AMPK and AKT, which was higher than both control groups (Fig. 2A and B) and PFK expression was similar to the AIN-93M and HFHF groups (Fig. 2C). The sorghum flour added to the HFHF diet increased the protein levels of PPARα relative to the HFHF group ($p < 0.05$) (Fig. 2D)

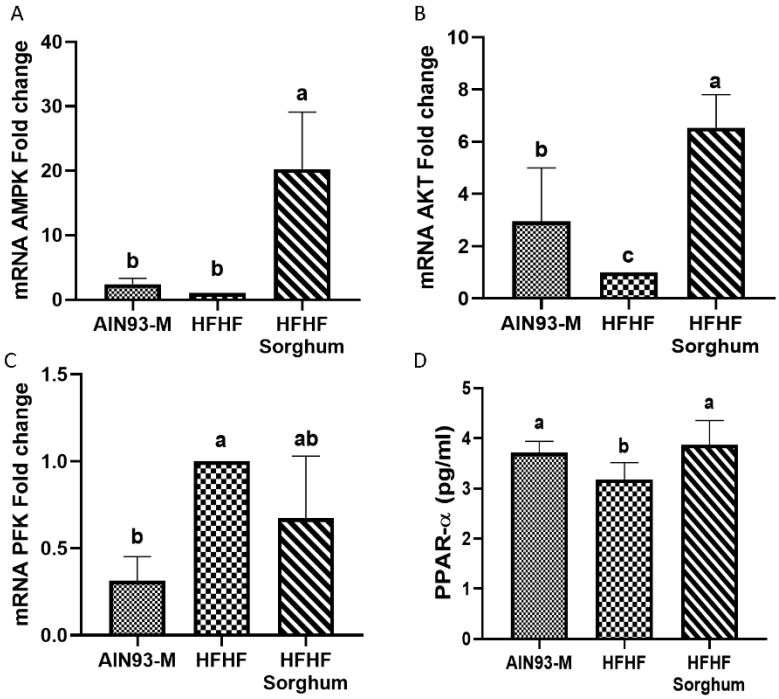


Fig. 2. Gene expression of biomarkers related to insulin resistance of animals fed with a HFHF diet and treated with BRS 305 sorghum flour after 10 weeks. Adenosine monophosphate-activated protein (AMPK) (A); protein kinase B (AKT) (B); phosphofructo kinase (PFK) (C); peroxisome proliferator-activated by receptor alpha (PPARα) (PEPCK) (D). Values are represented by means and standard deviation (n = 10), means followed by the same letter do not differ by the Newman-Keuls test at 5% proba- bility. AIN-93M: standard diet for rodents; HFHF: high-fat high-fructose diet; HFHF Sorghum: HFHF diet plus sorghum flour.

3.5 Histomorphometry measurements in the liver

The sorghum flour increased the percentage of cytoplasm compared to the control groups (Fig. 3A and B). The percentage of fat in the liver of animals fed an AIN93-M diet was 12.28% (Fig. 3A and B), classified as grade 1 liver steatosis (Fig. 3C). The HFHF group increased the per- centage of fat in the liver to 28.66% (Fig. 3A and B), classifying it in grade 2 liver steatosis (Fig. 3C). Besides, the consumption of sorghum reversed the liver steatosis to grade 1, with a percentage of 11.28% (Fig. 3A and B), similar to the AIN-93M control ($p > 0.05$). The sorghum flour added to the HFHF diet reduced inflammatory infiltrate, collagen type I, II, and total compare to the HFHF group (Fig. 3D)

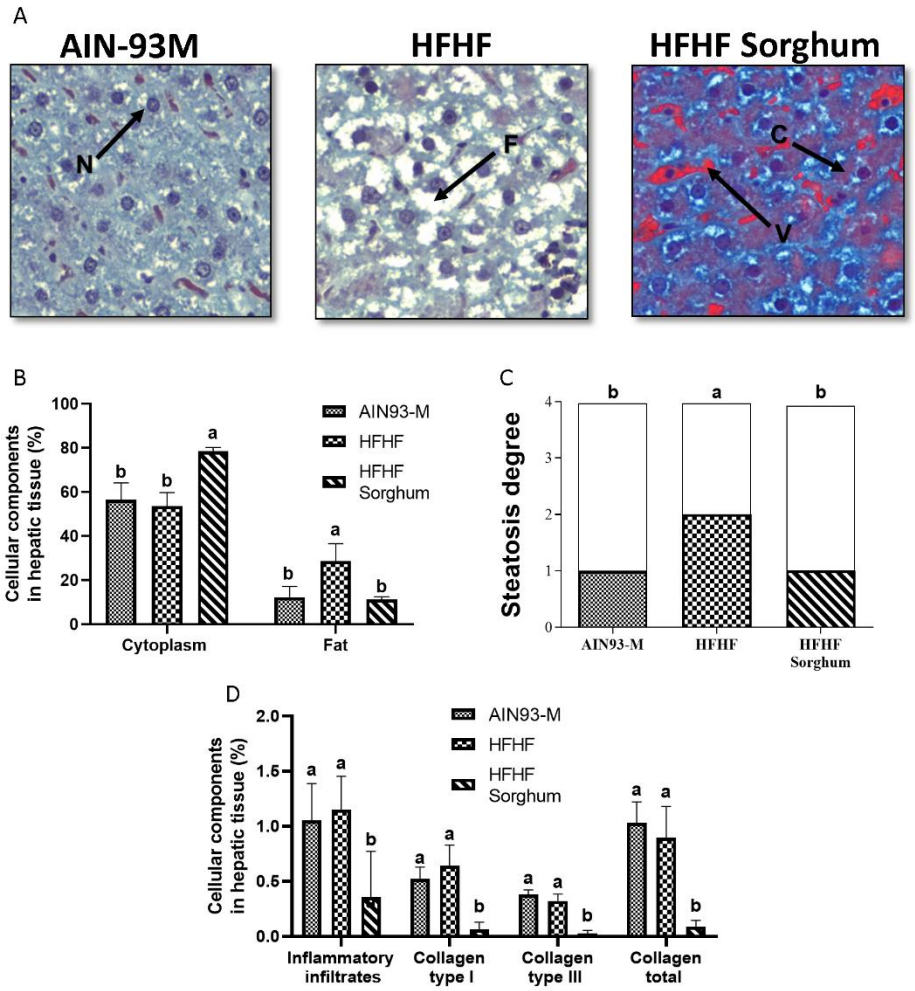
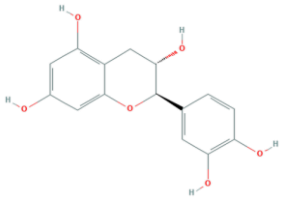
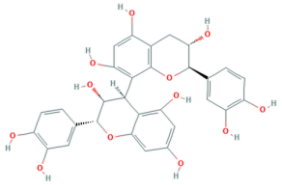
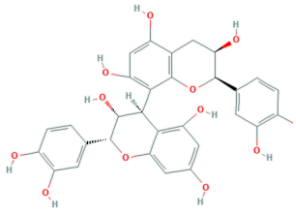
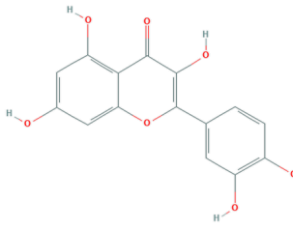
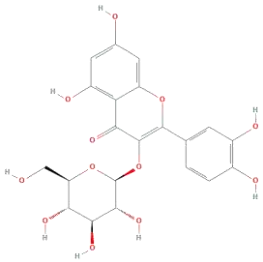
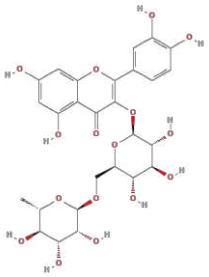


Fig. 3. Histological analysis of liver tissue of animals fed with a HFHF diet and treated with BRS 305 sorghum flour after 10 weeks. Histological images of liver from experimental groups (A); cellular components (B); degree of steatosis (C); cellular markers of hepatic steatosis (D). Values are represented by means and standard deviation (n = 10), means followed by the same letter do not differ by the Newman-Keuls test at 5% probability. Colorant: Gomori trichrome; N: Hepatocyte nucleus; F: Fat vesicles; C: cytoplasm; V: Blood vessels. Bar = 50 μm.

3.5 Molecular docking

The *in silico* analysis of the interaction between phenolic compounds Catechin, Procyanidin B1, Procyanidin B2, Quercetin, Quercetin 3-glucoside, Quercetin, and 3-rutinoside and PPARα showed that every phenolic compound founded in sorghum flour had interaction with PPARα (Supplementary Table S2), mainly quercetin-3-rutinoside, which had the highest interaction (Fig. 4).

Table S2. Estimated free energy binding (EFE) and chemical interactions among the phenolic compounds present in sorghum flour and PPARα.

Compound	Chemical structure*	PPARα	
		EFE	Ligant interaction
Catechin		-8,7	PHE A: 218; MET A: 320; THR A: 283; GLU A: 286; THR A: 279; TYR A: 334; VAL A: 332; LEU A: 331; GLY A: 335; ALA A: 333; MET A: 220; LEU A: 321; VAL A: 324; ALA A: 225; SER A: 323; ASN A: 221; LYS A: 222; TYR A: 214; ASP A: 372
Procyanidin B1		-7,4	ILE A: 228; ASN A: 236; PRO A: 237; PRO A: 238; LYS A: 232; SER A: 230; ASN A: 235; GLY A: 231; VAL A: 227; SER A: 234; ALA A: 233; VAL A: 223; LYS A: 224
Procyanidin B2		-7,3	VAL A: 223; VAL A: 227; LYS A: 224; SER A: 230; GLY A: 231; PRO A: 238; PRO A: 237; ASN A: 236; ILE A: 228; SER A: 234; ALA A: 233; LYS A: 232; ASN A: 235; VAL A: 227
Quercetin		-8,5	ILE A: 317; PHE A: 318; SER A: 280; TYR A: 314; TYR A: 464; LEU A: 460; LEU A: 456; GLN A: 277; VAL A: 444; HIS A: 440; PHE A: 273; CYS A: 276; ILE A: 272; MET A: 355; GLU A: 269; LEU A: 347; PHE A: 351; LEU A: 344; ILE A: 354
Quercetin 3-glucoside		-8,6	GLU A:282; ASN A: 219; GLU A: 286; PHE A: 218; ALA A: 333; CYS A: 275; MET A: 355; MET A: 330; CYS A: 276; VAL A: 332; LEU A: 321; VAL A: 324; ASN A: 221; MET A: 220; MET A: 320; THR A: 279; THR A: 283; TYR A: 334
Quercetin 3-rutinoside		**-8,9**	MET A: 325; LEU A: 331; LEU A: 321; VAL A: 324; MET A: 220; THR 1: 283; GLU A: 286; ASN A: 219; ILE A: 272; ILE A: 339; ILE A: 241; LEU A: 247; GLU A: 251; ALA A: 250; LEU A: 254; ALA A: 333; VAL A: 332; TYR A: 334; CYS A : 275; CYS A: 278; CYS A: 276; THR A: 279; GLU A: 282; MET A: 330

EFE: Estimated free energy. Docking calculation were carried out using AutoDock Vina. Negative values mean spontaneous reaction. Bold values represent the strongest interactions. *Chemical structures were obtained in Pubchem: https://pubchem.ncbi.nlm.nih.gov/. PPARα: Peroxisome proliferator-activated α receptor.

4. DISCUSSION

The present study provides new knowledge about the properties of the dry heated BRS 305 hybrid sorghum flour, rich in condensed tannins and resistant starch (RS), on insulin sensitivity, glucose tolerance, and modulation of liver adipogenesis and lipogenesis in animals fed with a high-fat high-fructose diet (HFHF). We use the heat treatment on sor- ghum, since this method has been shown efficient in maintaining anti- oxidant levels (Cardoso et al., 2014) and resistant starch (Teixeira et al., 2016) compared to the wet heat treatment in sorghum.

The HFHF diet was effective in promoting metabolic changes, which increased tissue adiposity, triglycerides, and glycemic levels decreased insulin sensitivity and glucose intolerance. Furthermore, HFHF diet increased alanine aminotransferase (ALT) and uric acid levels, markers related to liver and kidney integrity, respectively (Ozer et al., 2008). However, the presence of BRS 305 sorghum flour in a HFHF diet decreased the plasma levels of ALT, uric acid, triglycerides (TG), gly- cemia, and insulin, improving insulin sensitivity and glucose intolerance. Further, sorghum flour decreased the degree of liver steatosis and adiposity and increased PPARα, an important marker for mitochondrial β-oxidation (Wan et al., 2020).

The HFHF diet contributes to a pro-inflammatory state associated with possible damage to hepatocytes. In our study, we observed an in- crease in plasma ALT levels in the HFHF group, which are more common in hyperlipidemia and hyperglycemia conditions (Kim et al., 2008). The decrease in TG and glycemia by sorghum flour may have contributed to the reduction in ALT levels in HFHF sorghum group, as well as recovery the insulin sensitivity in this group. The significant relationship between TG and glycemia and ALT activity is demonstrated in literature (Kim et al., 2008), as observed in our study. The maintenance of AST values between the experimental groups may be due to its short half-life of 12h compared to 40–60 h for ALT and the rapid normalization in the blood. In addition, ALT is cytosolic and AST is cytosolic and mitochondrial, making it difficult to detect (Ramaiah, 2007).

The HFHF group in our study showed a hyperglycemic effect, decreasing glucose tolerance and promoting insulin resistance (Arbex et al., 2018). However, the BRS 305 sorghum flour in a HFHF diet improved glucose tolerance, showing a hypoglycemic effect. The reduction of glucose and insulin plasma concentration, probably due to the compounds present in this sorghum hybrid, such as phenolics,

condensed tannins, dietary fiber, and resistant starch (RS). Dietary fibers and RS can reduce the rate of digestion and activity of the enzyme amylase preventing the increase of glucose level, insulin, and decreasing post-meal plasma glucose (Carvalho et al., 2019). Further, sorghum tannins can partially inhibit the activity of α-amylase (pancreatic and salivary) and α-glucosidase during enzymatic carbohydrate hydrolysis, reducing the absorption rate and consequently improving the glycemic response (Mkandawire et al., 2013), as observed in other study with animals (Arbex et al., 2018). Thus, sorghum flour rich in condensed tannin and RS improves glucose metabolism under high-fat high-fructose diet conditions.

Furthermore, BRS 305 Sorghum flour increased the expression of the AMPK gene in liver tissue, relative to control groups. AMPK is an energy status sensor that plays a significant role in cellular energy homeostasis, associated with insulin resistance and metabolic disorders; its activation stimulates glucose uptake through the translocation of GLUT2 and GLUT4 to the cell surface in the liver and muscle, which can reduce blood glucose levels, improve insulin sensitivity, and modulate glucose homeostasis (Enes et al., 2020). It is observed that AMPK activity is downregulated in obesity and metabolic syndrome and under conditions of an unbalanced diet, which generates an energy imbalance and leads to insulin resistance (Enes et al., 2020). In addition, sorghum flour increased levels of AKT mRNA, a protein with the ability to phosphor- ylate and activate many metabolic targets, which can stimulate glucose uptake and glycogen synthesis (Ren et al., 2018). The AKT mediates most of the metabolic effects of insulin, regulating lipolysis and decreasing the levels of circulating fatty acids in adipose tissues (Jeong and Kim, 2019). These results show that BRS 305 sorghum flour has effects on the modulation of glycolysis and gluconeogenesis, associated with the activation of AKT and AMPK, and may represent an essential to control glucose metabolism.

The presence of BRS 305 sorghum flour in the HFHF diet decreased the level of uric acid compared to the HFHF diet. The uric acid, a biomarker of metabolic disorder, can directly stimulate the accumula- tion of liver fat, promoting pro-inflammatory and pro-oxidative effects, causing endothelial dysfunction and intracellular oxidative stress (King et al., 2018). The increase in uric acid in the HFHF diet probably occurred due to fructose, which is phosphorylated into fructose 1-phos-phate by fructokinase C, reducing intracellular levels of ATP (adenosine triphosphate),

GTP (guanosine triphosphate), and phosphate, leading to the accumulation of adenosine monophosphate (AMP) and fructose 1-phosphate (King et al., 2018). The drop in intracellular phosphate activates the enzyme AMP deaminase, which converts AMP into inosine monophosphate (IMP), resulting in the replacement of purine nucleotides that culminates in the formation of uric acid, using amino acid precursors (King et al., 2018). The phenolic acids (Arbex et al., 2018) present in sorghum can interfere in this metabolic pathway, reducing the levels of uric acid.

The food intake of the HFHF and HFHF sorghum groups was lower than the AIN-93M group. However, the final weight of the animals was similar. This result can be explained by the higher energy density of the HFHF diet compared to the AIN-93M, which increases satiety, reducing consumption, but without changing body weight. Furthermore, the consumption of sorghum flour was effective to reduce adiposity in ani- mals, probably due to its RS content. The RS can improved lipid meta- bolism disorder and inhibit the accumulation of TG into adipose tissue since after intake of resistant starch the fat synthesis speed is signifi- cantly reduced (Shen et al., 2015). Moreover, the RS can regulate the hormones leptin and adiponectin, which are involved in regulating food intake and body weight control (Stolarczyk, 2017). Thus, the con- sumption of sorghum could improve the lipid metabolism disorder caused by the HFHF diet and consequently decrease serum TG, reducing adiposity. In addition, phenolic compounds present in sorghum can also help to reduce adiposity by the ability to inhibit the expression of adipogenic genes, as observed by Arbex et al. (2018).

Sorghum flour in the HFHF diet increases the concentration of PPARα, decreasing liver lipogenesis. The effects of sorghum on PPARα occurs due to the interaction between the phenolic compounds of sor- ghum and the active site of PPARα, mainly quercetin-3-rutinoside, as the in silico analyzes demonstrated. Despite not being the phenolic com- pound present in greater quantity in dry heated whole sorghum, quercetin-3-rutinoside demonstrated the greatest interaction with PPAR-alpha in in silico analyzes. However, it was observed that all sor- ghum condensed tannins interact with PPAR-alpha, which probably explains the beneficial effects of sorghum on the genetic expression of this marker and the others related to the pathway. PPARα is a key regulator of hepatocyte metabolism, especially the β-oxidation of fatty acids (Navidshad and Royan, 2016). This effect of sorghum flour is promising since PPARα increases the expression of genes involved in the peroxisomal

and mitochondrial β-oxidation pathways, as well as trans- port proteins (CPT-1) (Mello et al., 2016), playing an essential role in the oxidation of liver fatty acids decreasing TG. de Sousa et al. (2018) showed that the extruded sorghum flour can increase PPARα even in a diet rich in saturated fat. This effect was attributed to 3-deoxyanocyani- din (3-DXA) (luteolinidine, apigeninidine, 7-methoxy-apigeninidine, and 5-methoxy-luteolinidine).

Thus, in this study, the intake of sorghum flour in an HFHF diet prevent lipogenesis de novo and promote β-oxidation. These effects were confirmed since sorghum flour decrease liver steatosis caused by HFHF diet, grade 2 (28.7% liver lipid) to grade 1 (11.3% liver lipid), decreasing lipotoxicity in the animal, as well as increase the percentages of cytoplasm and reduced inflammatory infiltrate, collagen type I/II and total. Inflammatory infiltrates, collagen type I/II, and total represents the typical fibrotic markers characterized by failure of degradation and excessive synthesis of extracellular matrix components (Zaki et al., 2019). Sorghum phenolic compounds are able to decrease the deposition of the excessive extracellular matrix, thus reversing the progression of liver fibrosis. The AIN93-M group had steatoses grade 1, which could be explained by the composition of the diet, which had 77.2% carbohy- drate, generating an increase in the synthesis of hepatic fatty acids and channeling excess acetyl resulting from glucose catabolism for fat biosynthesis in the liver by a process known as lipogenesis de novo (Ferramosca et al., 2013). Furthermore, the reduction of glycemia and postprandial hyperinsulinemia may decrease the storage of fat, reducing the synthesis of fatty acids from carbohydrates (Ye, 2013).

Then, in this study, we observed that the consumption of HFHF increased glucose and insulin levels, which promoted insulin resistance. This condition increased liver lipogenesis and excessive accumulation of free fatty acid that results in liver steatosis. Sorghum flour increased PPARα, which recovering insulin sensitivity and glucose tolerance and collaborate to reducing the degree of liver steatosis and adiposity. Further, the consumption of sorghum reduced plasma levels of ALT, uric acid, TG, glucose, and insulin, confirming the beneficial effects of sorghum on insulin resistance and hepatic metabolism (Fig. 5).

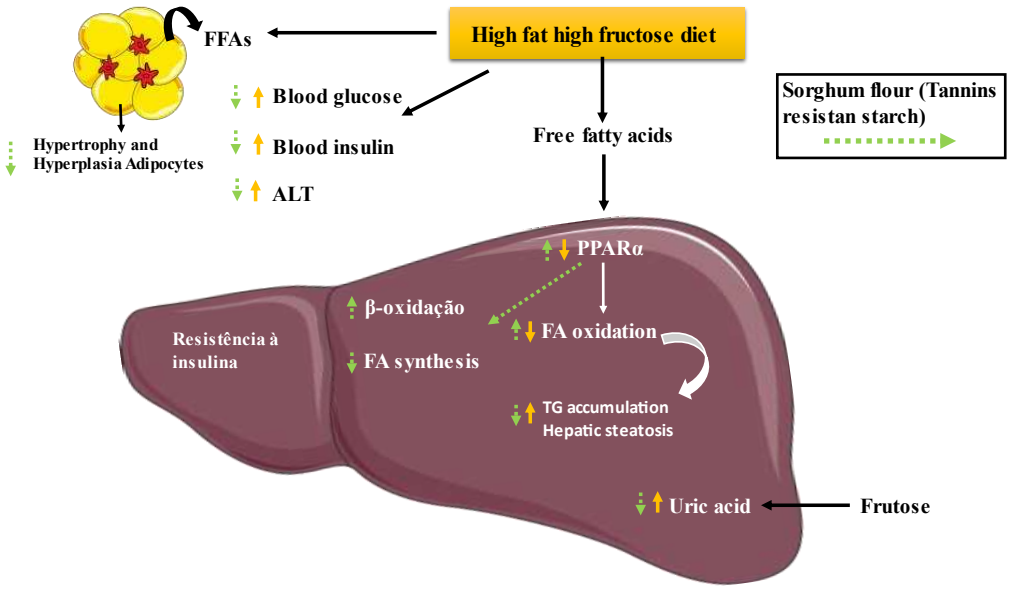


Fig. 5. Schematic diagram proposing the mechanisms of dry heat sorghum flour, BRS 305 hybrid to improving the meta- bolic changes caused by high-fat high- fructose diet (HFHF) in adult Wistar rats, after 10 weeks of intervention. Consump- tion HFHF diet induce hypertrophy and hy- perplasia of adipose tissue that increase monocyte infiltration into adipose tissue and lipolysis, leading to the secretion of glycerol and free fatty acids (FFA) from the adipose tissue into the circulation (Nieto-Vazquez et al., 2008). In addition, the HFHF diet increased glucose and insulin levels, which promoted insulin resistance. In the liver, in- sulin resistance increased liver lipogenesis and excessive accumulation of FFA resulted in liver steatosis. Sorghum flour reduced plasma levels of ALT, uric acid, TG, glyce- mia, and insulin, recovering insulin sensi- tivity and glucose tolerance. Further, sorghum flour decreased the degree of liver steatosis and adiposity and increased PPARα, an essential marker for inducing β-oxidation. FA: Fatty acids; PPAR-α: Peroxisome proliferator-activated α receptor; ALT: alanine aminotransferase; TG: triglycerides;

5. CONCLUSION

The intake of sorghum flour improves glucose metabolism by act directly on insulin sensitivity and glucose tolerance. In addition, sor- ghum flour promotes the control of adipogenesis and liver lipogenesis, reducing the accumulation of fat in the liver and the adipose tissues of animals fed an HFHF diet, which in turn may have contributed to the improvement of insulin resistance and glucose metabolism. These observed results may have been due to the composition of sorghum, as a rich source of resistant starch and condensed tannins that reestablished the normal metabolism of the animals that had been altered by the HFHF diet. Thus, sorghum demonstrates a good potential to control the fatty liver and insulin resistance and to prevent adiposity.

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Dry heated sorghum BRS 305 hybrid flour as a source of resistant starch and tannins improves inflammation and oxidative stress in *Wistar* rats fed with a high-fat high-fructose diet.

Abstract

This study aimed to evaluate the effect of dry heated sorghum BRS 305 hybrid flour, as a rich source of resistant starch and tannins, on inflammation and oxidative stress in animals fed with a high-fat high-fruc- tose diet. Phase 1 (8 weeks): male Wistar rats were divided into a group fed with an AIN-93 M diet (n = 10) and a group fed with a high-fat (35%) high-fructose (20%) (HFHF) diet (n = 20). Phase 2 (intervention 10 weeks): the control group was continued with the AIN-93 M diet (n = 10) and the HFHF group was divided into HFHF (n = 10) and sorghum flour (n = 10) groups. Sorghum flour decreased the NO, Akt, p65-NFκB, TLR4, and lipid peroxidation in the liver. Furthermore, sorghum flour improved SOD and CAT activities and the total antioxidant capacity of plasma. The phenolic compounds found in sorghum flour interacted in silico with AKT and p65-NFκB, mainly quercetin-3-rutinoside that showed the highest inter- action with AKT (EFE −8.0) and procyanidins B1 and B2 that showed the highest interaction with p65- NFκB (EFE −8.9). The consumption of BRS 305 sorghum with a high tannin and resistant starch content improved inflammation and oxidative stress by inhibition of p65-NFκB activation in rats fed a high-fat high-fructose diet.

Keywords: nitric oxide, Akt, NFκB, phenolics compounds, TLR4, *Sorghum bicolor L*

1. Introduction

Foods rich in fat and sugar are commonly consumed in modern society, creating an imbalance between energy intake and expenditure and contributing to the accumulation of visceral fat and lipotoxicity in tissues, including the liver (Zaki, Fattah e Hassan, 2019). This diet is represented in experimental studies as high-fat and high-fructose (HFHF) diet. It demonstrated that high fructose consumption decreases satiety, favoring a positive energy balance, increases adipogenesis, leading to visceral fat accumulation, leading to insulin resistance, inflammation, and lipid metabolism impairment, increasing arterial blood pressure, and vascular and liver damage (Lelis

et al., 2020). In the same way, a high-fat diet causes metabolic alterations by increasing fat deposits, especially intra-abdominal fat, increasing the plasma levels of free fatty acids and leptin (Nemati et al., 2017). Therefore, the consumption of a diet with a high level of fat and fructose, characteristic of the current Western diet, is directly related to the development of metabolic changes and diseases, such as obesity, diabetes mellitus, coronary diseases, cancer, among others (Nemati et al., 2017).

This HFHF diet increases the influx of free fatty acids (FFA) into adipose, muscle and liver tissue, and endothelial cells (Zaki, Fattah e Hassan, 2019). The FFA activates toll-like receptor-4 (TLR-4), causing downstream activation of nuclear factor kappa B (NFκB), a proinflammatory transcription factor, that once activated, migrates into the nucleus where it induces transcriptional expression of multiple proinflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and interleukin-6 (IL-6). Besides, FFA can be undergone to β-oxidation, which can stimulate the formation of reactive oxygen species (ROS), such as the nitric oxide (NO), that in turn activates NFκB and increasing the inflammation (Julieta et al., 2016; Sousa, de et al., 2019; Suárez-Cuenca et al., 2019).

Besides more, the TLR-4 activation induces the PI3K/Akt (phosphatidylinositol 3-kinase/phospho-protein kinase B) pathway that activates NADPH oxidase, which induces greater oxidative stress and activates NFκB, further increasing the inflammation process (Jiang et al., 2017; Khan et al., 2019). Besides, HFHF diet may modify the response of the immune cells, increasing the platelet/lymphocyte ratio (RPL) and the neutrophil/lymphocyte ratio (RNL), which also can stimulate oxidative stress (Julieta et al., 2016; Sousa, de et al., 2019; Suárez-Cuenca et al., 2019).

A diet that includes whole grains is a strategy to reduce the complications resulting from an unbalanced diet (Enes et al., 2020; Sousa, de et al., 2019). Sorghum (Sorghum bicolor L.) is among the five most produced types of cereals in the world with the capacity to grow in dry and arid regions, generating lower production costs (Rosa, 2012). Sorghum genotypes have a varied composition of phytochemicals with antioxidant activity, such as phenolic acids, tannins, anthocyanins, and dietary fibers, such as resistant starch (RS), which has prebiotic properties (Sousa, de et al., 2019). These compounds can act to reduce the risk of chronic diseases and non-communicable diseases (Lopes et al., 2018; Morais Cardoso, de et al., 2017; Sousa, de et al., 2018).

Among the sorghum genotypes, the BRS 305 hybrid consisted of a light brown pericarp with pigmented testa, without dhurrin, but with a high content of tannins (Martinez et al., 2020) and RS (Teixeira et al., 2016). Its extruded form has demonstrated a protective effect in changed metabolic conditions, like an improvement of oxidative stress and inflammation in patients with chronic kidney disease (Lopes et al., 2018). Other studies observed that BRS 305 hybrid whole sorghum flour improved the inflammation and oxidative stress in animals with oxidative stress induced by paracetamol (Silva et al., 2020), and Sodium fluoride (Martinez et al., 2020). However, the impact of BRS 305 whole sorghum flour on rats fed with a high-fat high fructose diet (HFHF), a diet that mimics the current classic Western diet, has not been investigated.

Given the compounds present in the sorghum BRS 305 hybrid, it is believed that this grain may be an alternative in controlling the metabolic changes caused by the HFHF diet. Then, we hypothesize that BRS 305 sorghum flour, as a rich source of resistant starch, condensed tannins, and phenolic compounds can improve inflammation and oxidative stress. Thus, the present study aimed to evaluate the effects of the BRS 305 whole sorghum hybrid flour on inflammation and oxidative stress in adult Wistar rats induced to metabolic changes by the HFHF diet.

2. Material and methods

2.1. Material

Whole sorghum grains of the BRS 305 hybrid, with brown pericarp, high tannin content (Martinez et al., 2020), and resistant starch (Teixeira et al., 2016) were developed by Embrapa Milho e Sorgo (Sete Lagoas, MG, Brazil) in August 2015. The grains were harvested, selected, and sieved manually and impurities were removed.

The whole sorghum grains were submitted to dry heat treatment to maintain antioxidants activity and resistant starch content, compared to wet heat treatment, as has been shown in previous studies (Cardoso et al., 2014; Teixeira et al., 2016). Thus, the grains were placed in aluminum trays and exposed to 105 °C in an air circulation oven for 30 min and spread over a maximum layer of 0.5 cm in height (Martinez et al., 2020). After heat treatment, the dry grains were ground in a knife mill (Brabender, Duisburg, Germany) with a 1.0 mm stainless steel sieve to obtain the whole sorghum flour

2.1.2. Chemical Analysis of Sorghum Flour

The data from the analysis of the composition and condensed tannins was based on our previous study (Martinez et al., 2020). The resistant starch content (RS) was determined through simulated digestion with pancreatic α-amylase and amyloglucosidase, using a commercial kit (Resistant star assay kit AACC 32-40, Megazyme), following the instructions of the manufacturer. Phytic acid content was determined using the K-PHYT Phytic Acid (Phytate) / Total Phosphorus kit (Megazyme), following the manufacturer's instructions. The content of total phenolic compounds in the sorghum extract was determined using the Folin-Ciocalteu method (Singleton, Orthofer E Lamuela-Ravento, 1999).

2.1.3. Identification of phenolic compounds

The 1g of sorghum flour was added in 5 mL of a methanol/water solution (50:50 v / v), mixed in the vortex for 1 min, and placed in a water bath with sonication at 24°C for 20 min. Then it was mixed again for 1 min and centrifuged (4000g,15 min). The supernatant was filtered with a 0.20 µm Teflon syringe and stored at - 20 °C until use.

The extracts and standards were analyzed by an Agilent 1220 Infinity liquid chromatography (LC) (Agilent Technologies, Santa Clara, CA, USA), coupled to an Advion expression LC compact mass spectrometer (Advion, Ithaca, NY, USA). Then,10 µL samples were injected and passed through a 3.5 µm 2.1 x 100 mm X Bridge Shield RP18 column (Waters, Milford, MA, USA) at 0.6 mL/minute with the controlled temperature at 40 °C (Silva, da et al., 2019). The mobile phase was ultrapure water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The polyphenols were eluted using linear gradients of 84.4% A in 1.50 min, 81.5% A in 2.25 min, 77.0% A in 6.25 min, 55.0% A in 1.25 min, 46.0% A in 2.25 min, 94.0% A in 2.25 min, and maintaining 94.0% A for 2.25 min, for a total run time of 18 min. From the column, the flow was directed to an ultraviolet (UV) detector of variable wavelength (265 and 278 nm), and later to an Advion LCMS mass spectrometer, and ESI mass spectrometry was performed in negative ionization mode using selected ion monitoring with a scan time of 200 ms. The temperature and voltage of the capillaries were 250°C and 180 volts, respectively, the voltage of the ESI source and the temperature of the gas were 2.5 kilovolts and 250 ° C, with a flow of desolvation gas at 240 L / hour. Advion Mass Express™ software (Advidon, Ithaca, USA) was used to

control LC, CMS instrumentation, and data acquisition. Individual polyphenols were identified and confirmed by comparing the m/z and LC retention times with authentic standards. Analysis of MS and UV data was performed using the Advion Data Express™ software.

2.2. Biological assay

Thirty Wistar rats (Rattus norvegicus) with 45 to 50 days of age, obtained from the central Bioterium of Biological and Health Sciences at Federal University of Viçosa, were used. The animals were distributed in individual stainless-steel cages, in an environment with a controlled temperature at 21 ± 2º C with a 12-hour photoperiod automatically controlled. The animals received distilled water and their respective experimental diets ad libitum.

The project was approved by the Animal Use Ethics Committee of the Federal University of Viçosa (CEUA / UFV), protocol No. 38/2019. All experimental procedures with animals were carried out under ethical principles in animal experimentation.

2.3. Experimental design

The experiment was divided into two phases. In phase I, metabolic changes were induced for eight weeks, adapted from Marineli et al. (2015). The animals were divided into two groups: lean control (155.98 ± 17.05g; n = 10), which received a standard diet for rodents AIN93-M (Reeves et al., 1993), and the HFHF group (162.67 ± 19.07g; n = 20), which received a diet rich in fat (31% from lard and 4% from soybean oil) and fructose (20%). In phase II, the treatment was carried out for ten weeks, according to Marineli et al. (2015). The animals in the lean control group were maintained on the AIN-93M diet (349.94 ± 30.71g; n = 10), and HFHF were redistributed into two groups, a HFHF control group (366.88 ± 36.90g; n = 10), which kept HFHF diet, and HFHF sorghum group, which received HFHF diet plus sorghum flour (368.44 ± 34.11g; n = 10). Randomization was performed in a non-random manner. The body weight of animals was used to maintain homogeneity between groups.

At the end of phase II, the animals were anesthetized with 100% isoflurane (Isoforine, Cristália) and euthanized by cardiac puncture. The blood was collected in tubes with EDTA anticoagulant and centrifuged 1006g for 10 min to obtain plasma.

The liver was collected, washed in saline (PBS), weighed, and immediately immersed in liquid nitrogen and stored at -80º C.

2.4. Experimental diets

The experimental diets, AIN93-M (lean control group) and HFHF (HFHF control group) were prepared according to Reeves; Nielsen; Fahey Jr. (1993) and Shapiro et al. (2011), respectively. The HFHF group received a diet with a high content of lipids (4% soybean oil plus 31% lard), and high content of fructose (20%) (Shapiro et al., 2011). The HFHF Sorghum group received a diet replacing 50% dietary fiber, 100% corn starch, 22.5% soybean oil, and 19.8% protein from sorghum flour, totaling 195.4g of flour per kg of diet (Sousa, de et al., 2018). The HFHF and HFHF sorghum diets were isoproteic, isocaloric, and isoglycidic (Table 1). After preparation, the diets were packed in dark polyethylene bags and stored in a freezer (-18ºC ± 1ºC), to minimize the oxidation of fatty acids from sorghum flour.

Table 1. Composition of experimental diets

Ingredients (g/kg diet)	AIN93-M	HFHF	HFHF Sorgo**
Albumin*	136.6	136.6	108.8
Dextrinized starch	155.0	45.0	46.5
Corn starch	463.5	135	-
Sucrose	100.0	28.6	31
Soybean oil	40.0	40.0	31.1
Sorghum	-	-	195.4
Lard	-	310.0	310.0
Fructose	-	200.0	200.0
Microcrystalline Cellulose	55.8	55.8	27.9
Mineral mix	35.0	35.0	35.0
Vitamin mix	10.0	10.0	10.0
L-cystine	1.8	1.8	1.8
Choline bitartrate	2.5	2.5	2.5
Macronutrients			
Protein (%)	12.6	12.6	12.4
Carbohydrate (%)	82.8	47.1	46.2
Lipids (%)	4.6	40.3	42.4
Energetic density (Kcal/g)	3.8	5.3	5.2

*Albumin with 88% of protein content. ** Chemical composition of sorghum in g/100g is carbohydrates: 58.93g; total dietary fiber: 14.28g; lipids: 4.56g; protein: 12.11g; moisture: 9.09g; antioxidant capacity (µmol Trolox Equivalent/g): 296.41; condensed tannins (mgEC.g-1): 59.53 (Martinez et al., 2020). AIN-93M: standard diet

for rodents; HFHF: high-fat high-fructose diet; HFHF Sorghum: HFHF diet plus sorghum flour.

2.5. Food consumption, biochemical and biometric data

Food intake and animal weight were measured weekly; the feed efficiency ratio (FER) was obtained through the relationship between body weight gain (g) and the amount of diet (g) consumed by the animals. In contrast, the energy efficiency ratio (EER) was obtained by the final body weight gain (g) divided by the total energy consumption (kcal) multiplied by 1000 (Sousa, de et al., 2018).

An inelastic measuring tape was used to measure waist circumference and naso-anal length. The body mass index (BMI) and the Lee index of the animals were calculated, as previous study (Novelli et al., 2007) and Neutrophil/Lymphocyte ratio (RNL) and Platelet/Lymphocyte ratio (RPL) were calculated as the ratio between the number of neutrophils and lymphocytes, and between the number of platelets and lymphocytes, respectively.

2.6. Oxidative stress, antioxidant enzyme activity, and total antioxidant capacity

The liver tissue homogenate was prepared in proportion 1:10 with 50 mM phosphate buffer with 1mM EDTA, pH 7.4, containing protease inhibitor (Protease Inhibitor Cocktail powder, Sigma Aldrich). Total proteins were quantified using the Bradford (1976). Oxidative stress was assessed by determining the malondialdehyde (MDA) content obtained by analyzing substances reactive to thiobarbituric acid (Buege e Aust, 1978) and nitric oxide using the Griess method (Green et al., 1982). The antioxidant enzyme superoxide dismutase (SOD) activity was analyzed by determining the inhibition of the auto-oxidation of the pyrogallol reagent (Marklund, 1985) and catalase by the decomposition of hydrogen peroxide (Aebi, 1984). The Total Antioxidant Capacity (TAC) of Plasma and liver was evaluated by the specific kit (Sigma Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions.

2.7. Quantification of cytokines, p65-NFκB, TLR4, and phospho-AKT1 [pS473] in the liver

The concentration of TNF and IL-10 was quantified in the liver of the animals using Elisa kits Rat kit TNF alpha Uncoated ELISA (Invitrogen, Austria) and Rat IL-10

kit ELISA (Elabscience, USA), respectively, following the manufacturer's recommendations.

Nuclear protein extraction from liver samples was performed using a specific kit (NE-PER™ Nuclear and Cytoplasmic Extraction Reagents - Thermo Scientific), following the manufacturer's instructions. Quantification of p65-NFκB and TLR4 was performed using a Rat NKκB-p65 ELISA kit (E-EL-R0674, Elabscience, USA) and Rat TLR4 ELISA kit (E-EL-R0990, Elabscience, USA), respectively.

The concentration of phospho-protein kinase B (AKT1 [pS473]) was determined by immunoassay, according to the manufacturer's instructions (Sigma Aldrich) using the Phospho-AKT1 [pS473] ELISA Kit, Ultrasensitive. Absorbance was measured using a spectrophotometer at 450 nm (Multiskan, ThermoFisher Scientific, MA, USA) and the protein concentration was calculated using a standard curve.

2.8. Molecular Docking

The main phenolic compounds of sorghum were used as potential ligands for the proteins involved in inflammation, evaluated by molecular coupling. The Protein Data Bank (PBD) website (http://www.rcsb.org/pdb/home/home.do) was used to obtain the 3D crystal structures of Akt (PDB: 3CQU) and p65-NFκB (PDB: 1oy3). The binding sites of inhibitors or co-crystallized substrates were selected as the center of the anchorage area. Catechin, Procyanidin B1, Procyanidin B2, Quercetin, Quercetin 3-glucoside, Quercetin, and 3-rutinoside were used as ligands, and their structures were recovered from the PubChem Compound database (https://pubchem.ncbi.nlm.nih.gov /).

The binder files were opened in AutoDock Tools to add partial loads of Gasteiger and detect the root of each rotating link in the set of structures. The dimensions of the survey space, the center point, and the flexible torsions were assigned with AutoDock Tools (Garrett et al., 2009), and the coupling calculations were performed using AutoDock Vina (Oleg e Arthur J., 2010). Several different runs per ligand were performed, and the pose with the highest binding affinity, with the least binding energy (BE) being saved. Protein-ligand interactions and binding modes were visualized in Discovery Studio Client (Dassault Systèmes Biovia Corp) (Rebollo-Hernanz, Zhang, Aguilera, Martín-Cabrejas e Mejia, de, 2019).

2.9 Statistical Analysis

The normality of the data was assessed by the Kolmogorov-Smirnov test. The data that did not present a normal distribution were transformed into Log_{10} before the parametric statistical analysis. The results found were submitted to analysis of variance (ANOVA), followed by the Newman-Keuls test at 5% probability. All statistical analyzes were performed using the Graphpad Prism version 7.0 program.

3. Results

3.1. The BRS 305 hybrid sorghum flour resistant starch and phenolic composition

The content of resistant starch, phytic acid, total phenolics, and phenolic profile of sorghum flour is listed in Table 2. Procyanidin B2, catechin, and procyanidin B1 were the main phenolic compounds present in the sorghum flour. Further, the sorghum flour showed a good source of resistant starch and phenolic compounds, mainly catechin and procyanidins B1 and B2.

Table 2. Total phenolics, phytic acid, resistant starch contents and phenolic profile of sorghum flour

Chemical composition	Sorghum flour*
Resistant starch (g/100g)	37.2 ± 1.5
Phytic acid (g/100g)	1.1 ± 0.1
Total phenolics (mg GAE/g)**	15.5 ± 0.6
Catechin (nmol/g)	45.5 ± 2.2
Procyanidin B1 (nmol/g)	29.9 ± 7.8
Procyanidin B2 (nmol/g)	47.5 ± 5.5
Quercetin (nmol/g)	2.0 ± 0.5
Quercetin 3-glucoside (nmol/g)	0.1 ± 0.0
Quercetin 3-rutinoside (nmol/g)	0.1 ± 0.0
Condensed tannins (mgEC/g)***	59.53 ± 1.8

* Values are shown by means and standard deviation

**mg GAE/g: gallic acid equivalent/100g

***mgEC/g: milligrams of catechin per gramme of sample

3.2. Effect of sorghum flour on food consumption, biometric measurements, and adiposity

The animals started phase I and phase II with similar weights (Table 3). There were no differences in the body weight gain, body mass index (BMI), energy intake, and the Lee index among all groups after ten weeks. The total food consumption was higher in the AIN93-M group when compared to the HFHF groups. The intake of whole

sorghum flour promoted the daily consumption of condensed tannins (41.6 ± 1.4 mg kg−1 d−1), resistant starch (260.7 ± 10.9 mg kg−1 d−1), and total phenolics (10.8 ± 0.3 mg−1kg−1 d−1) in the HFHF group (Table 3).

Table 3. Food consumption and biometric measurements of Wistar rats submitted to metabolic changes after ten weeks

Variables	AIN93-M	HFHF	HFHF Sorgo
Body weight gain (g)	65.1 ± 22.7[a]	63.5 ± 31.0[a]	58.8 ± 18.7[a]
Lee index (g/cm³)	292.8 ± 14.5[a]	299.0±11.9[a]	292.5 ± 14.8[a]
FER	4.7 ± 1.57[a]	5.4 ± 1.9[a]	5.2 ± 1.5[a]
EER	11.7 ± 3.4[a]	11.6 ± 5.3[a]	11.0 ± 3.0[a]
BMI (g/cm^2)	0.6 ± 0.1[a]	0.7 ± 0.1[a]	0.6 ± 0.1[a]
Food consumption (g)	1367.0±69.1[a]	1045.6±130.2[b]	1040.0±60.7[b]
Energy intake (kcal)	5286.0±77.7[a]	5351.0±338.6[a]	5282.0±293.7[a]

Values are represented by means and standard deviation (n=10). Means followed by the same letter do not differ by the Newman-Keuls test at 5% probability. AIN-93M: standard diet; HFHF: High-fat high-fructose diet; HFHF Sorghum: HFHF diet plus sorghum flour. FER: feed efficiency coefficient; EER: energy efficiency ratio; BMI: body mass index

3.3. Influence of sorghum flour on total antioxidant capacity of plasma and biomarkers of oxidative stress in the liver

The total antioxidant capacity (TAC) in the plasma was higher ($p < 0.05$) in the animals that received HFHF plus whole sorghum flour when compared to the AIN93-M and HFHF groups (Figure 1A). However, whole sorghum flour did not increase TAC in the liver relative to the HFHF group (Figure 1B). For antioxidant enzymes, sorghum flour increased ($p < 0.05$) the activity of SOD and CAT compared to the HFHF group (Figure 1C and D). However, the HFHF and AIN93-M groups showed no differences in SOD determination (Figure 1C). The NO (Figure 1E) and MDA (Figure 1F) concentration increased ($p < 0.05$) in the HFHF group compared to the other groups. Sorghum flour added to the HFHF diet decreased NO and MDA concentration ($p < 0.05$) became similar to the AIN93-M group (Figures 1E and F).

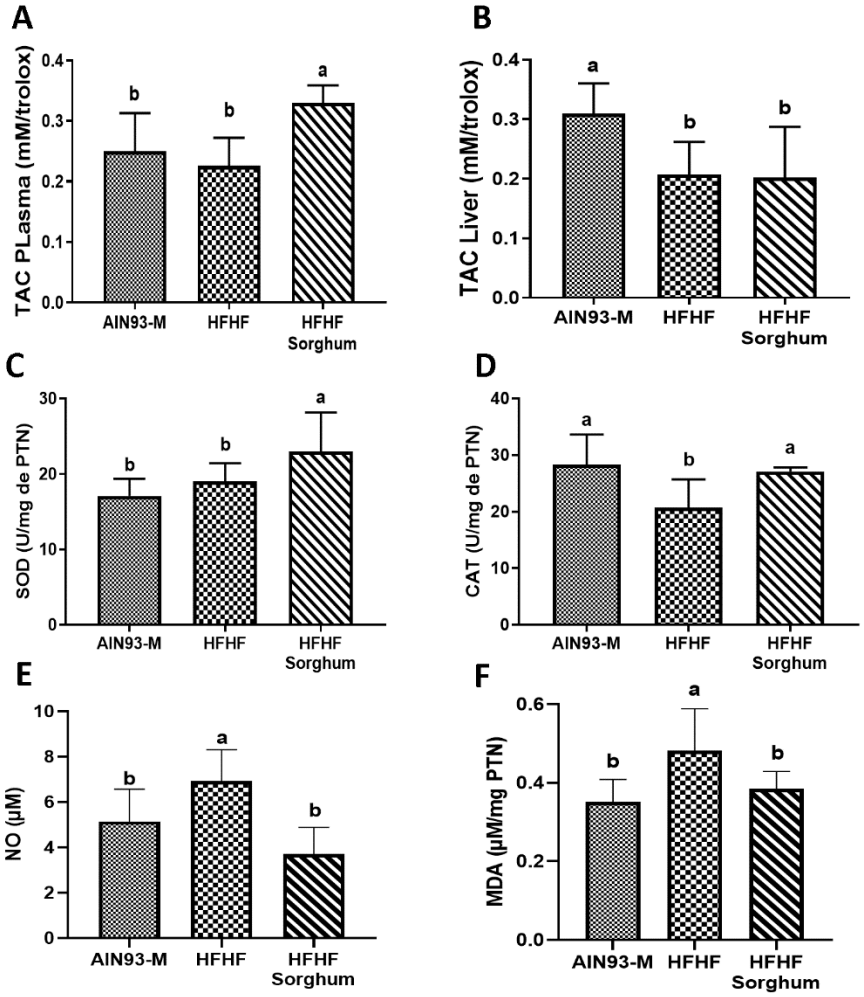

Figure 1. Sorghum flour increases total antioxidant capacity in plasma and antioxidant enzymes and reduces oxidative stress in the liver of Wistar rats fed with a high-fat high-fructose diet, after 10 weeks of treatment. Total antioxidant capacity (TAC) in plasma (A) in the liver (B), superoxide dismutase (SOD) (C), catalase (CAT) (D), nitric oxide (NO), (E) and malondialdehyde (MDA) (F) in the liver. Values are represented by means and standard deviation (n = 10). Means followed by the same letter do not differ by the Newman-Keuls test at 5% probability. AIN-93M: standard diet for rodents; HFHF: high-fat high-fructose diet; HFHF Sorghum: HFHF diet plus sorghum flour.

3.4. Effects of sorghum flour on inflammatory markers in the liver

Whole sorghum flour added to the HFHF diet reduced the ratio of neutrophil/lymphocyte (RNL) (Fig. 2A), the platelet/ lymphocyte (RPL) (Fig. 2B), the phosphorylated Akt protein (Fig. 2C), and the nuclear concentration of p65-NFκB (Fig. 2D) in the liver tissue, compared to the HFHF group. The con- sumption of a diet rich in saturated fat and fructose was effective in increasing the concentration ($p < 0.05$) of the pro- inflammatory cytokine TNF and the receptor TLR4 in the liver of the animals compared to the AIN93-M control. However, sorghum flour added to the HFHF diet did

not reduce the levels of TNF compared to HFHF (Fig. 2E) and tended ($p < 0.05$) to decrease TLR4, compared to HFHF, since the value was similar to that in the AIN93-M group (Fig. 2F). The anti- inflammatory cytokine IL-10 showed no difference among the experimental groups (Fig. 2G).

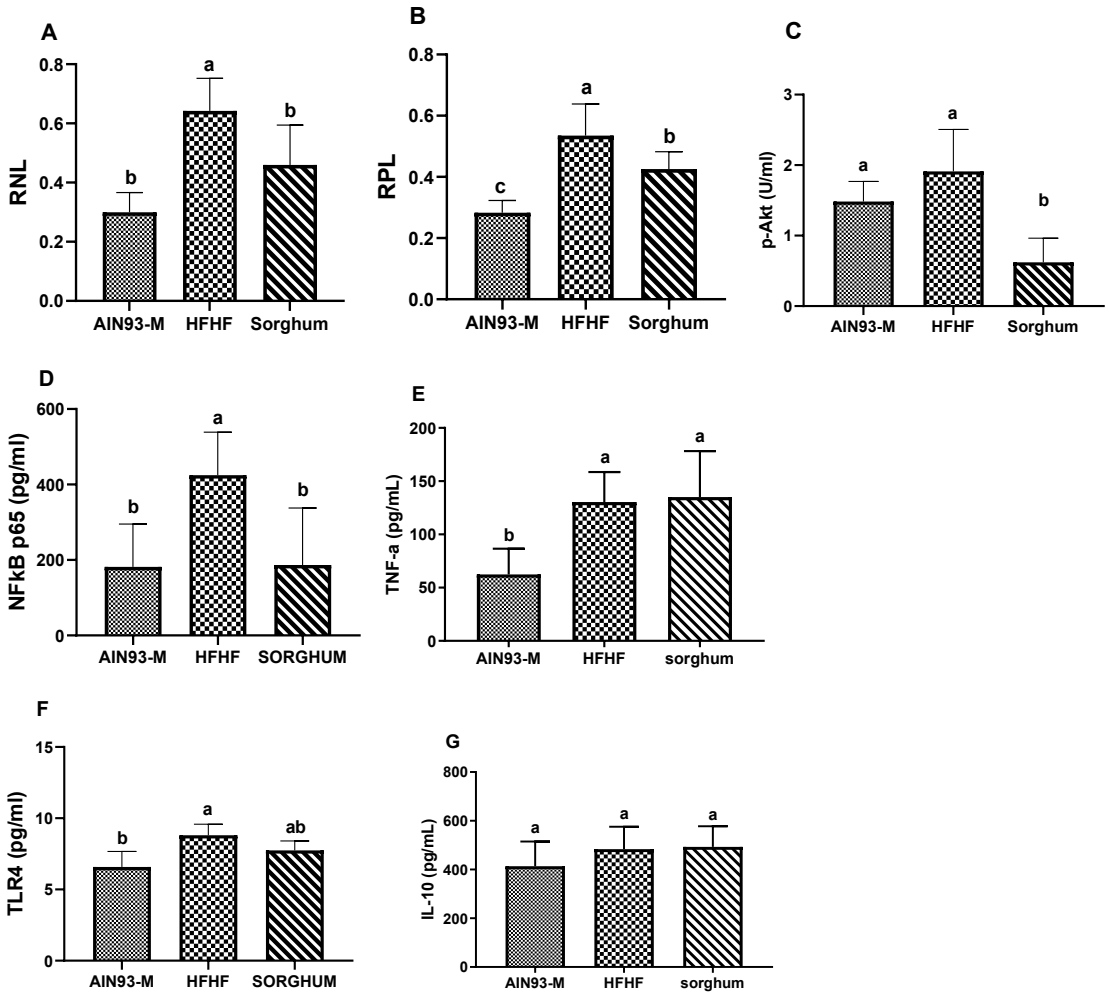

Figure 2. Sorghum flour reduces inflammatory markers in the liver of Wistar rats fed with a high-fat high-fructose diet after 10 weeks. Platelet/Lymphocyte ratio (RPL) (A), Neutrophil/Lymphocyte ratio (RNL) (B), Tumor necrosis factor (TNF) concentration (C), nuclear factor kappa B p65 concentration (NFκB p65) (D), phosphorylated phospho-protein kinase B p-Akt concentration (E), Toll-like receptor 4 (TLR4) (F), and interleukin 10 (IL-10) concentration (G) in the liver of the animals. Values are represented by means and standard deviation (n = 10). Means followed by the same letter do not differ by the Newman-Keuls test at 5% probability. AIN-93M: standard diet for rodents; HFHF: high-fat high-fructose diet; HFHF + Sorghum: HFHF diet plus sorghum flour.

3.5. Molecular docking

The molecular in silico analysis of the interaction between phe- nolic compounds (catechin, procyanidin B1, procyanidin B2, quercetin, quercetin 3-glucoside, and Quercetin 3-rutinoside) and inflammatory markers showed that every phenolic com-

pound found in sorghum flour interacted with AKT and p65- NFκB, since all interactions used low binding energy (esti- mated free energy), occurring spontaneously (Table S1†). However, quercetin-3-rutinoside was the phenolic compound with the highest interaction with AKT (EFE −8.0) (Fig. 3A) and procyanidins B1 (Fig. 3B) and B2 (Fig. 3C) had the highest interaction with p65-NFκB, because it demonstrated the lowest binding energy (EFE −8.9).

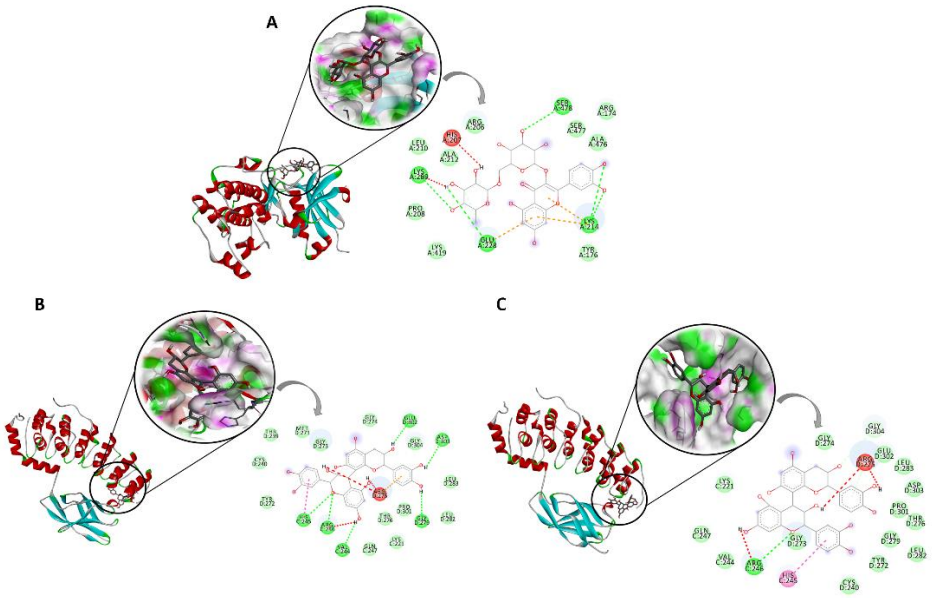

Figure 3. The in-silico interaction of the phenolic compounds with pro-inflammatory markers. Interaction between: quercetin-3-rutinoside and AKT (A), Procyanidin B1 and p65 NFκB (B), Procyanidin B2 and p65 NFκB (C). Dates analyzed by AutoDock Vina® and visualized by Discovery Studio 2016 Client®. p65 NFκB: nuclear factor-kappa B. Akt: phospho-protein kinase B.

4. Discussion

The present study investigated the effect of BRS 305 hybrid sorghum flour as a rich source of tannins and resistant starch (RS) on inflammation and oxidative stress markers in *Wistar* rats fed with a high-fat high-fructose diet. Sorghum flour was able to modulate inflammation and oxidative stress triggered by the HFHF diet, inhibiting the activation of p65 nuclear factor kappa B (p65-NFκB) and reducing the concentrations of pAkt. Besides, sorghum flour increased the total antioxidant capacity of plasma, the activity of antioxidant enzymes (SOD and CAT), reduced the concentration of nitric oxide (NO) in the liver, as well as, neutrophil/Lymphocyte ratio (RNL) and platelet/lymphocyte ratio (RPL) in blood.

In this study, we used sorghum with dry heat treatment because it comes close to the way sorghum is consumed by people daily, for example when used in culinary preparations, such as cakes, bread, and cookies (Rashwan et al., 2021,). Besides, studies have observed that the loss of resistant starch, carotenoids, and phenolic compounds in sorghum with dry heat treatment are minimal, with some of the phenolic compounds, such as luteolinidin, apigeninidin, and 3-deoxyanthocyanidins, the values did not change. Interestingly, the content of vitamin E was increased compared to raw sorghum flour. (Teixeira et al., 2016; Cardoso et al., 2014)

The total food consumption in the HFHF diet was lower compared to the AIN-93 M group. The higher caloric density in the HFHF diets promoted satiety and similar energy intake to the AIN-93 M group, which did not interfere with weight gain among experimental groups. Similar results were found by Enes et al., 2020 and Marineli et al., 2015. However, although the HFHF diet did not influence the body weight of animals, it was found that this diet increased the production of inflammatory markers and oxidative stress, such as those involved in the NFκB pathway.

NFκB is a transcription factor that regulates gene expression in response to oxidative stress and inflammation. We observed that the HFHF diet promoted an increase of p65-NFκB signal- ing that may have interacted with several parallel pathways, such as the signaling cascade started by phosphatidylinositol 3-kinase (PI3K) and phospho-protein kinase B (Akt) (Jiang et al., 2017). The HFHF diet increased phosphorylation of Akt that may have activated the PI3K/Akt pathway, which could have contributed to the activation and translocation of p65-NFκB to the nucleus. Sorghum flour was able to decrease the levels of pAkt, which probably collaborated to the down-regulation of p65-NFκB, inhibiting this inflammatory pathway (Jiang et al., 2017). These results were con- firmed by in silico analysis that showed a higher interaction between the main phenolic compounds of sorghum flour, especially procyanidin B1–B2 dimers and quercetin-3-rutino- side, and Akt and p65-NFκB, which may reduce phosphoryl- ation and consequent activation of these proteins and the associated inflammatory pathway. Furthermore, the reduction of RNL and RPL by sorghum confirms its ability to reduce inflammation (Suárez-Cuenca et al., 2019) under HFHF diet conditions.

Saturated fat and fructose may increase the growth of pathogenic bacteria, increasing the levels of LPS/endotoxin, which can bind to TLR4 and initiate

downstream inflammation pathways, like NFκB, and promote the secretion of proinflammatory markers (de Sousa et al.,2019). In our study, sorghum flour had a TLR4 protein concentration similar to those in AIN93-M and HFHF control groups. This effect of sorghum to maintain TL4 values similar to the AIN-93 group may have occurred due to its anti- oxidant and prebiotic effect. Procyanidin B1 may bind to TLR4, in the intestine, through competitive interaction with LPS, which suppresses the activation of NFκB pathways (Xing, et al., 2015). Furthermore, the RS present in sorghum composition is a dietary fiber that can be fermented by the intestinal micro-biota, increasing the short-chain fatty acids production (SCFA), such as butyrate, which can reduce the inflammatory pathway by p65-NFκB inhibition. Besides, the RS can also stimulate the growth of beneficial bacteria, reducing endotoxemia levels and consequent activation of TLR4 (de Sousa et al., 2018).

The most prominent phenomenon in the inflammation process is the increase of the NO levels due to the activation of induced nitric oxide synthase (iNOS) by pro-inflammatory cyto- kines. Inhibition of NO production has been widely used as a biomarker to assess the anti-inflammatory capacity (Julieta, et al., 2016). In our study, the animals with metabolic changes induced by the HFHF diet, when consumed BRS 305 sorghum hybrid flour, showed lower hepatic levels of NO compared to the HFHF group. This anti-inflammatory effect probably occurs due to the presence of phenolic compounds (mainly procyanidin type B) in BRS 305 sorghum hybrid flour, which inhibits the NFκB activation pathway and consequently NO production, as observed in other studies(de Sousa et al., 2019; Julieta, et al., 2016; Kim et al., 2012) highlighting the anti-inflammatory effect of sorghum.

The consumption of a high-fat high-fructose diet in our study probably led to an increase in the influx of free fatty acids (FFA) in adipose, muscle and liver tissues, and endothelial cells. This condition can alter the β-oxidation of FFA, modifying the number and function of mitochondria in different tissues, resulting in mitochondrial dysfunction, which may affect the electron transport chain, causing an electron flow towards oxygen and production of ROS, such as NO, and causing lipid oxidation, and increasing the levels of MDA. In this study, we observed an increase of the total antioxidant capacity (TAC) in the plasma and antioxidant SOD and CAT enzymes, in addition to a reduction in MDA levels. The phenolic compounds such as phenolic acids, condensed tannins and flavonoids, and phytic acid present in sorghum composition, probably improve the

mitochondria effects, like those observed in a previous study in cell culture (Rebollo-Hernanz et al., 2019). Furthermore, phenolic compounds have a known antioxidant activity by scavenging a wide-ranging selection of ROS and scavenge radicals and chelate metal ions, for example, quercetin chelates iron ions. Phenolic compounds also inhibit enzymes responsible for ROS generation, such as NADPH oxidase (NOX) and xanthine oxidase, upregulating endogenous antioxidant enzymes like SOD, CAT, and glutathione (Yahfoufi et al., 2018), contributing to the reduction of oxidative stress in cells, as observed in this study and by others authors (de Morais Cardoso et al., 2017).

Thus, in our study, BRS 305 whole sorghum flour showed the antioxidant potential to sequester free radicals and decrease lipid peroxidation through MDA reduction, further increasing the total antioxidant capacity of plasma and the antioxidant enzymes SOD and CAT, and also inhibited NFκB activation by inhibiting Akt phosphorylation and reducing TLR4 causing a reduction in nitric oxide (Fig. 4).

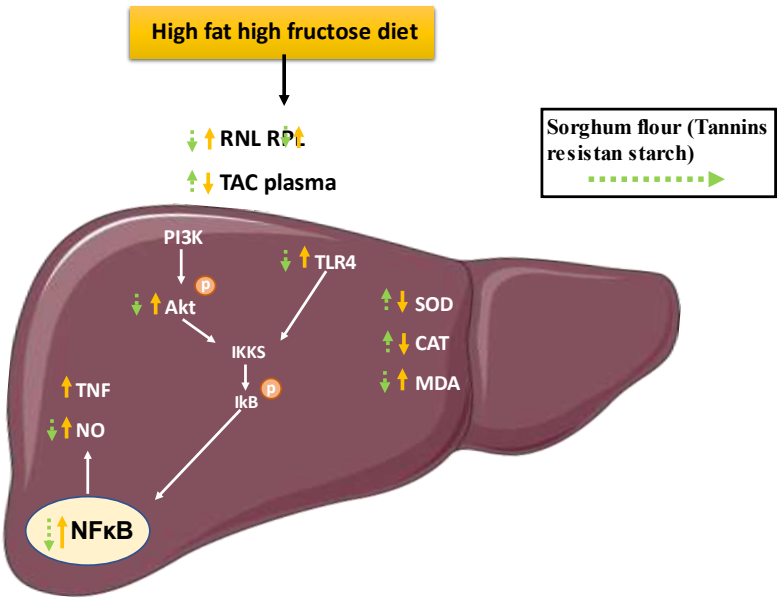

Fig. 4 Schematic diagram proposing the mechanisms by which dry heat-treated sorghum flour can affect the metabolic changes promoted by high-fat high-fructose diet (HFHF) in adult Wistar rats, after 10 weeks of intervention. The HFHF diet increased the production of nitric oxide and pro-inflammatory cytokines, leading to a reduction in the anti- oxidant enzymes SOD and CAT and an increase in the platelet/lympho- cyte ratio and the neutrophil/lymphocyte ratio and the levels of tumor necrosis factor in the liver of the animals. Furthermore, the HFHF diet increased TLR4 and p-Akt protein, activating the p65 subunit of NFκB, contributing to increased inflammation. Sorghum flour had an anti- oxidant effect since it increased the total antioxidant capacity of

plasma and the antioxidant enzymes SOD and CAT; and inhibited NFκB p65 activation by the PI3K/Akt pathway through inhibition of Akt phosphoryl- ation and reduction of TLR4, decreasing nitric oxide. ↑: increase; ↓: reduction; NO: nitric oxide; RPL: platelet/lymphocyte ratio; RNL: neutro- phil/lymphocyte ratio; SOD: superoxide dismutase; TAC: total anti- oxidant capacity; TNF: tumor necrosis factor; TLR4: toll-like receptor 4; NFκB: nuclear factor-kappa B.

5. CONCLUSION

The dry heated whole sorghum flour of the BRS 305 hybrid, a source of resistant starch and phenolic compounds, demon- strated the potential for use as a functional food ingredient. It reduced inflammatory markers in adult Wistar rats fed with a high-fat high-fructose diet, such as the transcription factor p65-NFκB by inhibition of Akt phosphorylation. Besides, the consumption of BRS 305 sorghum flour reduced oxidative stress, inhibiting the production of nitric oxide and increasing antioxidant enzymes, contributing to the increase in total anti- oxidant capacity. Thus, dry heated whole sorghum flour of the BRS 305 hybrid can be an alternative grain with good compo- sition, which can act beneficially on inflammation and oxi- dative stress, reversing the malefic effects of a diet high in fat and

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Sorghum flour BRS 305 hybrid has the potential to modulate the intestinal microbiota of rats fed with a high-fat high-fructose diet

ABSTRACT

Aim

The present study aimed to investigate the effect of dry heated whole sorghum BRS 305 hybrid flour on the gut microbiota modulation and gut health of rats fed with a high-fat high-fructose diet (HFHF).

Methods

In phase I (8 weeks), 45–50-day old, male Wistar rats, were separated into the AIN93-M group (n = 10; fed with normal diet) and HFHF group (n = 20; fed with diet rich in saturated and simple carbohydrate). In phase II (10 weeks), we maintained the AIN-93-M group, and the HFHF group was divided into the HFHF group (n = 10) and HFHF plus sorghum flour group (n = 10).

Results

The consumption of sorghum flour increased the circular muscle layer and propionic acid when compared to the HFHF group. The sequencing of the 16S rRNA gene of the cecal microbiota presented no changes in the α-diversity and β-diversity between. However, the sorghum group exhibited higher relative abundance of Firmicutes and higher Firmicutes/Bacteroidetes ratio compared to the other experimental groups, and lower abundance of Bacteroidetes, compared to the HFHF group. Despite, sorghum increased the abundance of the genera *Roseburia* and *Lachnospiraceae_NK4A136_group* compared to the HFHF group. No differences were observed in total goblet cell number, crypt thickness and height, circular muscle layer, secretory IgA, and butyric acid between all groups.

Conclusion

In conclusion, the consumption of sorghum flour can modulate the gut microbiota composition, abundance of SCFA-producing bacteria, and intestinal morphology even with consumption of an HFHF diet.

Keywords: *Sorghum bicolor* (L.) Moench; SCFAs; bioactive compounds; gut microbiome; diversity analysis.

1. INTRODUCTION

Diet is one of the main modulators of the intestinal microbiota composition, which directly affects the biological processes, epithelial integrity and intestinal homeostasis of the host [1, 2]. The type and amount of proteins, fats and carbohydrates present in diets and people lifestyle widely affect the microbiota composition among individuals [2, 3]. Evidence indicates that high fructose diets can shape the intestinal microbiota by increasing the Firmicutes-to-Bacteroidetes ratio and Proteobacteria one of the best sources of lipopolysaccharide (LPS) [1]. On the other hand, the intake of saturated fat can increase intestinal permeability and Gram-negative bacteria, and reduce Gram-positive bacteria [4], thus increasing the passage of bacterial products, such as LPS, through the intestinal barrier to the systemic circulation, which characterizes endotoxemia [4, 5].

The consumption of high-fat high-fructose diet (HFHF) increases body fat mass and negatively affects glucose metabolism, which results in excessive accumulation of free fatty acids (FFA) and liver steatosis [6]. These affect the inflammatory properties, which can stimulate the formation of reactive oxygen species (ROS), induce greater oxidative stress and activate NFκB, further increasing the inflammation process [7]. Furthermore, the HFHF diet may damage the gut barrier function and induce gut dysbiosis by increasing Firmicutes and reducing Bacteroidetes phylum [1].

Considering the high risk of the consumption of the HFHF diet, nutritional strategies should be investigated to prevent/treat these negative effects. A diet composed mostly of foods rich in dietary fiber and complex carbohydrates, such as resistant starch (RS) and dietary fiber that can be fermented by the intestinal microbiota, thus stimulating the growth of certain bacteria in the colon, producers of

short-chain fatty acids (SCFAs), such as acetate, propionate and butyrate, exercises beneficial effects against the HFHF diet, decreases inflammation and intestinal permeability, and preserves the mucosal integrity and colonic homeostasis [1, 8, 9]

In this context, sorghum (Sorghum bicolor (L.) Moench) is a promising cereal, highly efficient in the use of water and soil nutrients, which allows it to be cultivated in dry and/or warm areas where the production of other cereals would not be economically viable. Due to its efficiency, sorghum production cost is low and profitable [10]. Thus, among the sorghum genotypes, the BRS 305 hybrid has high content of dietary fiber, condensed tannins [11] and RS [6], which provide high antioxidant and anti-inflammatory capacity and the ability to modulate the composition of the intestinal microbiota and parameters related to diseases, including obesity, diabetes and dyslipidemia [6, 7, 12, 13].

Furthermore, it was demonstrated that the consumption of sorghum flour BRS 305 hybrid improved glucose metabolism, modulated adiposity and reduced liver steatosis and lipogenesis [6]. Moreover, its consumption reduced inflammatory markers, such as the transcription factor p65-NκB, by inhibiting Akt phosphorylation. It also decreased oxidative stress by increasing antioxidant enzymes and increasing the total antioxidant capacity [7] in Wistar rats fed with a high-fat high-fructose diet for 10 weeks. In another study on extruded sorghum flour, the SC 319 genotype improved intestinal dysbiosis by increasing Bacteroidetes and decreasing Firmicutes in Wistar rats fed a high-fat diet for 8 weeks [9].

Although the protective effect of sorghum has been observed on metabolic changes caused by HFHF diets, the effect of BRS 305 hybrid whole sorghum flour as a rich source of resistant starch and condensed tannins on the modulation of intestinal microbiota has not been investigated yet. Thus, the present study aimed to evaluate the effect of BRS 305 hybrid whole sorghum flour with high-fat high-fructose diet on gut microbiome composition and colon histomorphometry in Wistar rats fed with HFHF diet.

2. MATERIAL AND METHODS

2.1. Material

The BRS 305 genotype, with high tannin content [11], resistant starch [6] and light brown pericarp, was produced by Embrapa Milho e Sorgo, Sete Lagoas, MG, in

August 2015. The seeds were manually selected and sieved to remove impurities. Sorghum was subjected to dry heat treatment. The grains were placed in aluminum trays and exposed to 105°C, in an air circulation oven (Nova Ética, model 400/6ND, São Paulo, Brazil) for 30 minutes [11]. After heat treatment, the grains were ground in a knife mill (Brabender®, Duisburg, Alemanha) with a 1.0 mm stainless steel sieve to obtain the flour. Studies have demonstrated that this treatment is more efficient in maintaining the antioxidant levels [14] and resistant starch [15], compared to the wet heat treatment.

The chemical composition of BRS 305 sorghum flour, performed in our previous articles, demonstrate a composition of 58.9g carbohydrates; 14.3g total dietary fiber; 4.6g lipids; 12.1g protein; 9.1g moisture; 59.53 mg condensed tannins; 37.2 g resistant starch [11].

The chemical analysis, inflammatory markers, and biological parameters were previously carried out and already published [6, 7, 11].

2.2. Animals study and Diets

The experiment was divided into two phases, phases I (to induce metabolic changes) and II (treatment). In Phase I (8 weeks), thirty Male Wistar rats (Rattus norvegicus), 45–50 days old, were randomized by body weight, into two groups: the normal control group (wt 155.98 ± 17.05g; n = 10), fed with diet AIN93-M (Reeves et al., 1993), and the HFHF group (wt 162.67 ± 19.07g; n = 20), fed with a high-fat-high-fructose diet (4% soybean oil plus 31% lard) and fructose (20%), (SHAPIRO et al., 2011). During the treatment (Phase II; 10 weeks), the control group (AIN-93M; wt 349.94 ± 30.71 g, n = 10) was maintained, and the animals from the HFHF group were redistributed into: HFHF diet (wt 366.88 ± 36.90g; n = 10), which maintained the high-fat high-fructose diet, and the treatment group, the HFHF + sorghum flour group (wt 368.44 ± 34.11g; n = 10) (replacing 50% fiber, 100% corn starch, 22.5% soybean oil, and 19.8% protein from sorghum flour).

After preparation, the diets were packed in dark bags of polyethylene, properly labeled, and stored in a freezer (-18ºC ± 1ºC), to minimize the oxidation of fatty acids. All diets were isoproteic, with concentrations of protein (12.6; 12.6; 12.4%), carbohydrate (82.8; 47.1; 46.2%), lipids (4.6, 40.3, 42.4%), and energetic density (3.8, 5.3, 5.2 Kcal/g) for AIN93-M, HFHF, sorghum groups, respectively (Table 1). In all

phases, the animals were distributed in individual stainless-steel cages, in a temperature-controlled environment, at 21 ± 3ºC, with an automatically controlled 12-hour photoperiod and received water and diet ad libitum [7].

At the end of phase II, the animals were anesthetized with 100% isoflurane (Isoforine, Cristália) and euthanized by cardiac puncture. The colon segment was collected, fixed in 10% formaldehyde and kept at room temperature for histological analysis. Cecum weight was measured and collected in a sterile microtube immersed in liquid nitrogen and stored at -80°C until the analysis.

This project was approved by the Animal Use Ethics Committee of the Universidade Federal de Viçosa (CEUA/UFV; protocol no. 38/2019). All experimental procedures were carried out in compliance with the ethical principles in animal experimentation.

Table 1. Composition of experimental diets

Ingredients (g/kg diet)	AIN93-M	HFHF	HFHF Sorgo**
Albumin*	136.6	136.6	108.8
Dextrinized starch	155.0	45.0	46.5
Corn starch	463.5	135	-
Sucrose	100.0	28.6	31
Soybean oil	40.0	40.0	31.1
Sorghum	-	-	195.4
Lard	-	310.0	310.0
Fructose	-	200.0	200.0
Microcrystalline Cellulose	55.8	55.8	27.9
Mineral mix	35.0	35.0	35.0
Vitamin mix	10.0	10.0	10.0
L-cystine	1.8	1.8	1.8
Choline bitartrate	2.5	2.5	2.5
Macronutrients			
Protein (%)	12.6	12.6	12.4
Carbohydrate (%)	82.8	47.1	46.2
Lipids (%)	4.6	40.3	42.4
Energetic density (Kcal/g)	3.8	5.3	5.2

AIN-93M: control diet for rodents; HFHF: diet rich in saturated fat and fructose; HFHF + sorghum flour: HFHF diet added of sorghum flour. *Chemical composition of sorghum in g/100g: carbohydrates: 58.9g; total dietary fiber: 14.3g; lipids: 4.6g; protein: 12.1g; moisture: 9.1g (Martinez *et al.*, 2020). **Albumin with 88% of protein content. The food intake and weight of the animals were measured weekly.

2.3. Gut health

2.3.1. Intestinal permeability

In the last week of the experiment, the animals were fasted for 12 h and, subsequently, 0.5 mL of a solution containing 19 mg of lactulose and 9 mg of mannitol was administered by gavage. After administration, the animals were placed in individual metabolic cages where they fasted for 5h, and the 24-hour urinary volume was collected, measured, recorded and stored at -80ºC [16].

To determine the concentrations of lactulose and mannitol, the collected urine was filtered on 0.45 µm millipore filters and placed in vials for analysis of high-performance liquid chromatography (HPLC). The mobile phase used was water in 0.005 mol/L of sulfuric acid, mobile phase flow of 0.6 mL/min, with a sample injection volume of 20 µL [17]. We use lactulose and mannitol (Sigma-Aldrich, São Paulo/SP, Brazil) as internal standards. The areas obtained by the curves were calculated and converted into g/L to calculate the percentage of urinary excretion of lactulose and mannitol and the proportion of lactulose/mannitol [18].

2.3.2. Quantification of short-chain fatty acids

Stool samples (about 500 mg) were homogenized in 1 mL of Milli-Q water for analysis, with the aid of vortex, and centrifuged at 12,000g, for 10 min. The supernatant was removed, and the other steps were performed as described by Siegfried, Ruckemann and Stumpf (1984).

Subsequently, the samples were analyzed by HPLC, using a Dionex Ultimate 3000 Dual detector HPLC apparatus (Dionex Corporation, Sunnyvale, CA, USA), coupled to a Shodex RI-101 refractive index (IR) detector, maintained at 40ºC, and Phenomenex Rezex ROA ion exclusion column, 300 × 7.8 mm (Phenomenex Inc. Torrance, CA, USA), maintained at 40ºC, injection volume 20 µl. Sulfuric acid 5 mM with flow of 0.7 mL/min was used as the mobile phase. Acetic, propionic and butyric acids (Sigma-Aldrich, São Paulo/SP, Brazil) were used as standards in the calibration curve.

2.3.3. Intraluminal pH of cecum content

The caecal luminal contents were removed, weighed and diluted at a ratio of 1:10 for the analysis of the cecal fecal pH. About 0.4 g of cecal feces was homogenized in 4 mL of distilled water by vortexing. Then, the glass electrode of the pH was inserted for reading [19].

2.3.4 DNA Extraction and Sequencing

Genomic DNA was extracted from fecal samples, following a mechanical disruption and phenol/chloroform extraction protocol [20]. The platform, Environmental Sample Preparation and Sequencing Facility (ESPSF) was used to load the samples onto an Illumina flow cell for paired-end sequencing reactions at the Argonne National Laboratory (Lemont, Illinois, USA), at the Argonne National Laboratory (Lemont, Illinois, USA). PCR amplicon libraries targeting the hypervariable V4-region of the 16S rRNA gene were produced using the primers 515F (5'GTGYCAGCMGCCGCGGTAA3') and 806R (GGACTACNVGGGTWTCTAAT3') and a barcoded primer set adapted for the Illumina MiSeq platform (Illumina, San Diego, California, USA) [21, 22].

Customized sequencing primers and procedures were used for sequenced of Amplicons on a 151bp x 12bp x 151bp MiSeq [21].

The sequences obtained for all samples in the present study were submitted to Sequence Read Archive (SRA) on the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sra) under the accession number PRJNA658699, and the data processing and analysis were performed using the Mothur software, v.1.40.0 [23]. Chimera sequences were detected and removed, using UCHIME [24]. The SILVA database v.132 [25] was used for aligned the sequences with the 16S rRNA gene and performed taxonomic classification

The Operational Taxonomic Units (OTUs) were grouped with a 97% sequence similarity cutoff. The coverage of all samples was assessed by the Good's coverage estimator (Bacteria > 97%). The samples were normalized for the lowest number of sequences produced by any sample. The standardized data table was used for calculating alpha and beta diversity and the relative abundance of OTUs. The Chao, Shannon and Simpson indexes was used for estimates of alpha-diversity. Beta-diversity between dietary groups was assessed by Principal Coordinate Analysis (PCoA) based on the Bray-Curtis dissimilarity index. The metagenome functional predictive analysis of any variations in the genetic capacity of the microbiota was carried out using the PICRUSt software system. The feature abundance was normalized by the 16S rRNA gene copy number and identified using the Greengenes database. The Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog prediction was also calculated

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2.4. Quantification of IgA

A homogenate of the cecal contents was prepared at a concentration of 1:5 (w/v) for the determination of Immunoglobulin A (IgA), according to standard procedures, Rat immunoglobulin A ELISA kit (Cat.NO EA0032Ra). About 200mg of cecal content was used in 800µl of phosphate-buffered saline solution and homogenized with the aid of a vortex. Mucosal immunity was assessed based on the concentration of IgA in the cecum content, using an enzyme-linked immunosorbent kit (ELISA) [26].

2.5. Colon Histomorphometric Analysis

A glass razor and stained with hematoxylin/eosin was using for Semi-serialized 3 µm thick histological colon fragments obtained in an automatic microtome (Reichert-Jung®). The slides were examined under a CX31 photomicroscope (Olympus, Japan). Twenty random fields per animal were selected to measure crypt thickness, crypt height and total goblet cell number of circular and longitudinal muscle layers (CML and LML, respectively). Crypts with visible and definite connective epithelium were used [27]. The images of the histological sections were captured with 20x objective, and the measurements were carried out with the aid of the ImagePro-Plus application® version 4.5 (Media Cybernetics, Rockville USA).

2.6. Statistical Analysis

Data of food consumption, body weight gain, intestinal permeability, pH fecal, colonic histomorphometric characteristics, and SCFA concentrations were initially submitted to a Kolmogorov-Smirnov normality test. The results were submitted to analysis of variance (ANOVA), followed by the Newman-Keuls test at 5% probability. Correlations between biological markers and gut microbial were assessed by Pearson's correlation test. $P<0.05$ was considered as statistically significant. The analysis were performed in Graphpad Prism version 9.0.

The Chao, Shannon and Simpson Indexes were used to calculate the bacterial richness within the samples, while the differences between the groups were analyzed by ANOVA, followed by a post hoc of Duncan. Differences between Beta diversity values were analyzed by the Pairwise PERMANOVA test. Statistically significant p-values associated with microbial clades and functions identified by LEfSe were

corrected for multiple comparisons, using the Benjamini Hochberg false discovery rate (FDR) correction. Statistical analysis was performed using SPSS version 20.0. The level of significance was established at ($p<0.05$).

3. RESULTS

3.1. Effect of sorghum flour on body weight gain and food intake

The body weight gain (g) corresponds to 65.1±22.7; 63.5±31.0; 58.8±18.7, and the total food intake (g) correspond to 1367.0±69.1; 1045.6±130.2; 1040.0±60.7, respectively for AIN93-M, HFHF, and sorghum groups and no significant differences were observed between the groups for these analyses ($p>0.05$).

3.2. Effect of sorghum flour on gut health

The HFHF and HFHF+sorghum groups presented lower cecum weight, compared to AIN-93M ($p<0.05$). No differences were observed in total goblet cell number, crypt thickness, crypt height (Fig. 1 and Table 2), IgA and longitudinal muscle layer ($p > 0.05$) between all groups (Table 2). The consumption of sorghum flour (HFHF + sorghum flour group) increased ($p< 0.05$) the circular muscle layer when compared to the HFHF group (Table 2).

Regarding the organic acids analyzed, the propionic acid increased ($p < 0.05$) in the cecal content of animals fed sorghum flour (HFHF + sorghum flor), compared to the AIN-93M and HFHF groups. Acetic acid presented higher values in the AIN93-M group than in HFHF and HFHF+sorghum. The levels of butyric acid were similar between all groups ($p > 0.05$) (Table 2).

The sorghum flour consumption increased the levels of urinary lactulose excretion ($p < 0.05$) (Fig. 2a). No significant differences ($p > 0.05$) were observed among experimental groups for urinary excretion of mannitol (Fig. 2b) and lactulose/mannitol ratio (Fig. 2c).

Table 2. Colon histological analysis and short-chain fatty acids in the cecal content

Variables	AIN-93M	HFHF	HFHF + Sorghum flour

Cecum weight (g/100g body weight)	1.23 ± 0.28[a]	0.91 ± 0.25[b]	0.88 ± 0.25[b]
pH cecum	9.01 ± 0.40 [a]	9.17 ± 0.25 [a]	8.82 ± 0.32 [a]
IgA (ng/ml)	851.49 ± 106.20 [a]	786.53 ± 89.47 [a]	833.20 ± 94.88 [a]
Total Goblet Cell Number (Unit)	24.64 ± 3.93[a]	23.72 ± 3.74[a]	26.66 ± 2.61[a]
Crypts thickness (µm)	19.24 ± 2.05[a]	19.52 ± 4.24[a]	19.01 ± 1.83[a]
Crypts height (µm)	135.09 ± 34.07[a]	161.44 ± 33.01[a]	146.82 ± 25.70[a]
LML (µm)	92.59 ± 38.86[a]	96.44 ± 55.09[a]	149.27 ± 29.96[a]
CML (µm)	31.20 ± 14.44[ab]	25.99 ± 8.36[b]	50.87 ± 14.27[a]
SCFA (mM/100g body weight)			
Propionic acid	1.12 ± 0.33[b]	1.04 ± 0.25[b]	1.687 ± 0.57[a]
Acetic acid	5.24 ± 1.29[a]	3.29 ± 0.71[b]	3.19 ± 1.03[b]
Butyric acid	1,12 ± 0,33 [a]	1,04 ± 0,25[a]	1,69 ± 0,57[a]

Values represent means ± SD (n=10). Means followed by the same letter at row had no difference by Newman-Keuls at 5% probability. The pH was measured using glass electrode of the pH; histologic slides were stained with hematoxin-eosin using objective of 20x; SCFA were determined by HPLC. AIN-93M: diet for rodents; HFHF: diet rich in saturated fat and fructose; HFHF + sorghum flour: HFHF diet added of sorghum flour. IGA: Immunoglobulin A; CML: circular muscle layer; LML: longitudinal muscle layer; SCFA: Short Chain Fatty Acids. Data were analyzed by one-way ANOVA.

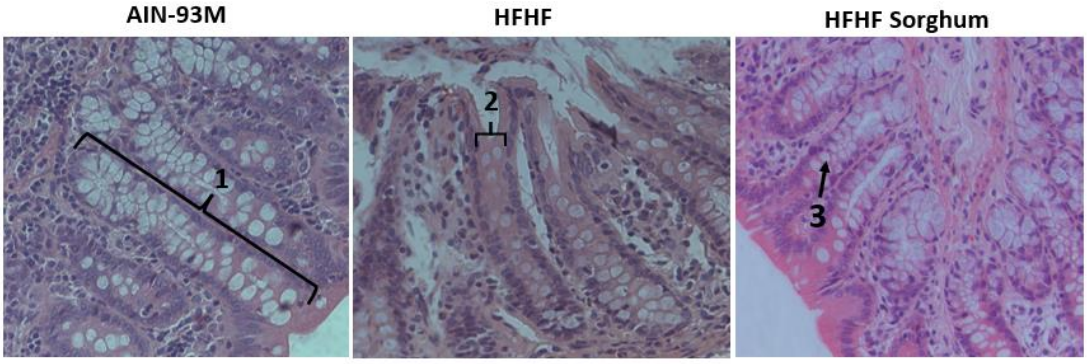

Fig. 1. Histological analysis of colon tissue of animals fed with a HFHF diet and treated with BRS 305 sorghum flour after 10 weeks. 1: Crypts height; 2: Crypts thickness; 3: Goblet Cell Number. Staining was carried out with hematoxylin and eosin. Bar: 20 µm. Objective: 20×.

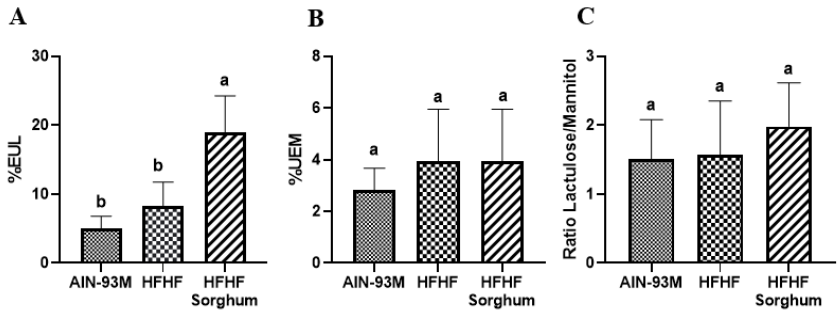

Fig. 2. Percentage of urinary excretion of lactulose and mannitol and the ratio between them. A: urinary excretion of lactulose (%EUL); B: urinary excretion of mannitol (%UEM); C: ratio lactulose/manitol. Values refer to means ± SD (n=10 Means followed by the same letter presented no difference by Newman-Keuls at 5% probability. Lactulose and mannitol were determined using HPLC. AIN-93M: diet for rodents; HFHF: diet rich in saturated fat and fructose; HFHF + sorghum flour: HFHF diet with the addition of sorghum flour.

3.2 Sorghum flour changes the intestinal microbiota pattern promoted by the HFHF diet

The sequencing of the 16S rRNA gene from the stool samples generated 794.417 raw sequences and obtained 608.745 sequences of good quality after filtering and cleaning. The Good's coverage obtained in the samples was > 99%, which indicates good sequencing coverage. The richness, diversity and dominance index (Chao1, Shannon and Simpson respectively) used to assess alpha diversity indicated no difference ($p>0.05$) between groups (Fig. 3a, 3b and 3c).

The gut microbial diversity differences among treatment and control groups are shown in Fig. 3. The β-diversity, assessed by principal coordinates analysis (PCoA) presented differences in the distance metrics between treatments (PERMANOVA, $p = 0.00009$, $r = 0.3561$) (Fig. 3).

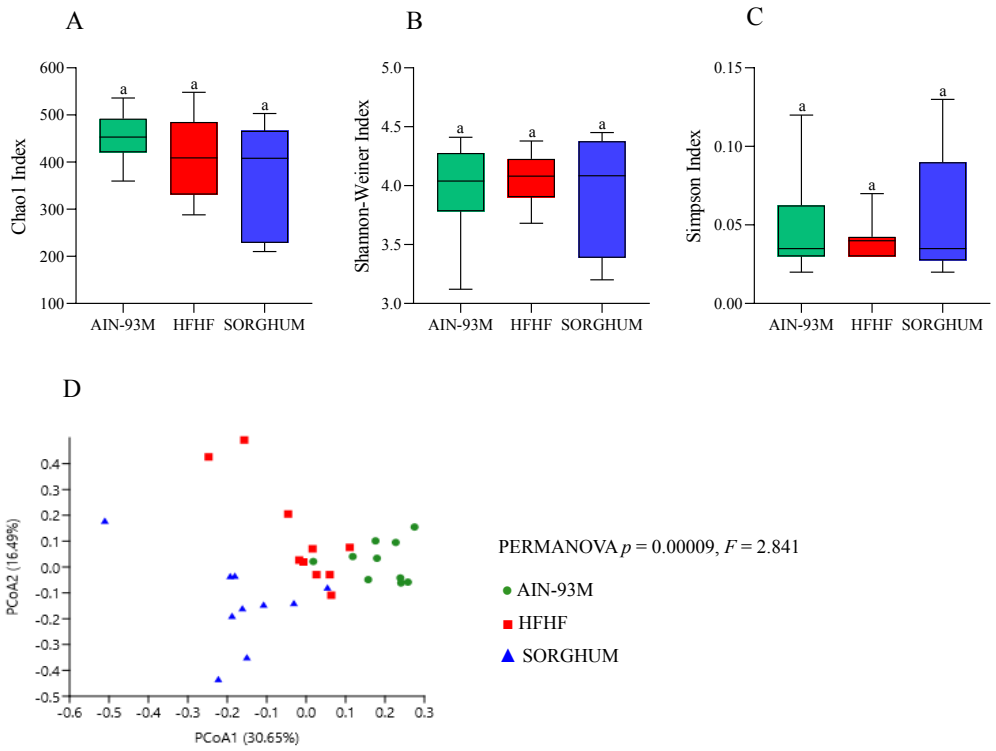

Fig. 3. Microbial diversity of the cecal microbiome of animals fed with a HFHF diet and treated with BRS 305 sorghum flour after 10 weeks. (a-c) Measure of α-diversity using the Chao, Shannon and Simpson indexes; d) Principal Coordinates Analysis (PCoA) based on Jaccard similarity distances of cecal microbial communities from the different treatment groups. Each dot refers to one animal, and the colors refer to the different treatment groups (n=10). AIN-93M: standard diet for rodents; HFHF: high-fat high-fructose diet; HFHF + sorghum flour: HFHF diet with the addition of sorghum flour.

The taxonomic classification of the samples comprised 18 phyla, 27 classes, 69 orders, 108 families and 225 genera. The sorghum group (HFHF + sorghum flour) presented greater abundance of Firmicutes ($p = 0.002$) compared to the other experimental groups, and the relative abundance of Bacteroidetes decreased in this group, compared to HFHF ($p = 0.008$) (Fig. 4a). It was observed that the Firmicutes/Bacteroidetes ratio was higher ($p < 0.05$) in the sorghum group (HFHF + sorghum flour) compared to the other experimental groups (Fig. 4b). No differences were found in the relative abundances of Actinobacteriota, Proteobacteria, Campylobacterota and Deferribacterota phyla between the all groups ($p>0.05$). The Desulfobacterota and Spirochaetota phyla presented lower abundance in the HFHF + sorghum flour group when compared to the HFHF group ($p = 0.012$, $p = 0.001$), respectively (Fig. 4a).

The sorghum group (HFHF + sorghum flour) presented reduced abundance of the genera *Muribaculaceae_ge* (5.01% vs 8.349%, p<0.0001) and *Bacteroides* (1.966% vs 2.508%, p = 0.045), when compared to the HFHF group. Furthermore, it exhibited increased abundance of *Lachnospiraceae_NK4A136_group* (4.163% vs 2.215%, p <0.0001), *Clostridia_UCG-014_ge* (3.141% vs 1.847, p = 0.001) and *Roseburia* (0.854 vs 0.396, p = 0.003). In the genera Colidextribacter, Streptococcus, Lachnoclostridium, *Faecalibaculum*, *Lactobacillus* and *Ruminococcus*, no differences were observed among the experimental groups. After FDR correction, significant differences were found between sorghum groups and the HFHF group for the families *Muribaculaceae* and *Ruminococcaceae* (Fig. 4c).

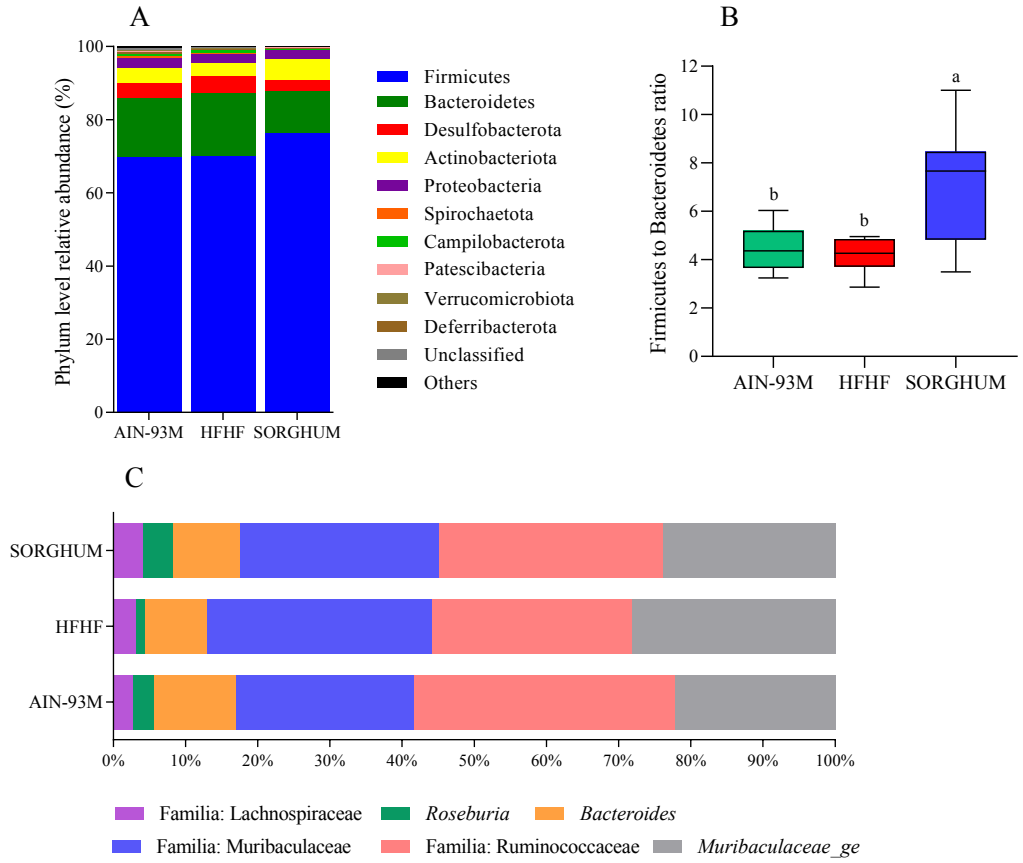

Fig. 4. Relative abundance of bacterial microbiota composition at phylum level of animals fed with a HFHF diet and treated with BRS 305 sorghum flour after 10 weeks. A) Relative abundance of each identified phylum; b) *Firmicutes*/*Bacteroidetes* ratio and c) Genus and Family level changes in each experimental group. n = 10/group. AIN-93M: diet for rodents; HFHF: diet rich in saturated fat and fructose; HFHF + sorghum flour: HFHF diet with the addition of sorghum flour. Only phyla with abundance > 0.2% in at least one group were displayed. Data were analyzed with a false discovery rate (FDR) correction.

The linear discriminant analysis effect size method (LEfSe) was used to investigate the significant bacterial biomarkers that could identify differences in the gut microbiota between the treatment groups. The sorghum group presented a significantly enriched unclassified member of Lachnospiraceae ($p < 0.05$) belonging to the phylum of Firmicutes. Besides, in the HFHF group, we observed that the member Muribaculaceae family was predominantly enriched in the Bacteroidetes phylum and the Lachnospirales order, and the Lachnospiraceae family, a member of the Firmicutes phylum, was significantly enriched ($p < 0.05$) in the AIN-93M (Fig. 5).

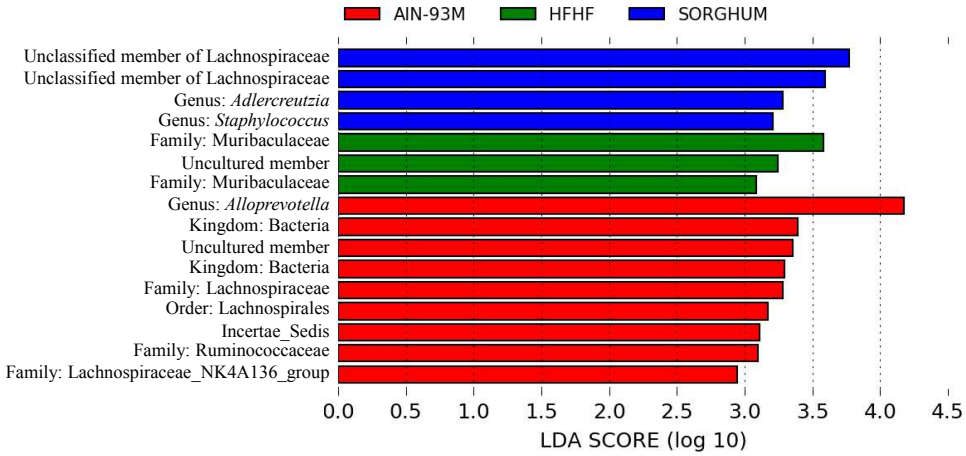

Fig. 5. LEfSe method used to compute Linear discriminant analysis (LDA) scores of the relative abundance difference between the experimental groups. AIN-93M: diet for rodents; HFHF: diet rich in saturated fat and fructose; HFHF + Sorghum: HFHF diet added of sorghum flour.

Pearson correlation analysis was used to assess the relationship between changes in gut microbial abundance, inflammatory markers, and biological parameters. When assessing correlations with propionic acid was significantly and negatively correlated with nuclear factor-kappa B (NFκB) ($r = -0.641$) and phospho-protein kinase B (p-Akt) ($r = -0.589$). *Roseburia* was significantly and positively correlated with propionic acid ($r = 0.474$), superoxide dismutase (SOD) ($r = 0.462$), catalase (CAT) ($r = 0.650$), and peroxisome proliferator-activated α receptor (PPAR-α) ($r = 0.650$), and were significantly and negatively correlated with alanine aminotransferase (ALT) ($r = -0.476$), Acid uric ($r = -0.607$), intraperitoneal glucose tolerance test (iGTT) ($r = -0.468$), triglycerides (TG) ($r = -0.585$), nitric oxide (NO) ($r = -0.468$) and nuclear factor-kappa B (NFκB) ($r = -0.495$). Change in *Lachnospiraceae_NK4A136_group* was also significantly and positively correlated with SOD ($r = 0.506$) and total antioxidant capacity in plasma ($r = 0.509$), and

significantly negatively correlated with change in TG (r = -0.533), NO (r = -0.538), NFκB (r = -0.382) and phospho-protein kinase B (p-Akt) (r = -0.560) (Fig. 6).

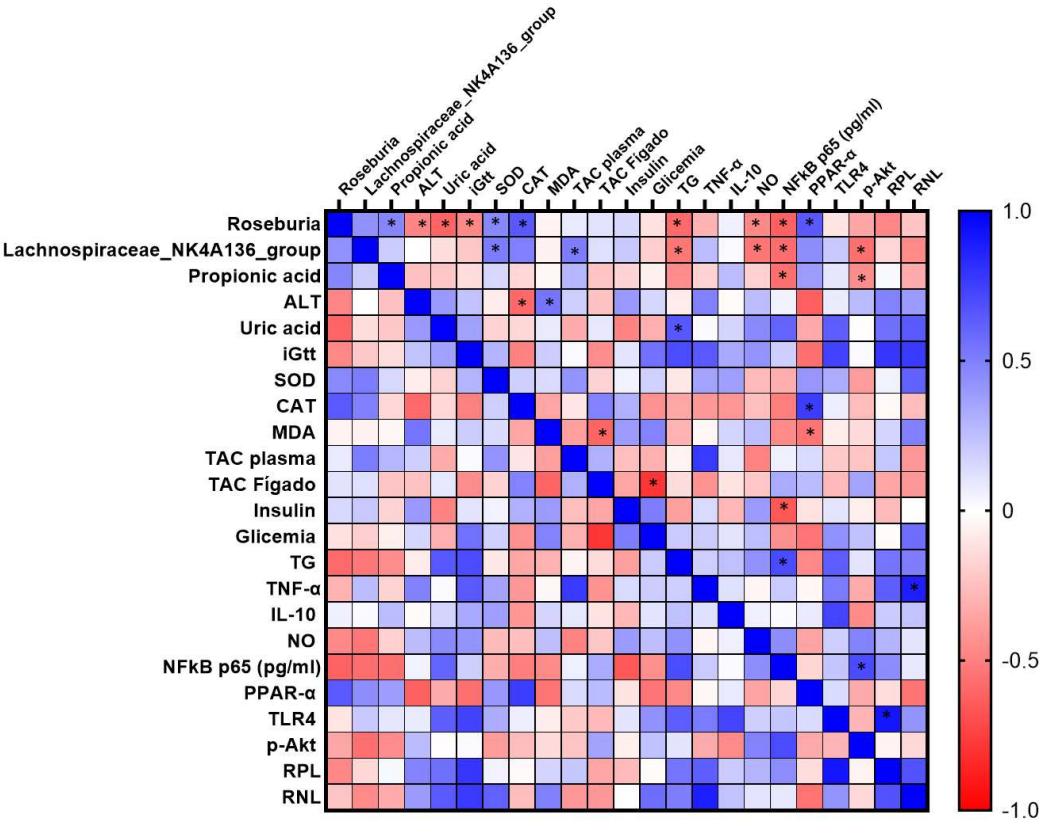

Fig. 6. Heat map of the Pearson rank correlations between biological and gut microbial outcomes of animals fed with a HFHF diet and treated with BRS 305 sorghum flour after 10 weeks. *: $p < 0.05$. ALT: alanine aminotransferase; iGTT, intraperitoneal glucose tolerance test; SOD: superoxide dismutase; CAT: catalase; MDA: malondialdehyde; TAC: total antioxidant capacity; TG: triglycerides; TNF: tumor necrosis factor; IL-10; NO: nitric oxide; NFκB: nuclear factor-kappa B; PPAR-α: peroxisome proliferator-activated α receptor; TLR4: toll-like receptor 4; Akt: phospho-protein kinase B; RPL: platelet/lymphocyte ratio; RNL: neutrophil/lymphocyte ratio.

Furthermore, the Sorghum group presented significance ($p < 0.05$) in the metabolism of the cofactors and vitamins of KEGG metabolic pathways identified, compared with the HFHF group, except for the tetrahydrofolate biosynthesis, which was lower ($p < 0.05$) (Fig. 7a). No differences were observed for cellular processes, amino acid metabolism or metabolic processes for the HFHF and sorghum groups (Fig. 7b, 7c and 7d).

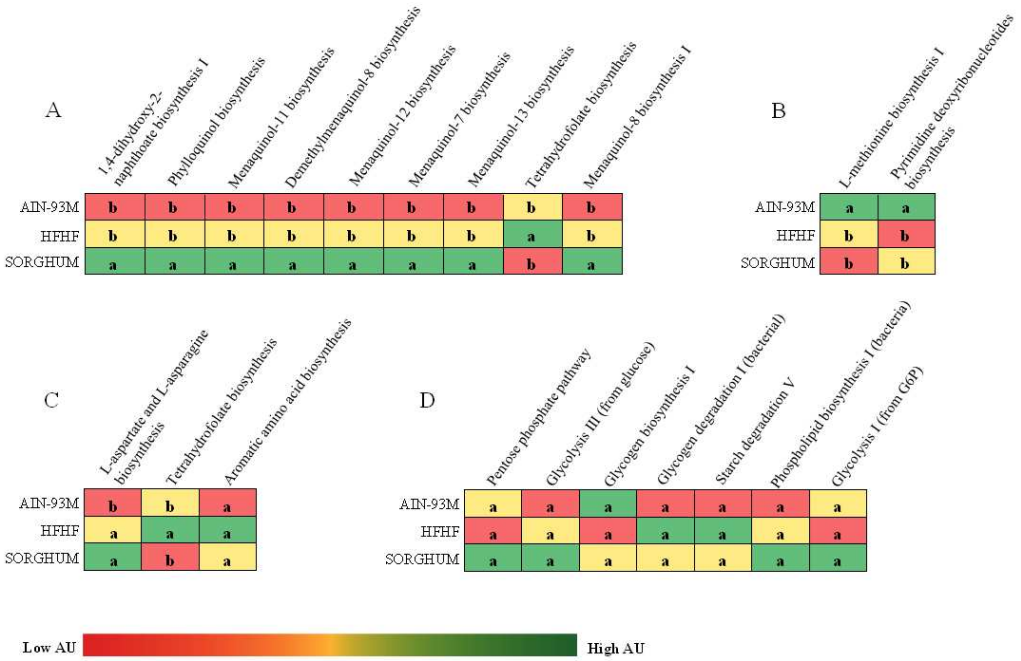

Fig. 7. Relative significance of differentially enriched microbial metabolic pathways in cecal microbiota after the consumption of animals fed with a HFHF diet and treated with BRS 305 sorghum flour after 10 weeks. a) Metabolism of cofactors and vitamins; b) Cellular Processes; c) Amino acid metabolism; d) Metabolic Processes. AIN-93M: diet for rodents; HFHF: diet rich in saturated fat and fructose; HFHF + Sorghum: HFHF diet with the addition of sorghum flour; G6P: glucose 6-phosphate. a–b Treatment groups with different letters are significantly different by ANOVA ($p<0.05$).

4. DISCUSSION

BRS 305 hybrid sorghum is cereal rich in dietary fiber and an excellent source of bioactive compounds, such as condensed tannins and resistant starch (RS) [6, 7, 11]. These compounds may reach the large intestine, where colon bacteria enzymatically act on their structure, sequentially producing metabolites with different physiological meanings [28]. This study brought new insights about the properties of the dry heated BRS 305 hybrid sorghum flour, which is rich in condensed tannins and resistant starch (RS), on gut microbiota modulation and gut health in animals fed with a high-fat high-fructose diet (HFHF).

Sorghum intake promoted no changes in the α-diversity and was effective in increasing the relative abundance of Firmicutes phylum, Firmicutes/Bacteroidetes ratio and the abundance of the genera *Roseburia*, *Lachnospiraceae_NK4A136_group*. Further, it increased the propionic acid levels and the circular muscle layer, while decreasing the abundance of the Bacteroidetes phylum compared to the HFHF group.

The HFHF diet did not change the structure of the intestine, since it was not able to change the crypt thickness, crypt height and total goblet cell number. Therefore, the sorghum group maintained these markers at normal levels. Similar results were obtained by Moraes et al., (2012) that evaluated the intake of a high-fat diet and sorghum (BRS 305 hybrid) for 35 days and there were no changes in the crypt depth of Wistar rats. On the other hand, Xie Et Al. (2020) observed that the consumption of a high-fat diet altered the colon epithelial barrier function by reducing crypt depth in 12-month-old mice C57Bl/6J, which led to the conclusion that these morphological changes may be due to the effect of the diet on the intestinal function. In our study, the sorghum diet increased the circular muscle layer, which is beneficial for intestinal motility. This result is probably due to the dietary fiber present in the sorghum flour, which increases intestinal motility, leading to hyperplasia and/or hypertrophy of muscle cells [31, 32].

The increase of propionic acid production by animals feed with sorghum can be related to the high content of dietary fiber, condensed tannins [11] and RS [6], which are associated with increasing SCFA levels by the fermentation of the microbiota [3, 8, 33]. In addition, we observed that the increase of propionic acid was correlated with NFκB and p-Akt concentration, which can be associated with the reduction of inflammation and oxidative stress markers after consumption of hybrid sorghum BRS 305 observed in our previously studies [6, 7].

No difference was observed in the richness, diversity, and species dominance of the intestinal microbiota in male Wistar rats after ten weeks, which demonstrates that the species diversity in the groups remained stable. In addition, the analysis of β-diversity evaluated by PCoA revealed that the composition of the intestinal microbiota did not differ between the groups. Sorghum changed the structure of the intestinal microbial community, especially by reducing the abundance of Bacteroidetes and increasing the abundance of Firmicutes and the ratio of Firmicutes to Bacteroidetes. Many Firmicutes are related to host body weight, but other Firmicutes are abundant in non-obese individuals [34]. There is a limited number of specific bacterial species of phylum Firmicutes and Bacteroidetes associated with obesity [35].

On the other hand, studies have shown that the abundance data of Firmicutes in the intestinal microbiota of healthy individuals ranged from 11% to 95%, and that of Bacteroidetes, between 0.6% and 86.6% [34, 36], which demonstrate that the

variations of these abundances were higher among studies. The increase of Firmicutes is associated with intestinal dysbiosis in the obese that, in turn, increased intestinal permeability. We observed that sorghum flour increased the percentage of urinary excretion of lactulose, possibly due to high abundance of Firmicutes [9, 34]. However, mannitol and lactulose/mannitol ratio were maintained, demonstrating that paracellular transport was not compromised.

Ruminococcaceae, which belongs to the phylum of Firmicutes, contains several butyrate-producing bacteria, performs anti-inflammatory activities and might exert potential physiological functions in the host health [37, 38]. Further, the *Muribaculaceae* family member of the Bacteroidetes phylum has a functional potential in the gut, capable of producing SCFAs (butyrate, acetate, and propionate) by degrading specific types of polysaccharides [39, 40]. In our study, the effects from sorghum did not interfere with butyrate and acetic acid production, as it did not differ between sorghum and HFHF groups. Furthermore, we observed that sorghum intake increased the abundance of Genera *Roseburia* and *Lachnospiraceae_NK4A136_group*, which are associated with reduced glucose intolerance, improved inflammation and oxidative stress [41–44]. This is consistent with our previous findings, in which the hybrid sorghum BRS 305 improved inflammation, oxidative stress and increased the antioxidant enzymes [7]. In addition, hybrid sorghum BRS 305 intakes, improved the insulin sensitivity and glucose tolerance, promoted the control of adipogenesis and hepatic lipogenesis, reducing the accumulation of fat in the liver and adipose tissues in animals fed a high-fat high-fructose diet [6].

According to the functional analysis of the microbiota, the results reveal that the sorghum group upregulated the pathways associated with the metabolism of cofactors and vitamins (e.g., 1,4-dihydroxy-2-naphthoate biosynthesis I, phylloquinol biosynthesis, menaquinol-(7, 8, 11, 12, 13) biosynthesis, demethylmenaquinol biosynthesis). Significant differences from the other groups tested in the KEGG microbial metabolic pathway analysis were observed. The pathways from the metabolism of cofactors and vitamins lead to the biosynthesis of menaquinone (vitamin K2), phylloquinone (vitamin K1) [45], which are essential for humans and animals, since they cannot synthesize it, but can usually receive a sufficient amount from bacteria growing in their intestines [46, 47].

5. CONCLUSION

The present study provides evidence that the consumption of sorghum flour, even associated with a high-fat high-fructose diet, impacts on gut microbiota and the host metabolism, modulating the intestinal microbiota by increasing the abundance of the genera Roseburia, and Lachnospiraceae_NK4A136_group. Further, the sorghum flour increases the propionic acid levels which correlate with anti-inflammatory properties and increases the circular muscle layer, which can benefit the intestinal motility. Therefore, these results emphasize the benefits of sorghum consumption, which may encourage further studies, as well as its consumption by the population.

Acknowledgements

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Compliance with Ethical Standards

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

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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6. GENERAL CONCLUSIONS

The present study provides evidence that the intake of whole sorghum flour from the hybrid BRS 305, a source of resistant starch, dietary fiber and phenolic compounds, improves glucose metabolism, acting directly on insulin sensitivity and glucose tolerance, also promoting control of hepatic adipogenesis and lipogenesis, reducing the accumulation of fat in the liver and adipose tissue. Sorghum consumption efficiently reduced inflammatory markers such as NFκB-p65 transcription factor by inhibiting Akt phosphorylation and decreasing oxidative stress, reducing nitric oxide production and increasing levels of antioxidant enzymes, contributing to increased capacity total antioxidant.

In addition, sorghum flour increased levels of propionic acid and longitudinal muscle layer, which are beneficial for intestinal motility and have anti-inflammatory properties. Also, sorghum modulates the intestinal microbiota by increasing the abundance of the *Roseburia* and *Lachnospiraceae_NK4A136_group* genera, demonstrating its potential for use as a functional food ingredient acting significantly on the intestinal microbiota and host metabolism.

Thus, the chemical composition of dietary fiber and bioactive compounds present in sorghum flour of the hybrid BRS 305, even associated with a diet rich in saturated fat and fructose, has the potential to control fatty liver, insulin resistance and prevent adiposity by acting beneficially on inflammation, oxidative stress and intestinal health, reversing the harmful effects generated by the HFHF diet.

7. FINAL CONSIDERATIONS

In general, substituting 50% of the daily dietary fiber recommendations in animals for fiber from dry heated whole sorghum flour (BRS 305) in a high-fat saturated high-fructose diet (mimic the Western eating condition) for ten weeks increased antioxidant activity, improved inflammation, oxidative stress, improved glucose metabolism by act directly on insulin sensitivity and glucose tolerance, in addition, dietary fiber and resistant starch content improved gut health, and improved metabolic response. Therefore, further studies are needed to continue exploring the molecular pathways and mechanisms involved in the consumption of sorghum in the processes of metabolic alterations and the effect of intake of high-fat high-fructose diet. Thus, it is

essential to investigate the bioactive properties of whole sorghum BRS 305 on satiety and antioxidant response in the brain and adipose tissue in Wistar rats fed with high-fat high-fructose diet, as well as the effect of consumption of sorghum on acute and chronic in humans to evaluate glycemic response, satiety, weight loss, inflammation and intestinal health and verify the mechanisms involved and the effects on human host health.

to evaluate glycemic response, satiety, weight loss, inflammation and intestinal health

8. Anexo

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 38/2019, intitulado "**Bioatividade do sorgo (*Sorghum bicolor L.*) na resistência à insulina, estresse oxidativo inflamação e microbiota intestinal em ratos alimentados com dieta rica em gordura satura e frutose**", coordenado pela professora Hércia Stampini Duarte Martino do Departamento de Nutrição e Saúde, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTI, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTI, portanto sendo aprovado por esta Comissão em 03/09/2019, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 38/2019, named **"Sorghum (Sorghum bicolor L.) bioactivity on insulin resistance, oxidative stress, inflammation and intestinal microbiota in rats fed a high fat saturate and fructose diet"**, is in agreement with the a actual Brazilian legislation (Lei Nº 11.794, 2008), Normative Resolutions edited by CONCEA/MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific Purposes and Teaching) and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI therefore being approved by the Committee on September 09, 2019 valid for 12 months.

Sílvia Almeida Cardoso
Prof.ª Sílvia Almeida Cardoso
Presidente
Comissão de Ética no Uso de Animais – CEUA/UFV