HAIRA GUEDES LÚCIO

EFFECT OF HIGH BIOACTIVE COMPOUNDS FROM SORGHUM (*Sorghum bicolor* L.) INTAKE ON APPETITE NEUROENDOCRINE CONTROL, ANTIOXIDANT RESPONSE, AND INTESTINAL HEALTH IN VIVO

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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“Você não pode esperar construir um mundo melhor sem melhorar os indivíduos. Para esse fim, cada um de nós deve trabalhar para o seu próprio aperfeiçoamento e, ao mesmo tempo, compartilhar uma responsabilidade geral por toda a humanidade.”

Marie Curie

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ABSTRACT

LÚCIO, Haira Guedes, D.Sc., Universidade Federal de Viçosa, February, 2024. **Effect of high bioative compounds sorghum (*Sorghum bicolor* L.) intake on appetite neuroendocrine control, antioxidant response, and intestinal health in vivo**. Adviser: Hercia Stampini Duarte Martino. Co-advisers: Barbara Pereira da Silva, Mariana Grancieri, Valéria Aparecida Vieira Queiroz and Izabela Montezano de Carvalho.

The Western Diet along with a sedentary lifestyle, leads to obesity and comorbidities such as intestinal dysbiosis, diabetes, chronic kidney disease (CKD) and affects neuroendocrine satiety pathways. Sorghum is a cereal rich in bioactive compounds, and offer benefits to systemic and intestinal health. Objectives: To investigate the effect of two varieties of sorghum, rich in tannins and resistant starch, on appetite, satiety, antioxidant response, intestinal health and metabolic markers in animals and human model. Methods: For Study 1, 24 male Wistar rats (50-days-old) were used. For 8 weeks, they were divided into two groups: AIN93-M control (n=8) and high-fat-high-fructose diet (HFHF) (n=16). For the next 10 weeks, HFHF group was subdivided into HFHF (n=8) and HFHF + sorghum flour (n=8) replacing 50% of the daily dietary fiber recommendation. Data were analyzed using ANOVA and Newman-Keuls (α=0.05) with GraphPadPrism. Study 2 involved men with overweight during eight weeks. Participants consumed breakfast cereals with milk or a drink with 40g of sorghum (n=10) or 38g of extruded wheat (n=11), following a 500kcal/day restricted diet. Anthropometric measurements, blood, and fecal samples were collected for analysis. The Study 3 was a controlled, randomized, single-blind clinical trial involving CKD patients on hemodialysis. Volunteers were divided into two groups: symbiotic group (n=19) consuming probiotic milk containing *Bifidobacterium longum* + extruded SC319 sorghum, and control group (n=20) consuming pasteurized milk with extruded corn, for 7 weeks. Participants were assessed for anthropometric measures, gastrointestinal symptoms, and intestinal behavior, and blood and fecal samples were collected. For studies 2 and 3, short-chain fatty acid (SCFAs) production was analyzed, and α-diversity and β-diversity were estimated. Functional prediction analysis was performed by Kyoto Encyclopedia of Genes and Genomes. Statistical analyses conducted using GraphpadPrism and STAMP software, adopting α=5%. Results: Study 1: Sorghum reduced gene expression of leptin, resistin, and endocannabinoid receptor type 1 in adipose and brain tissues compared to the HFHF group. It also decreased

neuropeptide Y expression and increased the expression of sirtuin-1, heat shock protein 72, nuclear factor erythroid-derived 2, peroxisome proliferator-activated receptor alpha, superoxide dismutase, and catalase activity. *In silico* analysis showed interaction of 3-deoxyanthocyanins with PPARα, CB1, and leptin receptors. Study 2: Sorghum consumption led to intragroup weight loss and reduced body fat percentage without affect inflammatory markers. Sorghum consumption did not change SCFAs production, fecal pH, or α and β-diversity indices. However, sorghum consumption decreased *Clostridium_sensu_stricto* 1, *Dorea*, and *Odoribacter*, while increasing CAG-873 and *Turicibacter*. Study 3: Symbiotic drink meal reduced uremic toxins and improved gastrointestinal symptoms. It increased the production of short-chain fatty acids and Chao1 index, though Shannon and Simpson indices and beta diversity remained unchanged. Symbiotic drink also enhanced energy metabolism, amino acid metabolism, and ribonucleotide biosynthesis. Conclusion: Dry-cooked sorghum BRS305 modulated gene expression of neuroendocrine satiety pathways markers and improved antioxidant capacity in rats. Extruded sorghum SC319 improved intestinal microbiota composition, reduced body fat, and promoted weight loss. Extruded BRS 305 sorghum combined with a probiotic drink improved intestinal health and reduced uremic toxins and constipation in CKD patients.

Keywords: Sorghum bicolor L., satiety, antioxidant response, 3-deoxyanthocyanins, chronic kidney disease, intestinal health

RESUMO

LÚCIO, Haira Guedes, D.Sc., Universidade Federal de Viçosa, Fevereiro, 2024. **Effect of high bioative compounds sorghum (Sorghum bicolor L.) intake on appetite neuroendocrine control, antioxidant response, and intestinal health in vivo.** Orientadora: Hercia Stampini Duarte Martino. Co-orientadoras: Barbara Pereira da Silva, Mariana Grancieri, Valéria Aparecida Vieira Queiroz e Izabela Montezano de Carvalho.

A Dieta Ocidental associada ao sedentarismo leva à obesidade e comorbidades como disbiose intestinal, diabetes, doença renal crônica (DRC) e afeta vias neuroendócrinas da saciedade. O sorgo é um cereal rico em compostos bioativos, oferecendo benefícios à saúde sistêmica e intestinal. Objetivo: Investigar o efeito de variedades de sorgo, ricos em taninos e amido resistente, no apetite, saciedade, resposta antioxidante, saúde intestinal e marcadores metabólicos em modelo animal e humano. Metodologia: Para o estudo 1 foram utilizados 24 ratos *Wistar* machos (50 dias). Durante 8 semanas, eles foram divididos em dois grupos: controle AIN93-M (n=8) e dieta rica em gordura e frutose (HFHF) (n=16). Nas 10 semanas subsequentes, o grupo HFHF foi subdividido em HFHF (n=8) e HFHF + farinha de sorgo (n=8), substituindo 50% da recomendação diária de fibras. Os dados foram analisados utilizando ANOVA e Newman-Keuls (α=0,05) no GraphPadPrism. O estydi 2 envolveu homens com excesso de peso. Os participantes consumiram, durante oito semanas, cereais matinais contendo 40g de sorgo (n=10) ou 38g de trigo extrusado (n=11), seguindo dieta com restrição de 500kcal/dia. Medidas antropométricas, amostras de sangue e fezes foram coletadas para análise. O estudo 3 envolveu ensaio clínico controlado, randomizado, simples-cego com pacientes com DRC dialíticos. Os voluntários foram divididos: grupo simbiótico (n=19) consumindo leite probiótico com *Bifidobacterium longum* + sorgo SC319 extrusado; grupo controle (n=20) consumindo leite pasteurizado com milho extrusado, durante 7 semanas e avaliados quanto às medidas antropométricas, sintomas gastrointestinais e comportamento intestinal. Para os artigos 2 e 3, foi analisada síntese de ácidos graxos de cadeia curta (AGCCs), α-diversidade e β-diversidade. A análise de predição funcional foi realizada usando Enciclopédia de Genes e Genomas de Kyoto. Análises estatísticas foram realizadas

no GraphpadPrism e STAMP (α=5%). Resultados: Artigo 1: O consumo de sorgo reduziu expressão gênica de leptina, resistina, receptor endocanabinóide tipo 1 e neuropeptídeo Y no tecido adiposo e cérebro, aumentou expressão de sirtuína-1, proteína de choque térmico 72, fator nuclear 2 derivado de eritróide, receptor alfa ativado por proliferador de peroxissoma e atividade de superóxido dismutase e catalase. Análise *in silico* mostrou interação de 3-desoxiantocianinas com PPARα e receptores CB1 e de leptina. Artigo 2: O consumo de sorgo promoveu perda de peso e redução de gordura corporal sem alterar marcadores inflamatórios. O consumo de sorgo não alterou a produção de SCFAs, pH fecal e α e β-diversidade, e diminuiu *Clostridium_sensu_stricto* 1, *Dorea* e *Odoribacter*, enquanto aumentou CAG-873 e *Turicibacter*. Artigo 3: A refeição com bebida simbiótica reduziu toxinas urêmicas, melhorou sintomas gastrointestinais, aumentou a produção de AGCCs e índice Chao1, além do metabolismo energético, metabolismo de aminoácidos e biossíntese de ribonucleotídeos pela predição funcional. Conclusão: O sorgo cozido a seco BRS305 modulou expressão gênica de marcadores das vias neuroendócrinas da saciedade e melhorou capacidade antioxidante em ratos. O sorgo extrusado SC319 melhorou a composição da microbiota intestinal, reduziu a gordura corporal e promoveu a perda de peso. O sorgo BRS 305 extrusado combinado com bebida probiótica melhorou a saúde intestinal e reduziu toxinas urêmicas e constipação em pacientes com DRC.

Palavras-chave: Sorghum bicolor L., saciedade, resposta antioxidante, 3-desoxiantocianinas, doença renal crônica, saúde intestinal

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LIST OF ACRONYMS AND ABBREVIATIONS

3-DXAs: 3-deoxyanthocyanins

µL: microliter

AGE: advanced glycation end products

AIN-93M: diet formulated for maintenance of adult rodents.

ANOVA: analysis of variance

AOAC: Association of Official Agricultural Chemists

BMI: body mass index

BF: Body fat

CAT: catalase

CB1: endocannabinoid receptor type 1

CEUA/UFV: Ethics Committee of the Federal University of Viçosa

CFU: colony forming unit

CG: control group

CKD: Chronic Kidney Disease

dL: deciliter

DRI: Dietary Reference Intakes

DVS: Direct Vat Set

DXA: dual-energy X-ray absorptiometry

EDTA: ethylenediaminetetraacetic acid.

EFE: estimated free energies

FDR: false discovery rate

FOS: fructooligosaccharides

H2O2: hydrogen peroxide

HCl: hydrochloric acid

HD: hemodialysis

HFHF: high-fat high-fructose diet

HPLC: High performance liquid chromatography

HSP72: heat shock protein 72

IAA: indole-3 acetic acid

IL-6: interleukin 6

IL-10: interleukin 10

IS: indoxyl sulfate

Kcal: kilocalorie

KEGG: Kyoto Encyclopedia of Genes and Genomes

Kg: kilogram

LEfSe: Linear Discriminant Analysis Effect Size

LEP-r: leptin receptor

MG: Minas Gerais

mg: milligram

mRNA: messenger RNA

NPY: neuropeptide Y

NRF2: erythroid-derived nuclear factor 2

OTU: operational taxonomic unit

PAC-QOL: Patient Assessment of Constipation Quality of Life

PCoA: Principal Coordinate Analysis

PCR: reactive C protein

p-CS: p-cresyl sulfate

PERMANOVA: Permutational multivariate analysis of variance

PPARα: peroxisome proliferator-activated receptor alpha

PPAR-γ: peroxisome proliferator-activated receptor gamma

RAGE: advanced glycation end products receptors

RI: refractive index

ROS: reactive oxygen species

RT-qPCR: RealTime PCR System

SAD: sagittal abdominal diameter

SB: sorghum group at baseline

SCFA: short chain fatty acids

SD: standard deviation

SE: sorghum group at endpoint

SFA: satured fat

SG: symbiotic group

SIRT-1: sirtuin 1

SOD: superoxide dismutase

TNFα: tumor necrosis factor α

VFA: volatile fatty acids

WB: wheat group at baseline

WC: waist circumference

WE: wheat group at endpoint

WHO: World Health Organization

WHtR: Waist-to-height ratio

ZnSOD: enzyme superoxide dismutase activated by zinc

LIST OF SYMBOLS

°C: Celsius

%: Percentage

SUMMARY

1. INTRODUCTION

Diets based on the consumption of foods rich in nutrients, unsaturated fatty acids (MUFAs and PUFAs) and dietary fiber have been replaced by those rich in saturated fats and refined sugars, which has shaped the Western dietary pattern (Marventano et al., 2018). This dietary pattern, which includes excessive consumption of ultra-processed foods, associated with a sedentary lifestyle, becomes risk factors for the development of chronic diseases, such as chronic kidney disease, diabetes mellitus and high blood pressure, as well as impact the intestinal health of individuals (Christie et al., 2020a; Khoshbin e Camilleri, 2020).

Satiety hormones are responsible for controlling food intake through hunger and satiation mechanisms, which is the feeling of satisfaction or fullness that occurs after eating, controlling appetite and stopping the feeling of hunger. The main related hormones are ghrelin and leptin, which can be controlled both by gastrointestinal response mechanisms to food intake and by the activity of their brain receptors, such as type 1 endocannabinoid receptors (Cummings e Overduin, 2007). Gastrointestinal responses to nutrients can be modified by changes in food intake, so that increased food consumption reduces gastrointestinal sensitivity to the satiating effects of nutrients and peripheral and central hormones (Feinle-Bisset, 2014). Furthermore, the consumption of a diet rich in fat and/or fructose is associated with leptin resistance and the hyperactivation of type 1 endocannabinoid receptors, which, in turn, are associated with increased food intake, favoring the emergence of metabolic changes and, or obesity (Drori et al., 2020; Silvestri e Marzo, Di, 2013; Tam et al., 2017), systemic oxidative damage and inflammation, which can culminate in the appearance of chronic non-communicable and neurodegenerative diseases (Saiyasit et al., 2020).

The excessive accumulation of body fat, especially visceral fat, secretes a variety of pro-inflammatory substances that can lead to systemic inflammation and lead to changes in the composition and/or function of the intestinal microbiota, resulting in intestinal dysbiosis in a bidirectional way (Maurizi et al., 2018; Turnbaugh et al., 2006). Therefore, the composition of the intestinal microbiota is influenced by the dietary pattern, especially the consumption of saturated fats, normally increased in individuals with obesity (Barber et al., 2021). For example, it has been suggested that the increase of pathogenic microbiota suppresses the oxidation of muscle fatty acids, favoring body adiposity, inflammation, and the emergence of insulin resistance. This inflammation

can contribute to the development of several diseases, including type 2 diabetes and cardiovascular disease (Poudyal et al., 2012; Vernochet et al., 2014).

Among the co-morbidities associated with a dietary pattern rich in saturated fats and/or obesity and other chronic diseases such as type 2 diabetes mellitus and hypertension, we can highlight chronic kidney disease. The presence of uremic compounds resulting from deficiencies in the glomerular filtration capacity, common in chronic kidney disease patients, may also be a triggering factor for intestinal dysbiosis. These compounds increase luminal pH, favor the growth of pathogenic bacteria in the body and cause irritation in the intestinal mucosa and, consequently, an increase in its permeability (Plata et al., 2019). The metabolic changes caused by chronic kidney disease led to changes in the composition and functions of the intestinal microbiome which, by breaking the intestinal epithelial barrier through increased intestinal permeability, allows the passage of toxic by-products, such as lipopolysaccharide that fall into the blood circulation, contributing for inflammation and metabolic endotoxemia (Castillo-Rodriguez et al., 2018; Fernandez-Prado et al., 2017).

The frequent consumption of whole grains promotes numerous benefits to intestinal health, including production of short-chain fatty acids (SCFA), secretion of intestinal hormones, immunological homeostasis and restoration of intestinal permeability. Many cereals can be considered prebiotics, as they are composed of complex carbohydrates that are resistant to degradation in the small intestine, but are metabolized by microbes in the colon, where they are fermented into short-chain fatty acids (SCFA), gases and other products (Flint, 2012; O'Connor et al., 2017; Slavin, 2013a).

Sorghum grains *Sorghum bicolor* (L.) are considered an important source of energy, carbohydrates and proteins and contain a variety of bioactive compounds. Among the carbohydrates present in sorghum, resistant starch stands out, which plays the role of dietary fiber in the intestine, promoting an increased feeling of satiety and exerting beneficial effects on intestinal health (Barros, Awika & Rooney, 2012). The SC319 sorghm genotype is an excellent source of bioactive compounds including flavonoids, tannins, anthocyanins, vitamin E and carotenoids, which contribute to its high antioxidant capacity (Anunciação et al., 2017). The BRS305 sorghum genotype is a cultivar rich in condensed tannins, 3-deoxyanthocyanins and resistant starch (Lopes et al., 2018). Several studies have demonstrated positive effects of resistant starch in modulating the intestinal microbiota (DeMartino e Cockburn, 2020; Keenan

et al., 2015; Llopart et al., 2017; Yoshida et al., 2019; Zaman e Sarbini, 2016), in improving insulin sensitivity (Bindels et al., 2017) and reducing adiposity (Keenan et al., 2015). Furthermore, the effects of sorghum consumption in human nutrition have demonstrated improvements in the intestinal microbiota (Sousa, de et al., 2019a), as well as effects on increasing satiety, reducing cecal pH and the activity of the enzymes β-glucosidase and β -glucuronidase (Llopart et al., 2017). Among the bioactive compounds identified in sorghum highlight the phenolic acids, flavonoids, condensed tannins, polycosanols, phytosterols, stilbenes and phenolamides. These compounds can exerts antidiabetic, cholesterol-lowering, anti-inflammatory and antioxidant effects (Medina Martinez et al., 2021a; b).

In this sense, sorghum BRS305 when submitted to a dry heat treatment maintains such as a cereal rich in condensed tannins and resistant starch (Medina Martinez et al., 2021b), which is associated with intestinal modulation and functionality since the non-digestible carbohydrate present in food is fermented in the colon, producing short-chain fatty acids. Condensed tannins and 3-deoxyanthocyanins, also present in SC319 and BRS305 sorghum, respectively, acts as antioxidant compounds that can improve the body's response to pro-oxidant stimuli from a diet rich in fat and fructose (Medina Martinez et al., 2021a; b), as well as assisting in the inflammatory response Sorghum SC319 is a genotype with high antioxidant capacity due to its chemical composition, which presents bioactive compounds such as phenolic acids, flavonoids, tannins, 3-deoxyanthocyanidins and vitamin E (Anunciação et al., 2017; Cardoso et al., 2015). Condensed tannins and 3-deoxyanthocyanins also act as antioxidant compounds in chronic kidney patients (Lopes et al., 2018a), and in weight loss (Anunciação et al., 2018). Furthermore, the presence of kaferins and the resistant starch content of the grain reduce the digestibility (Barros, Awika e Rooney, 2012), which can promote satiation and restore the physiological activity of receptors and proteins that act as satiety regulators.

The present study investigated for the first time, BRS305 sorghum genotype on satiety centers and antioxidant response of brain and adipose tissue in animal model, as well as the combination of sorghum with a probiotic *Bifidobacterium longum* on intestinal health of CKD patients. This research was the first one to investigate the effects of extruded SC319 sorghum on inflammatory markers and intestinal health markers of Brazilian man with overweight.

2. HYPHOTESIS

H1: BRS305 whole sorghum flour subjected to dry cooking, rich in tannins and resistant starch, subjected to dry cooking, modulates positively the satiety and adipogenesis pathways and reestablishes the antioxidant response after ingestion of a diet rich in saturated fat and fructose, in rats Wistar.

H2: In men with overweight, the consumption of extruded SC319 sorghum, rich in tannins, associated to a 500 kcal/day reduction, reduces anthropometric measures such as body weight, body fat percentage, waist circumference, inflammatory markers, anthropometric measures; intestinal health, with growth of beneficial bacterial genera, modulating the intestinal microbiota, increasing the short chain fatty acids production and reducing fecal pH.

H3: In individuals with chronic kidney disease on hemodyalisis, the consumption of a symbiotic meal containing extruded BRS 305 sorghum-based breakfast cereal added with probiotic milk containing Bifidobacterium longum, improves intestinal health, with growth of beneficial bacterial genera, modulating the intestinal microbiota, reducing gastrointestinal symptoms, increasing the short chain fatty acids production and reducing the uremic toxins production.

3. OBJECTIVES

3.1. General objective

To investigate the effect of two varieties of sorghum (*Sorghum bicolor* L.), rich in tannins and resistant starch, on apetite, satiety, antioxidant response, intestinal health and metabolic markers in animals and human model.

3.2. Specific objectives

- To evaluate the effect of BRS 305 whole sorghum flour, subjected to dry cooking, on the antioxidant response of the brain and adipose tissue of *Wistar* rats fed a diet rich in saturated fat and fructose;

- To investigate the effect of whole sorghum flour BRS 305, subjected to dry cooking, on neuroendocrine control of appetite and satiety in *Wistar* rats fed a diet rich in saturated fat and fructose;

- To analyze the impacts of the consumption of sorghum SC319 associated with calorie restriction on inflammatory markers in overweight and/or obese men;

- To evaluate the effects of sorghum SC319 consumption associated with calorie restriction on markers of intestinal health in man with overweight.

- To investigate the effect of consuming extruded BRS 305 sorghum, plus associate with probiotic milk, on modulating the microbiota and intestinal health of chronic kidney disease patients on hemodyalisis;

- To investigate the effect of consuming extruded BRS 305 sorghum, associate withplus probiotic milk, on biochemical and uremic markers in chronic kidney disease patients on hemodyalisis.

4. LITERATURE REVIEW

This research have three different lines of investigation that included to evaluated the effects of BRS305 sorghum genotype submitted do dry heat treatment on satiety centers and antioxidant response of brain and adipose tissue of rats fed a high fat high fructose diet; the effects of extruded SC319 sorghum associated to a caloric restriction of 500 kcal/day on inflammatory markers and intestinal health markers of Brazilian obese man; and the effects of BRS305 extruded sorghum associated to a probiotic *Bifidobacterium longum* on intestinal health of CKD patients.

So, the literature review was divided into chapters, in order to facilitate reading and understanding for the reader, relating the topics of this literature review with the objectives of the different studies carried out in this research.

4.1. CHAPTER 1

4.1.1. DIETARY PATTERN, OBESITY, NEUROENDOCRINE CONTROL OF APPETITE, INFLAMMATION AND OXIDATIVE STRESS

A diet high in fat and sugar and low in dietary fiber is referred to as the “Western diet”. This eating behavior has become common in populations in developed countries, where people in general are consuming “saturated” fats in amounts exceeding 30% of their daily energy intake. This dietary pattern is often associated with a high intake of highly palatable ultra-processed foods, which in turn are also rich in sugars (Abdalla, 2017). The increased consumption of foods with high caloric density and containing large amounts of saturated fatty acids and fructose is directly related to the global epidemic of overweight and obesity. Furthermore, excessive consumption of these foods is related to the emergence and worsening of diseases such as dyslipidemia, diabetes mellitus, coronary heart disease, and non-alcoholic fatty liver disease. In turn, these diseases have high morbidity and mortality rates (Dandona et al., 2005; Zivkovic, German e Sanyal, 2007).

In addition to the effects on the risk of developing chronic non-communicable diseases, high-fat diets are capable of modulating endocannabinoid levels independently of their fatty acid composition (Silvestri e Di Marzo, 2013). In animals, high-fat diets trigger binge eating patterns and result in an increase in intestinal motility, anandamide (AEA) and 2-arachidonoylglycerol (2-AG) levels, possibly increasing cannabinoid receptor stimulation. This increase in stimulation of the CB1 endocannabinoid receptor results in an increase in the synthesis of fatty acids,

increasing the expression of the lipogenic transcription factor sterol regulatory element binding protein-1c (SREBP-1c), triggering greater production of acetyl coenzyme-A carboxylase-1 and fatty acid synthase. Increased levels of AEA and 2-AG in response to high fat diets occur due to decreased activity of monoacylglycerol lipase (MGL) and fatty acid amide hydrolase (FAAH) and increased action of N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), which occurs independently of food intake (Christie et al., 2020a; Naughton et al., 2013; Di Patrizio, 2021).

Fructose consumption has been shown to significantly induce weight gain (10%) in male Sprague-Dawley rats over an 8-week treatment period, even balancing caloric intake with the control group. The same study observed that the supply of fructose for 6 to 7 months was able to increase body weight by 1.27x when compared to the group fed a standard diet and adipose tissue (especially abdominal fat) in male and female Sprague-Dawley rats (Bocarsly et al., 2010). Moreira et al., (2022) offered a diet rich in fat and fructose to adult male Wistar rats for 8 weeks and observed an increase in body weight in animals fed this type of diet (Moreira et al., 2022). From the ninth week onwards, these authors began an intervention offering oil and chia seed, which is a pseudocereal, associated with this dietary pattern and the consumption of chia was able to positively modulate metabolic disorders and body weight in these animals. In overweight and obese humans, the daily consumption of 25% of energy from fructose for 10 weeks, offered as a sweetened drink, culminated in an increase in visceral adiposity when compared to the consumption of an isocaloric drink sweetened with glucose (Stanhope & Havel, 2009).

One hypothesis raised to explain the association between excessive fructose consumption and obesity is directly linked to leptin resistance. Leptin is a hormone produced mainly in white adipose tissue and is involved in satiety mechanisms. Diet-induced obesity has been strongly associated with chronic hyperleptinemia, characterized by elevated, rather than reduced, levels of circulating leptin in adipose tissue. Studies developed in animal models suggest that chronically increased levels of circulating leptin desensitize hypothalamic leptin receptors, a behavior like what happens in the case of insulin resistance. Under these conditions, the hypothalamus becomes increasingly less responsive to leptin, the sensation of hunger persists, and food intake does not decrease, although the energy reserve in the form of adipose tissue is abundant (Gruzdeva et al., 2019; Mendoza-Herrera et al., 2021).

Hyperleptinemia may be associated with decreased phosphorylation of signal transducer and activator of transcription 3 (STAT3), which decreases the anorectic response associated with leptin, and also with impaired signaling of the leptin receptor and deficiencies in leptin transport through the blood-brain barrier (El-Haschimi et al., 2000; Scarpace et al., 2007). Also, in addition to fructose's ability to modulate the release of satiety hormones in the periphery, it affects several satiety regulators in the hypothalamus. Lindqvist et al. suggested that the gene expression of peptide YY (PYY), neuropeptide Y (NPY) and proopiomelanocortin (POMC) were significantly reduced after consuming a high-fructose diet (Lelis et al., 2020; Lindqvist, Baelemans e Erlanson-Albertsson, 2008).

Long-term ingestion of a high-fat diet (HFD) culminates in pathologies in the cerebral hippocampus that play a role in cognitive function. A diet high in fat and calories can lead to the accumulation of nutrients in the hypothalamus, triggering inflammatory responses, leading to a chronic inflammation in the hypothalamus, which negatively impacts the function of neurons responsible for controlling food intake and satiety in reason to hypothalamus be a critical brain region that regulates appetite, metabolism and energy expenditure (Lee et al., 2020). The hypothalamic inflammation in obesity is characterized by the activation of immune cells, the release of pro-inflammatory cytokines and neuronal dysfunction. Factors such as nutrients excess, leptin resistance, and the presence of free fatty acids contribute to this inflammation (Kim et al., 2019).

Excess of free fatty acids can activate inflammatory signaling pathways in glial cells and neurons. Hypothalamic inflammation is closely linked to leptin resistance, a key phenomenon in obesity. Inflammatory cytokines can interfere with leptin signaling in hypothalamic neurons (De Git & Adan, 2015). For example, activation of the JNK (c-Jun N-terminal kinase) pathway by inflammation can inhibit the phosphorylation of the leptin receptor, preventing its action. Furthermore, inflammation can lead to decreased expression of leptin receptors in hypothalamic neurons, reducing leptin sensitivity and contributing to dysregulated food intake (Gragano et al. 2017).

Leptin resistance is common in obese individuals and reduces the effectiveness of the leptin signaling that promotes satiety, contributing to excessive food intake and the maintenance of obesity. Furthermore, leptin resistance is associated with increased hypothalamic inflammation (Pimentel et al., 2014). Obesity conditions also increase the production of inflammatory cytokines such as TNF-α, IL-

6 and IL-1β, which can cross the blood-brain barrier or be produced locally in the hypothalamus, exacerbating inflammation (Vong et al., 2011).

Previous studies have shown that inflammation, oxidative stress, microglial hyperactivity, neuronal cell death, synaptic dysplasticity, and loss of dendritic spine in the hippocampus were observed after 12 weeks of HFD feeding in animals, which consequently developed cognitive impairment (Chunchai et al., 2018; Hao et al., 2016; Pratchayasakul et al., 2015; Saiyasit et al., 2020). Woodie & Blythe (2018), when offering a so-called product rich in fructose to rodents, observed effects on insulin dysregulation, development of hyperlipidemia and reduced cognitive performance in these animals (Woodie & Blythe, 2018). Substantial evidence suggests that increased oxidative stress and altered apoptosis, caused by ingestion of a diet rich in lipids and/or fructose, contribute to the pathogenesis of neurodegenerative diseases. In this context, free radicals are implicated in the progression and development of cognitive deficits by interrupting synaptic transmission, mitochondrial function, neuroinflammation and axonal transport, recognized as contributing factors to neurological diseases (Tan Norhaizan, 2019).

As noted, it is known that oxidative stress induced by a high-fat diet plays a prominent role in damaging brain function, culminating in the development of neurodegenerative diseases. Although there is an antioxidant defense system composed of enzymes and factors with antioxidant activities such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) glutathione reductase (GR), nuclear factor erythroid-derived 2 (NRF2) (Batandier et al., 2020; Langley et al., 2020; Rosa et al., 2017; Shal et al., 2018) the high lipid deposition caused by this diet can induce high levels of reactive oxygen species (ROS), which include superoxide anions, hydroxyl radicals, hydrogen peroxide (H2O2) etc. (Maurizi et al., 2018). The brain, in turn, is highly sensitive and vulnerable to oxidation due to the presence of large amounts of unsaturated fatty acids and ROS that serve as substrates for lipid peroxidation (Li et al., 2021; Maurizi et al., 2018; Si et al., 2021).

The accumulation of ROS leads to a weakening of the antioxidant defense system and an increase in oxidative stress. As a result, there is peroxidation of fatty acids, which is also considered a biomarker of oxidative stress in neurodegenerative diseases, along with nitration and carbonylation of proteins and oxidative damage to RNA and DNA. The central nervous system is also adversely affected by high levels of circulating free fatty acids and the lipotoxic effects caused by metabolic changes,

such as high levels of triglycerides or cholesterol (Langley et al., 2020; Li et al., 2021). Additionally, upon reaching the brain, these fatty acids interact with several transcription factors to trigger signaling pathways, such as peroxisome proliferator-activated receptor (PPAR), which is a ligand-activated transcription factor that acts by activating lipid regulatory proteins. Particularly, PPARα plays a crucial role in regulating the gene expression of fatty acid β-oxidation factors such as catalase, acyl-CoA oxidase 1 (ACOX1) and carnitine-palmitoyl transferase-I (CPT-I) (Tan e Norhaizan, 2019; Zheng e Cai, 2019).

On the other hand, the consumption of whole grains, foods that are sources of dietary fiber and other bioactive compounds, can help in the recovery of metabolic disorders caused by the consumption of a diet rich in fructose and saturated fat, as is the case with dysfunctions in leptin metabolism and brain receptors related to satiety, as well as combating brain oxidative stress (Ampatzoglou et al., 2015; Kopf et al., 2018). It is known that exacerbated adipogenesis affects metabolism in different ways, stimulating cerebral and systemic oxidative stress, triggering low-grade inflammatory reactions and favoring the emergence of diseases, such as chronic non-communicable or neurodegenerative diseases, in the long term (Kuri-Harcuch et al., 2019; Moseti, Regassa e Kim, 2016a). The bioactive compounds present in whole grains can improve the antioxidant response and reduce the oxidative stress exacerbated in these conditions, minimizing the inflammatory condition caused by the Western dietary pattern (Martinez et al., 2021a). In this sense, sorghum can be included in the diet, as it is a cereal with a high content of dietary fiber and bioactive compounds such as 3-deoxyanthocyanins and condensed tannins. New in vivo research has been developed and points to sorghum as a cereal capable of reducing oxidative stress and systemic inflammation, as well as improving glucose and lipid metabolism, demonstrating promise in the management and recovery of metabolic changes caused by this eating behavior.

4.1.2. NEUROENDOCRINE CONTROL OF APPETITE AND SATIETY

The neuroendocrine control of appetite and satiety is an intricate process that involves a network of molecular signals and neuronal circuits in the brain, mainly in the hypothalamus. Among the key elements of this system, two main pathways stand out: The type 1 endocannabinoid receptor pathway, which acts through a complex system to regulate appetite and metabolism (Lu & Makie, 2020), and the melanocortin

pathway, which acts in a coordinated manner to regulate food intake and energy expenditure, contributing to the maintenance of energy balance (Cone, 2021).

4.1.2.1. NEUROENDOCRINE CONTROL OF APPETITE/SATIETY AND ENDOCANABINOID SYSTEM (CB1 RECEPTOR PATHWAY)

Satiety can be defined as the suppression of hunger at the end of food intake (Smith, 1998), and represented by the period without hunger between meals (Strubbe & Woods, 2004). This period represented by satiety is variable and its end coincides with the reappearance of the feeling of hunger, which is normally accompanied by the consumption of the next meal, thus resuming the food intake cycle. Satiety can be affected by both physiological and psychological mechanisms that, in turn, trigger afferent signals to the brain from multiple sites in the GIT (stomach, pancreas and intestine (Hargrave & Kinzig, 2012).

Among the physiological factors, we can highlight short-term signals from the gastrointestinal tract (GIT) after meals (Zac-Varghese et al., 2010) and long-term signals from adipose tissue (the body's energy reserve) (Woods, 2005). Short-term signals from the GIT are transmitted mainly via the vagal and spinal nerves to the nucleus of the solitary tract (Schwartz, 2000; Powley et al., 2005). Long-term signals, also known as adiposity signals, reach the arcuate nucleus via the median eminence or by crossing the blood-brain barrier. There are several integrations and convergences between these signals mediated by neural connections between the arcuate (ARC) nucleus, neurotransmissor signals (NTS) and vagal afferent fibers (Boguszewski et al., 2010). Among the short-term signals released in the GIT, the main ones are hormones. These hormones released by the gastrointestinal tract or pancreas can exert an orexigenic (as is the case with ghrelin) or anorexigenic (as is the case with peptide YY (PYY) and glucagon-like peptide (GLP-1) effect), with their secretion differentially stimulated by fats and carbohydrates (Gibbons et al., 2013).

Young adult C57BL-6 mice fed a high-fat diet (60% of total energy value) for 12 weeks had their nodose ganglia removed after euthanasia and the cells were cultured and added to ghrelin solution to evaluate the regulation of components of the endocannabinoid system mediated by endocannabinoids and ghrelin. It was observed that obesity induced by the consumption of a high-fat diet can alter the effect of ghrelin on the expression of arcuate nucleus receptors, such as CB1, growth hormone secretagogue receptor (GHSR) and vanilloid 1 transient receptor potential (TRPV1) in

nodose neurons, which can affect afferent vagal signaling (Christie et al., 2020a). Furthermore, fat intake has been shown to be capable of increasing blood levels of PYY to similar levels (Van Der Klaauw et al., 2013) or higher than carbohydrates (Gibbons et al., 2013; Yang et al., 2009), increasing GLP-1 in humans (Christie et al., 2020b; Gibbons et al., 2013).

The endocannabinoid system is a signaling system related to physiological processes, including food intake, fat accumulation and energy homeostasis, and is one of the most conserved systems among vertebrate species. In this context, the activation of the endocannabinoid system favors food/energy intake, as well as energy conservation and inhibits energy expenditure. In this regard, the endocannabinoid system regulates appetite, both centrally and peripherally, namely by controlling leptin and ghrelin signaling (Howlett et al., 2002).

This system comprises the cannabinoid receptors CB1 and CB2, as well as their lipid-derived ligands, such as: N-arachidonoyl-ethanolamine (AEA) and 2-arachidonoyl-glycerol (2-AG), and enzymes related to fatty acid metabolism (Fowler, Doherty & Alexander, 2017). Since the discovery (around 1990) of the first 2 endocannabinoids, 2-arachidonoylglycerol (2-AG) and N-arachidonoylethanolamine (anandamide, AEA), several molecules with similar structures, mainly N-acylamides, but also chain glycerol esters long fatty acids, were found (Witkamp, 2016, 2018).

The molecular targets of endocannabinoids include G protein-coupled receptors (GPCRs) with limited homology to cannabinoid receptors, but also entirely different receptors, including PPARα. Some of these “endocannabinoid-like” molecules, for example, oleoylethanolamine, have been shown to play specific roles in energy homeostasis, including the regulation of appetite and/or satiety. The formation and degradation pathways of endocannabinoid compounds are directly related to the fatty acid renewal routes (Lu & Makie, 2020). The CB1 and CB2 receptors are GPCRs and share 44% homology and 68% identity for the transmembrane domains. According to the contrasting expression of these two receptors, it was initially assumed that CB1 is mainly expressed in the brain, while the CB2 receptor is mainly peripheral and expressed by immune cells (Grabied & Dehghani, 2017).

The endocannabinoid system also participates in the control of energy balance, acting in the regulation of food intake. Fasting is capable of stimulating the activity of the endocannabinoid system and food intake acts to inhibit the activity of this system. Furthermore, pathological obesity may be related to chronic hyperactivity of

the endocannabinoid system (Tam et al., 2012). These mechanisms mainly involve the cannabinoid receptor type 1 (CB1), which is present in the alimentary duct (blocking CB1- inhibits the secretion of ghrelin by the stomach), in the limbic system (endogenous CB1 endocannabinoid agonists present in the hippocampus inhibit phosphorylation induced by ghrelin, the transcription factor cyclic AMP (cAMP) response element binding protein (CREB)), and in the hypothalamus.

Leptin is an adipocyte-derived hormone that acts as an important regulator of food intake and energy homeostasis. Leptin regulates food intake, body mass and reproductive function and plays a role in fetal growth, pro-inflammatory immune responses, angiogenesis, and lipolysis. Leptin also orchestrates complex biological effects through its receptors, expressed both centrally and peripherally. Metabolic changes, such as leptin deficiency or resistance, can result in the emergence of obesity, diabetes, and infertility in humans (De Vos et al., 1995; Obradovic et al., 2021).

Adipocytes are the primary source of circulating leptin (Caron et al., 2018). Other tissues, such as endocrine cells of the gastrointestinal tract, muscle and brain, have also been reported to express leptin (Friedman et al., 2019). After leptin synthesis and secretion by fat cells in white adipose tissue, it binds and activates its cognate receptor (LEP-R). The distribution of leptin receptors facilitates the pleiotropic effects of leptin, playing a crucial role in regulating body mass through a negative feedback mechanism between adipose tissue and the hypothalamus. Leptin resistance is characterized by reduced satiety, excessive consumption of foods rich in saturated fat and simple carbohydrates, associated with an increase in total body mass. This often leads to obesity, which reduces the effectiveness of using exogenous leptin as a therapeutic agent. Therefore, combining leptin therapies with leptin sensitizers may help overcome this resistance and, consequently, obesity (Ottaway et al., 2015).

Leptin messenger RNA (mRNA) levels are associated with the size of adipocytes in the postprandial period of individuals (Zhang et al., 2017). In rodents, leptin mRNA levels are increased in gonadal and retroperitoneal (intra-abdominal) adipose tissues when compared to inguinal (subcutaneous) adipose tissues in young adult animals (Zheng et al., 1997; Zhang et al., 2002). However, leptin gene expression is extremely sensitive to acute negative energy balance, regardless of long-term energy balance (adiposity). Short-term fasting decreases leptin mRNA and plasma concentrations by more than 60% before having any significant effect on total fat stores or adipocyte size (Zhang et al., 1997; Zheng et al., 2017).

Plasma leptin concentrations are directly related to adipose tissue across a wide range of adiposity in mammals. This association between plasma leptin levels and the percentage of body fat, in conditions of obesity, is associated with hyperleptinemia, which plays a role in maintaining the state of leptin resistance. In this condition, CB1-r is hyperactivated and contributes to the establishment of leptin resistance, which is when the brain becomes less receptive to leptin (Obradovic et al., 2021). However, blockade of the peripheral CB1 cannabinoid receptor can reverse hyperleptinemia in mice with diet-induced obesity by antagonizing leptin production in adipose tissue and promoting leptin clearance by the kidney (Tam et al., 2012). Because circulating leptin can reach the medio basal hypothalamus, normalization of plasma leptin can directly lead to re-sensitization of hypothalamic leptin receptors, resulting in increased signaling by endogenous leptin. Alternatively, circulating leptin may regulate the sensitivity of hypothalamic leptin receptors indirectly, through vagal afferent neurons. Leptin receptors are expressed on vagal afferents innervating the stomach and duodenum, and their activation has been reported to promote satiety (Tam et al., 2017).

Regarding the consequences related to disturbances caused in the activity of satiety centers, the presence of obesity, the accumulation of body fat and the presence of metabolic changes such as glucoses metabolism, which can culminate in diabetes mellitus, are pointed out as factors of risk for developing chronic kidney disease, especially in adults. In this sense, dietary aspects, such as the Western dietary pattern, which is rich in saturated fats, fructose and sodium, have been identified as risk factors for the emergence of this disease (Silva Junior et al., 2017).

The presence of excess weight and obesity is also associated with renal hemodynamic, structural and histological changes, in addition to metabolic and biochemical changes that lead to kidney disease. Adipose tissue is dynamic and is involved in the production of "adipokines" such as leptin, adiponectin, tumor necrosis factor-α, monocyte chemotactic protein-1, transforming growth factor-β, and angiotensin-II. In conditions of hyperleptinemia, there is an activation of the sympathetic nervous system that results in an increase in blood pressure and inhibition of the synthesis of nitric oxide, which is a potent vasodilator. Furthermore, the accumulation of adipose tissue, especially the increase in visceral adiposity, leads to renal compression and, consequently, an increase in intrarenal pressure (Kopple, 2010; Kopple & Feroze, 2011; Silva Junior et al., 2017).

Increased food intake and the accumulation of fats in the body, in turn, lead to changes in energy metabolism and satiety, resulting in increased production of AGEs, hyperleptinemia, and hyperactivation of AGEs, leptin and type 1 endocannabinoid receptors. Furthermore, it is also related to the reduction of the body's antioxidant response, favoring oxidative stress and inflammation, and may even be associated with the development of neurodegenerative diseases. Therefore, the use of foods that present bioactive compounds that exert antioxidant and inflammatory effects (Ibars et al., 2022), as well as a high content of dietary fiber and/or resistant starch, such the BRS305 sorghum, can improve the metabolic disorders (Martinez et al., 2021a and 2021b), promoting satiety, reducing food intake and improving the response antioxidant.

In view of the above, understand the effects and disorders caused by the consumption of a diet rich in fat and fructose on satiety centers, which include the metabolism of leptin, resistin and peptide YY, as well as on adipogenesis, expressed mainly by PPARγ, and on Brain control of appetite, regulated by both the leptin receptor and the endocannabinoid receptor 1, is essential to seek strategies to modulate and possibly reverse these metabolic disorders, which can culminate in other diseases, such as chronic kidney disease. In this sense, the inclusion of sorghum, a cereal rich in condensed tannins and 3-deoxyanthocyanins, can be a strategy used to remodel the response of these satiety centers and appetite regulation, control adipogenesis and body fat, as well as improve of the brain oxidative response to this eating behavior.

4.1.2.2. NEUROENDOCRINE CONTROL OF APPETITE/SATIETY AND ENDOCANABINOID SYSTEM (MELANOCORTIN PATHWAY)

The hypothalamic inflammation promoted by obesity interferes with the function of pro-opiomelanocortin (POMC) and neuropeptide Y/agouti related peptide (NPY/AgRP) neurons, essential for regulating appetite and satiety. Also can impair the function of POMC neurons, which produce alpha melanocyte-stimulating hormone (α-MSH), an anorectic peptide that promotes satiety. Thus, hypothalamic inflammation not only affects appetite control, but also impacts energy metabolism and glucose homeostasis (Lindberg et al., 2021) and is associated with insulin resistance, both in the brain and peripherally. Insulin resistance in the hypothalamus may contribute to systemic metabolic dysfunction, including glucose homeostasis. Furthermore, inflammation can affect the function of hypothalamic centers that regulate energy expenditure, resulting in a lower metabolic rate and contributing to weight gain (Cone, 1999).

The POMC-expressing neurons are mainly located in the arcuate nucleus of the hypothalamus (ARH). POMC is a polypeptide precursor that, once cleaved by specific enzymes, generates several bioactive peptides, including α-MSH (Gui et al., 2023). The release of α-MSH by these neurons is a critical step in mediating anorexigenic effects, reducing the food intake and their effects are potentiated by transcript regulated by amphetamine and cocaine (CART) neuropeptide coexpressed by POMC neurons (Zhang et al., 2021). Thus, CART works in synergy with α-MSH to promote satiety and inhibit food intake. The combined effects of these two peptides are mediated through their interaction with melanocortin receptors, specifically melanocortin receptors 4 (MC4R) and 5 (MC5R) (Sweeney et al., 2023). These receptors are coupled to the G protein and, when activated by α-MSH, initiate an intracellular signaling cascade that results in the inhibition of food intake and increased energy expenditure. MC4R is widely distributed in the brain, while MC5R is found in smaller quantities, but both play essential roles in regulating appetite and energy metabolism (Nakhate et al., 2019).

The POMC-αMSH axis is influenced by several peripheral signals that reflect the energetic state of the organism. Among these signals, leptin and insulin stand out, hormones that signal abundance of energy. Leptin, secreted by adipose tissue, and insulin, released by the pancreas, act directly on POMC neurons to promote POMC expression and α-MSH release. These hormones activate POMC neurons through

specific receptors, triggering a cascade of events that culminates in the release of α-MSH and the activation of MC4R and MC5R receptors, resulting in the inhibition of food intake (Cone, 2021).

Leptin and insulin play critical roles in regulating the activity of POMC neurons. In experimental models feed wiht a standard diet, leptin binds to the leptin receptor (ObR) on POMC neurons, activating the JAK2/STAT3 pathway, which promotes the transcription of genes encoding anorectic peptides, including POMC (Liu et al., 2023). Insulin, in turn, activates the PI3K/Akt signaling pathway, which also promotes POMC expression and α-MSH release. These signaling pathways converge to increase the activity of POMC neurons and the release of α-MSH, resulting in the activation of MC4R and MC5R receptors and the inhibition of food intake (Vohra et al., 2022).

Signaling through MC4R is particularly important for appetite regulation. Genetic studies in humans and animals have demonstrated that mutations in the MC4R gene are associated with severe obesity, highlighting the importance of this receptor in maintaining energy balance. When α-MSH binds to MC4R, a signaling cascade is activated that involves the activation of adenylate cyclase and an increase in cyclic AMP (cAMP) levels. This increase in cAMP activates protein kinase A (PKA), which in turn phosphorylates several downstream targets, resulting in the inhibition of food intake and increased energy expenditure (Baldini e Phelan, 2019).

In addition to the classical cAMP signaling pathway, MC4R activation may also involve other signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathway, which plays a role in modulating gene expression associated with appetite regulation. Signaling through MC4R not only influences feeding behavior, but also basal metabolism and thermogenesis, processes that contribute to the maintenance of energy balance (Ni et al., 2022). MC5R also plays a role in regulating appetite and energy expenditure. Activation of MC5R by α-MSH can influence the function of exocrine glands and lipid metabolism, suggesting a complementary role in the regulation of energy balance. Although the distribution of MC5R in the brain is more limited, its expression in other tissues indicates that it may have additional peripheral functions in regulating metabolism (Dores, 2014).

The anorexigenic effects mediated by α-MSH through melanocortin receptors are counterbalanced by orexigenic signals such as neuropeptide Y (NPY) and agouti-related peptide (AgRP). Both are produced by arcuate nucleus neurons that have opposite effects to those of POMC neurons. AgRP acts as an antagonist of MC4R

receptors, competing with α-MSH and inhibiting its anorectic effects. Thus, the balance between the activity of POMC/CART and NPY/AgRP neurons is crucial for the precise regulation of appetite and energy balance (Vohra et al., 2022).

In this context, the hypothalamic inflammation presented in conditions of obesity compromises the expression and function of POMC neurons, reducing the production of α-MSH, which impairs the body's ability to signal satiety and contributes to excessive food intake and weight gain. Furthermore, leptin resistance and obesity-associated hyperleptinemia are related to decreased MC4R receptors in the hypothalamus, reducing the effectiveness of satiety signals transmitted by α-MSH, especially exacerbated by chronic inflammation. This metabolic imbalance can result in negative feedback on the POMC-MSH axis, compromising the regulation of appetite and metabolism, perpetuating a vicious cycle of weight gain and difficulty losing weight. Thus, the activation of inflammatory pathways with bioactive compounds such as 3-deoxyanthocyanins can inhibit POMC expression or α-MSH release, reducing satiety signaling. Conversely, inflammation can increase the activity of NPY/AgRP neurons, which promotes hunger. Increased expression of NPY and AgRP due to inflammation contributes to hyperphagia, or excessive food intake (Chen et al., 2022; Souza et al., 2016).

4.1.3. ADIPOGENESIS

Adipogenesis is the process of cellular differentiation of preadipocytes into adipocytes. Preadipocytes derive from adipocyte progenitor cells that arise from various sources in the body (Ambele et al., 2020). The process of adipogenesis has been widely studied in vitro in a wide variety of cell culture systems, and it has been observed that different conditions and hormones regulate the development of adipocytes (Lee et al., 2019).

Adipocytes are the defining cell type of adipose tissue. Previously, adipocytes were considered only responsible for energy storage. However, adipose tissue is now recognized as a dynamic organ that contributes to several important physiological processes, such as lipid metabolism, systemic energy homeostasis, and whole-body insulin sensitivity. Adipocyte differentiation is a highly orchestrated process that can vary between different fat depots, as well as between sexes (Kuri-Harcuch et al., 2019; Sarjeant e Stephens, 2012).

There are two distinct classes of adipose tissue: white adipose tissue and brown adipose tissue. White adipose tissue is responsible for the largest amount of adipose tissue present in mammals, including adult humans, and is a critical local factor for energy homeostasis, insulin signaling and endocrine activity. In contrast, brown adipose tissue is primarily responsible for no shivering thermogenesis that is facilitated by UCP-1 (uncoupling protein 1), an inner mitochondrial membrane protein that translocate protons from the intermembrane space to the mitochondrial matrix (Lee et al., 2019).

Evidence has demonstrated that white and brown adipocytes require Peroxisome proliferator-activated protein receptor gamma (PPARγ), which is encoded by the PPAR-γ gene. PPARγ is a transcription factor induced during the differentiation of preadipocytes to adipocytes and is classified as two varied isoforms, for the differentiation and maturation process of this cell typ. PPAR-γ1 and PPAR-γ2. PPAR-γ2 is expressed exclusively in adipose tissue, while PPAR-γ1 can be expressed in cardiac, hepatic, intestinal or adipose tissue regions (Seiri, Abi & Soukhtanloo, 2019).

In adipocytes, PPAR-γ is responsible for the hydrolysis of triglycerides (TG) by activating lipoprotein lipase. It acts on the absorption of fatty acids through the mediation of adipocyte protein 2, the fatty acid transport protein and the fatty acid transporter CD36 (Ghaben e Scherer, 2019). Furthermore, it exerts functions in the metabolism of macronutrients in an impartial way, through the secretion of hormones such as adipocytokines, such as adiponectin and leptin, and these are correlated with insulin action. Adiponectin in adipose tissue activates the AMP-activated protein kinase (AMPK) pathway to increase insulin-stimulated glucose uptake. It also exerts several functions in inflammation, energy metabolism and cell proliferation through other signaling pathways, such as mammalian target of rapamycin (mTOR) and NF-κB in organs such as liver, muscle and pancreas (Moseti, Regassa & Kim, 2016b; Zhao et al., 2017). In inflammation, PPAR-γ can induce pro-inflammatory genes, such as: tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and interleukin 1 beta (IL-1β), or anti-inflammatory cytokines such as: interleukin 4 (IL - 4), interleukin 10 (IL - 10) and transforming growth factor beta (TGF - β), for stimulating the tissue repair process, inducing neutrophil apoptosis, efferocytosis, macrophage trafficking and activation of alternative M2 macrophages to cease the inflammatory process (Ambele et al., 2020).

The expansion of adipose tissue occurs through the recruitment and differentiation of new pre-adipocytes when there is a need to store excess fat and

occurs through the combination of adipocyte hyperplasia and hypertrophy and is driven by epigenetic and environmental factors. Adipocyte hyperplasia is the physiological form of adipose tissue expansion and when it occurs in an orderly manner, it protects against metabolic diseases, maintaining the normal function of adipocytes and the sufficient storage capacity of lipids within the adipose tissue. On the other hand, when only adipocyte hypertrophy occurs, there is a reduction in adipogenesis, leading to adipose tissue dysfunction and inflammation, culminating in an impaired lipid storage capacity, adverse secretome and crosstalk with other tissues, in addition to etopic accumulation of lipids (Hamarstedt et al., 2018; Kwaifa et al., 2020).

Under physiological conditions, adipocytes have the ability to effectively sequester lipids for their cellular interior, which prevents the accumulation of toxic lipids (lipotoxicity) in other tissues and organs, such as muscle, liver and heart, and this is directly related to preservation of metabolic function and all levels of pathology associated with obesity. In conditions of obesity, it has been shown that approximately 70-80% of individuals carry out disordered remodeling of adipose tissue at both the structural and functional levels, causing an inflammatory reaction (Forte et al., 2020).

Interferences in the hormonal control of adipose tissue functions, such as those caused by the consumption of a diet rich in fructose and saturated fat, can lead to inadequate fat deposits because of obesity. This weight gain alters lipid homeostasis to promote adipogenesis and lipid accumulation, which begin to occur in a disorderly manner, and are due to several mechanisms, such as an increase in the number of adipocytes, the size of adipocytes or changes in the endocrine pathways responsible for controlling development of adipose tissue, such as brain appetite and satiety centers, which then become known as obesogenic interference mechanisms. Changes in hormones that regulate appetite and satiety, as well as food preferences, alter the basal metabolic rate or energy balance, and this favors the storage of energy in the form of fat. Finally, the mechanisms may involve metabolic disorders such as insulin sensitivity, leptin resistance and lipid metabolism in the endocrine system, as well as stimulation of inflammation and oxidative stress (Darbre, 2017; Heindel & Blumberg, 2019).

4.1.4. DIET RICH IN SATURATED FAT AND FRUCTOSE AND AGEs

Advanced glycation end products (AGEs) constitute a group of compounds formed exogenously or endogenously by various pathways in the human body. AGEs

can be divided into two main groups according to their origin: endogenous and exogenous. Most endogenous AGEs are normally formed spontaneously by glycosylation in different tissues of the body, accumulate in the body under physiological metabolic conditions, increase progressively during normal aging, and even more rapidly with metabolic disorders such as hyperglycemia, diabetes, hypertension, cancer, dyslipidemias, and degenerative diseases (Garay-Sevilla et al., 2021; Twarda-clapa et al., 2022). Exogenous AGEs are mainly derived from dietary intake, and foods of animal origin with high fat and protein content have higher AGE levels than foods of plant origin, which have a high content of water, antioxidants and vitamins (Song et al., 2021). In general, they are formed non-enzymatically in the Maillard reaction after heat treatments, by condensation between carbonyl groups of reducing sugars and free amine groups of nucleic acids, proteins or lipids, followed by additional rearrangements producing stable and irreversible final products (Song et al., 2021; Twarda-clapa et al., 2022).

In recent decades, AGEs have aroused the interest of the scientific community due to growing evidence of their involvement in several pathophysiological processes, relationship with hyperglycemia, overweight and chronic diseases, such as diabetes and various inflammatory diseases such as obesity, cardiovascular diseases, metabolic syndrome and neurodegenerative disorders. They are recognized by several cellular receptors and trigger many signaling pathways related to inflammation and oxidative stress (Mouanness e Merhi, 2022; Twarda-clapa et al., 2022).

What studies have demonstrated is that changes in AGE concentrations were positively associated with anthropometric and biochemical changes related to excess weight (Rudman et al., 1981). In women, AGEs can increase ovarian aging by increasing oxidative stress, contributing to damage to ovarian function, initiating bone remodeling and increasing the risk of osteoporosis during menopause. The presence of estradiol and the consumption of natural compounds such as isoflavone have been reported to inhibit the production of AGEs (Twarda-clapa et al., 2022; Yoshikata et al., 2021).

The accumulation of AGEs in the body leads to the activation of several signaling pathways through a series of cell membrane receptors. AGEs activate receptors on the cell surface and induce several biological effects. It has been demonstrated that the AGES receptor (RAGE) is involved in the progression of obesity, correlating with adipose tissue inflammation, adipocyte hypertrophy and insulin

sensitivity. Thus, the RAGE ligand (AGE) is expressed in various cell types, such as adipocytes, macrophages and endothelial cells plays a role in the activation of this receptor and consequently in metabolic disorders. Oxidative stress contributes to the endogenous formation of AGEs. Reactive oxygen species (ROS) involved in oxidative stress can damage proteins, possibly accompanied by the production of more AGEs and increased macrophage infiltration (Song et al., 2021; Van Dongen et al., 2022).

A soluble form of RAGE (sRAGE) has the same AGE binding specificity as RAGE, so it can competitively bind to AGEs, thereby blocking activation of the initiation of the pro-inflammatory signaling cascade and oxidative stress. A high-fat diet increases the levels of RAGE ligands, which are abundant in adipose tissue, such as high mobility group box-1 protein (HMGB1) and carboxymethyl lysine (CML)-AGE, in the liver and adipose tissue (Feng et al., 2021; Garay-Sevilla et al., 2021). RAGE was inversely associated with leptin levels; inhibition of leptin action can increase RAGE expression in beta cells and lead to lower insulin secretion. The activation of RAGE by AGE suppresses the expression of the adiponectin gene through a mechanism dependent on the generation of ROS, and when AGE was prevented from binding to RAGE, adiponectin levels were restored (Garay-Sevilla et al., 2021). RAGE deficiency has been associated with resistance to obesity, increased expression of GLUT4 and adiponectin (Ribeiro et al., 2019).

One of the main mechanisms that inhibit the formation of AGEs are the inhibition of ROS formation, protection of the protein structure and degradation of AGEs. Currently, several natural compounds with antioxidant properties have good inhibitory activity against the formation of AGEs. Natural compounds are chemical substances that are extracted from plants or animals and have distinct pharmacological effects. Natural compounds that potentially inhibit the formation of AGEs are divided into the following six classes based on their structural properties: polyphenols, polysaccharides, terpenoids, vitamins, alkaloids, and peptides. Due to the diverse structures and functions of natural compounds, the mechanisms by which they inhibit the formation of AGEs are also diverse. Current studies suggest that the mechanisms by which AGE formation is inhibited fall into seven groups: covering protein glycation sites, scavenging oxidative free radicals, regulating AGE receptors, trapping active dicarbonyl compounds, chelating metal ions, inhibiting aldose reductase and reducing blood glucose levels (Song et al., 2021).

Therefore, BRS 305 whole sorghum flour, which is a food rich in condensed tannins has a high antioxidant capacity. This antioxidant potential can act by inhibiting the formation of AGEs and/or reducing their coupling to the RAGE receptor, playing a relevant role in inhibiting the formation of AGEs, in a situation of consumption of a diet rich in fat and fructose (HFHF).

4.2. CHAPTER 2

4.2.1. OBESITY AND GUT MICROBIOTA

Obesity is classified based on BMI >30kg/m² and is considered a chronic disease with multifactorial etiology, and the increase in its prevalence has been a cause for global concern. The use of BMI alone as a predictor of obesity is not recommended, as metrics that indicate the individual's adiposity are not adopted to calculate this measure. Therefore, the characterization of obesity itself is simple: excess adiposity (World Health Organization, 2000).

Adipose tissue is an endocrine organ and is responsible for producing adipokines that exert pro- or anti-inflammatory actions, and can also attract immune system cells. The excess fat present in obesity has been associated with adipose tissue dysfunction, compromising adipogenesis, and altering the proliferation/differentiation of adipose tissue. As a result, there is an increase in free fatty acids in the bloodstream, leading to obesity and the development of metabolic disorders. This impaired angiogenesis can lead to tissue hypoxia, which, associated with hormonal disorders, is associated with reactive oxygen species, oxidative stress and an increase in the pro-inflammatory response (increasing cytokines such as TNFα, IL-6 and CRP) and a reduction in the anti-inflammatory response (reduction of adiponectin), culminating in chronic low-grade inflammation. In this sense, in addition to promoting the accumulation of adipose tissue, the inflammatory condition caused by obesity is associated with the occurrence of disorders in lipid and glucose metabolism, exacerbated oxidative stress and a high susceptibility to various diseases, particularly chronic non-communicable diseases, such as cardiovascular diseases, type 2 diabetes mellitus and cancer (Cuevas-Sierra et al., 2019; Delzenne et al., 2011; Gérard, 2016).

Some studies have shown that an imbalance in the intestinal microbiota can contribute to the development of obesity. This happens because in conditions of obesity there is bidirectional signaling within the gut-brain axis, which is mediated by

metabolic, endocrine, neural and immune system mechanisms. The intestinal microbiota is a complex system of organisms, mainly different bacterial species. The interaction between the gut microbiota and the brain, known as “the gut-brain axis (GBA)” is a bidirectional connection through neural, immunological and endocrine pathways. Brain signaling through the autonomic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis influences many gastrointestinal processes, including transit and motility, mucus and fluid secretion, immune activation, intestinal permeability, and relative intestinal microbial abundance and gene expression patterns in certain intestinal microorganisms (Buhmann, Roux, Le & Bueter, 2014; Solas et al., 2017; Son, van et al., 2021).

The gut microbiota composition differs depending on adiposity and body mass. The gut microbial community carries out essential functions, including the synthesis of vitamins, defense against antigens and bacterial intruders, production of short-chain fatty acids (SCFAs), and modulation of the immune system. Moreover, SCFAs such as acetate, butyrate, and propionate are generated by gut microbiota microorganisms by the undigested carbohydrates and soluble dietary fiber, and serves as energy source for colonocytes (Ballini et al., 2020; Forte et al., 2020b; Gasmi et al., 2020). Butyrate, in particular, plays a crucial role in enhancing the integrity of the intestinal membrane by up-regulating the expression of ZO1 and claudin-1, pivotal proteins involved in tight junction formation. This heightened barrier integrity diminishes the passage of bacteria-derived lipopolysaccharides (LPS) into the systemic circulation, consequently reducing insulin resistance and enhancing overall glucose metabolism (Forte et al., 2020).

Further, the host and gut microbiota present a symbiotic relationship so that the host needs of the breakdown of undigested carbohydrates such as polysaccharides and dietary fibers, and the gut microbiota needs of these compounds to survive (Barber et al., 2021; Duca e Lam, 2014). On the other hand, unfavorable changes in the intestinal microbiota composition, known as intestinal dysbiosis are associated with increased intestinal permeability, which can culminate in endotoxemia, due to the passage of endotoxins and pathogens, favoring the inflammatory process promoted by obesity conditions (Weiss & Hennet, 2017). So, disorders on gut microbiota composition activates the adaptive and innate immunity of the intestine and increases the inflammatory level by displacing immunogenic bacterial products (Yoo et al., 2020).

The microbiota-gut-brain axis is extremely important in the management of obesity since the intestinal microbiota can be targeted brain through stimulation of the vague nerve and activation of immuno-neuroendocrine mechanisms (Sen et al., 2017; Torres et al., 2002). The food intake, appetite mechanisms and energy homeostasis are regulated by neuroendocrine signals that provide a reciprocal link between the gastrointestinal tract and the brain. Thus, the altered composition of the intestinal microbiota associated with obesity leads to increased fat deposition, insulin resistance and inflammation, unbalancing energy metabolism and the activity of satiety-related mechanisms (Hamamah et al., 2023; Liu et al., 2021; Suriano et al., 2023).

Associated to obesity metabolic conditions, a diet rich in lipids, cholesterol, sugars and salt, and with a low intake of dietary fiber, vitamins and minerals leads to intestinal dysbiosis in reason of changes in the microbial community due to the fact that different bacterial species use different substrates (Cotillard et al., 2013). Generally, unfavorable bacteria prefer refined carbohydrates as their primary sources of energy (Walker et al., 2011). Furthermore, diets with lipid content culminate in changes in mucosal homeostasis, inducing the wear of the protective layers of the intestinal mucosa and, therefore, increasing intestinal permeability and the levels of pro-inflammatory cytokines (IL-6, TNFα and Nf-κB), exacerbating systemic inflammation. Similar results were found for high cholesterol consumption (Kim e Park, 2012; Murakami et al., 2016; Zhuang et al., 2012).

4.2.3. CHRONIC KIDNEY DISEASE AND GUT MICROBIOTA

Chronic kidney disease (CKD) is a clinical syndrome secondary to definitive changes in renal function and/or kidney structure. It is characterized by its irreversibility and slow, progressive evolution. Another important aspect is that this pathology represents a higher risk of complications and mortality, especially related to cardiovascular events (Charles & Ferris, 2020). The main causes of CKD include diabetes, hypertension, chronic glomerulonephritis, pyelonephritis, chronic use of anti-inflammatories, autoimmune diseases, polycystic kidney, Alport disease, congenital malformations, and prolonged acute kidney disease (Kazancioğlu, 2013).

Two mechanisms can trigger chronic kidney disease: something that works as an initial trigger and the perpetuation mechanism. This initial stimulus may be a basal kidney problem, immune-mediated inflammation or a toxic insult. This renal injury can then be perpetuated by hyperfiltration and hypertrophy of the remaining nephrons.

Possible causes for this perpetuation may be hormones, cytokines or growth factors. This results in an increase in glomerular filtration pressure, thus causing changes in glomerular architecture and structure as well as changes in podocytes, which damages the filtration system. Ultimately, these continuous insults lead to nephron sclerosis and further decrease in kidney function (Charles & Ferris, 2020).

The diagnosis of chronic kidney disease is given to an adult patient when he or she presents, for a period equal to or greater than three months, a glomerular filtration rate (GFR) below 60 ml/min/1.73m², or a GFR higher than 60 ml /min/1.73m² (Bikbov et al., 2020). Some of the indicators of kidney damage are albuminuria, changes in kidney morphology, hematuria/leukocyturia, persistent hydroelectrolyte disorders, histological changes on kidney biopsy and the need for kidney transplantation (Ammirati, 2020).

CKD is categorized into five stages, according to the glomerular filtration rate). These are: I: > 90 ml/min/1.73m² (normal or high filtration); II: 60-89 ml/min/1.73m² (slightly reduced filtration); IIIA: 45-59 ml/min/1.73m² (moderately reduced filtration); IIIB: 30-44 ml/min/1.73m² (moderate to severely reduced filtration); IV: 15-29 ml/min/1.73m² (severely reduced filtration) and V:- < 15 ml/min/1.73m² (kidney failure) (Bikbov et al., 2020).

In general, CKD is very prevalent in the adult population. For example, the prevalence of the disease is higher in females (5.5%) than males (4.2%) and the prevalence is higher in older people, especially older than 70 years and in patients that present comorbities (Santos-Araújo et al., 2023). In the United States was 14.8% among adults in 2018 and has been increasing over time (United States Renal Data System, 2018). In Brazil, the prevalence estimate is still uncertain. In addition to its high worldwide prevalence, CKD is associated with a higher risk of cardiovascular disease, severity, and death. In fact, global data from 2013 showed that the reduction in GFR was associated with 4% of deaths worldwide, which represent 2.2 million deaths (Ammirati, 2020).

Furthermore, CKD results in changes in the composition of the intestinal microbiota and intestinal functionality which, by disrupting the intestinal epithelial barrier, leads to increased intestinal permeability and then to the production of endotoxins, which favors systemic inflammation and associated complications. The exacerbated presence of urea and uremic toxins can alter the intestinal microbiota, promoting dysbiosis and increasing intestinal permeability (Ramezani et al., 2016).

This occurs because there is an increase in the flow of urea into the intestinal lumen, which is then hydrolyzed by microbial urease, producing ammonium hydroxide. This compound increases local pH, which facilitates the growth of pathogenic microorganisms and irritation to the intestinal mucosa (Plata et al., 2019; Vaziri et al., 2013). As a result, endotoxins and other harmful luminal contents enter the tissues underlying the intestine and the systemic circulation, favoring the manifestation of other pathologies (Esgalhado et al., 2020; Plata et al., 2019).

Furthermore, there is evidence that the intestinal microbiota is involved in the development and progression of CKD, leading to inflammation, proteinuria, hypertension and diabetes (KANBAY et al., 2018). The human intestinal microbiota is a complex and dynamic ecosystem, composed mainly of bacteria, although Eukaryotic and Archaeal domains are also present. Three bacterial divisions, namely Firmicutes (Gram-positive), Bacteroidetes (Gram-negative) and Actinobacteria (Gram-positive) dominate the intestinal microbiota of adult humans, together representing more than 90% of all bacteria (Eckburg et al., 2005; Gérard, 2016; O'Hara e Shanahan, 2006; Valdes et al., 2018).

The intestinal microbiota performs essential functions that the human body cannot perform. It is, therefore, critical for maintaining normal gastrointestinal and immunological functions and efficiency in the digestion of bioactive compounds (Valdes et al., 2018). In addition to fermenting non-digestible food components, it synthesizes vitamins and other essential micronutrients, metabolizes toxins and carcinogens in the diet, ensures the maturation of the immune system, affects the growth and differentiation of enterocytes, regulates intestinal angiogenesis and protects against enteric pathogens (Bernalier-Donadille, 2010).

It is estimated that 20 to 60 g of dietary carbohydrates reach the colon daily, including resistant starch, cell wall polysaccharides, and non-digestible oligosaccharides (Adak e Khan, 2019). In this sense, dietary supplementation with prebiotics such as inulin and fructooligosaccharides can promote the growth of specific groups of bacteria beneficial to the body, including bifidobacteria. The phylum Bacteroidetes has many genes that encode carbohydrate-active enzymes, which allows it to switch between different energy sources, other groups of bacteria encode fewer carbohydrate-active enzymes and are notably more specialized (Liu et al., 2021). Furthermore, some food proteins (e.g., collagen and elastin), as well as several plant

metabolites (e.g., phenolic substances) can also reach the large intestine and can undergo bacterial transformations (Bibbò et al., 2016).

Intestinal dysbiosis is associated with diseases, susceptibility to infections and, more recently, several non-intestinal pathologies, including cardiovascular diseases, obesity, diabetes, chronic kidney disease, autoimmune diseases, liver diseases and even brain diseases (Oliveira et al., 2021; Weiss & Hennet, 2017b). Furthermore, it is associated with several proteinuric kidney disorders, such as IgA nephropathy and lupus nephritis. In addition, proteinuric diseases such as treatment-resistant nephrotic syndrome, are a strong factor in the development and progression of CKD (Cigarran Guldris, González Parra e Cases Amenós, 2017).

In CKD patients the intestinal dysbiosis was correlated to an increase in species of the genera Enterobacteriaceae and Pseudomonadaceae of the phylum Proteobacteria, Bacteroidaceae and Clostridiaceae; and a decrease in species of Lactobacillacea, Prevotellaceae and Bifidobacteriaceae (Vaziri et al., 2013). Surprisingly, species that expand in CKD are generally capable of inducing local and systemic inflammation directly and indirectly (Kanbay et al., 2018). On the other hand, the intestinal microbiota can also, through the control of immunological activity, affect the development of idiopathic nephrotic disease syndrome, since the increased activity of regulatory T cells (TREGs) has been shown to reduce them (Omenetti, 2015).

The composition of intestinal microbiota may also contribute to the development of hypertension, especially through changes in the community of microorganisms related to blood pressure control (Dionne et al., 2021) and diabetes, due to intestinal inflammation and subsequent low-level systemic inflammation degree. Factors related to the progression and development of diabetes (Wen & Duffy, 2017) which, in turn, can cause proteinuria via renal arterial hyalinosis and diabetic nephropathy, respectively (Kanbay et al., 2018).

The accumulation of toxic substances during CKD is common, and this accumulation results in numerous symptoms and clinical complications during the treatment of the disease. All those residues that derive from renal failure are called uremic toxins, even though their metabolism or production does not depend on the metabolism of urea or its biosynthesis in the kidney. Among these toxins, the most abundant are p-cresyl sulfate, indoxyl sulfate, free fraction of indole-3-acetic acetic (IAA), homocysteine, uric acid (Vanholder, Glorieux e Smet, 2003). Urea is the main metabolite produced in the kidney and is significantly increased in the plasma of these

patients. Therefore, one of the main metabolic markers of CKD is uremia. The presence of uremia is associated with the degree of kidney damage, dietary restriction and hydration status of kidney patients (Fong, 2008; Tantisattamo & Kalantar-Zadeh, 2020).

Lun et al., (2019) showed that 90 different toxic substances have been described as resulting from reduced kidney function or as consequences of increased urea concentration in the intestinal epithelium (Lun et al., 2019). In CKD patients there are significant quantitative and qualitative changes in the intestinal microbiota, related to the overgrowth of pathogenic bacterial species capable of using nitrogenous products, increasing the production of uremic toxins such as indoxyl sulfate (IS) and p-cresyl sulfate, which in turn induce inflammation and cause pathophysiological impact, resulting in structural and functional changes in intestinal health, which indirectly increase morbidity and mortality in patients, by leading to a failure in the immune system (Anders, Andersen & Stecher, 2013; Poesen et al., 2014).

Exposure of intestinal bacteria to urea, through secretions from the gastrointestinal tract, also results in the conversion of urea to ammonia via bacterial urease. This high concentration of urea causes overgrowth of bacterial families containing urease (Wong et al., 2014). In severe renal failure, the colon becomes the main route of uric acid and oxalate secretion. This may explain the expansion of bacterial families producing uricase and indole and p-cresyl forming enzymes (Kanbay et al., 2018). Changes in appetite and food intake that occur as a result of kidney failure also have effects capable of altering the microbiome. For example, reduced consumption of dietary fiber and resistant starch associated with changes in appetite may result in increased rates of progression of chronic kidney disease (CKD) (Vaziri et al., 2014). In general, the role of whole grains in modulating intestinal health in the face of several chronic non-communicable diseases has been investigated, with promising results (Flint, 2012; Slavin, 2013a). Therefore, the consumption of whole grains, such as sorghum, can help intestinal modulation, as its grains are sources of dietary fiber and other bioactive compounds, such as condensed tannins and resistant starch, which can have beneficial effects on health and the intestinal microbiota. In 2021, sorghum was considered suitable for human consumption by the Brazilian National Health Surveillance Agency (Brasil, 2021).

It is known that pathological conditions can lead to changes in the composition of the microbiota and that, when in intestinal dysbiosis, it can aggravate inflammatory

processes such as and/or favor the emergence of diseases. In this sense, offering foods with prebiotic potential, such as sorghum genotypes BRS305 and SC319 genotypes, can improve the state of intestinal health, modulating the intestinal microbiota, increasing the synthesis of short-chain fatty acids and reducing permeability and intestinal pH.

These changes caused by foods with prebiotic potential can help reduce the inflammatory process and production of toxins and cytokines present in chronic diseases such as obesity and chronic kidney disease. Furthermore, if associated with probiotics, forming symbiotic, they sorghum consumption can promote the growth of beneficial bacteria, favoring markers associated with intestinal health, such as those mentioned above.

So, this study can help fill in gaps regarding beneficial effects on the intestinal and, consequently, metabolic health of individuals with chronic diseases such as obesity and chronic kidney disease, which have a high prevalence throughout the world.

4.3. CHAPTER 3

4.3.1. SORGHUM

Sorghum [Sorghum bicolor (L.) Moench] is a plant from the Poaceae family and is one of the five most cultivated cereals in the world. It is an important source of carbohydrates, calories and proteins in many countries in Africa, Asia and Central America (Altwhab et al., 2015). However, research has shown potential benefits of sorghum consumption on human health, which serves as a stimulant for increasing its consumption by humans in different countries around the world (Bernardo et al., 2019; Cardoso et al., 2015; Lopes et al., 2018a; b; Xiao, Chen & Huang, 2017). It is a plant capable of growing in different agricultural conditions, including dry and arid regions or those that suffer severe temperature fluctuations (Intstormil, 2010; Rosa, 2012). These characteristics make it economically viable, as it can be produced in climatic conditions where the cultivation of other cereals is not adapted, at a lower cost (Bernardo et al., 2019; Prado et al., 2019; Vargas-Solórzano et al., 2014).

Sorghum also appears in a perspective to meet the growing demand for gluten-free foods and drinks, which are consumed by people with celiac disease or those intolerant to this type of protein (Anunciação et al., 2018; Queiroz et al., 2014; Ciacci et al., 2007). In Brazil, the main use of sorghum is still for the preparation of animal

feed and ethanol production, although, in the last decade, several studies carried out in the country, especially led by Embrapa Corn e Sorghum, have demonstrated the potential of this food as well in human nutrition (Anunciação et al., 2018; Arbex et al., 2018; Lopes et al., 2018b, 2019; Martinez et al., 2021a, 2022; Medina Martinez et al., 2021a).

The nutritional composition of sorghum is similar to corn, and contains starch, proteins, lipids, non-starch polysaccharides and phytochemicals, such as phenolic compounds, phytosterols and policosanol, in addition to dietary fiber, resistant starch and micronutrients, including vitamins and minerals (Stefoska-Needham et al., 2015; Taylor & Duodu, 2015). Furthermore, there are different cultivars, and their nutritional composition may vary according to the cultivation soil, as well as genetic variations of the grain, as is the case of sorghum BRS 305. Sorghum BRS 305 is a hybrid that presents, in addition to the macronutrients, a high content of resistant starch and condensed tannins (Martinez et al., 2021a). The structure of the sorghum grain is made up of pericarp, endosperm and germ. The color of the pericarp is controlled by the R and Y genes, which, depending on dominance or recessivity, change the color of the grain, ranging from white to purple (Dykes, 2019). Some varieties of the plant have a fourth structure called pigmented test, located between the pericarp and the endosperm, which is responsible for the presence of condensed tannins. The grain proportion and composition characteristics depend on the genotype and cultivation conditions (Morais Cardoso, de et al., 2017; Wu et al., 2019).

The main bioactive compounds present in sorghum grains are phenolic acids (caffeic and ferulic) and flavonoids (especially 3-deoxyanthocyanidins and condensed tannins). These compounds are responsible for the antioxidant action and are found mainly in the bran (pericarp) of the grains. The tannins present in sorghum are condensed, also known as proanthocyanidins. They are formed by catechins and are only found in varieties that have a pigmented forehead. The presence of tannins is decisive regarding the genotypes of the species, so that type I is the one that does not have a pigmented forehead, consequently it is free of tannins; type II, which has a pigmented forehead and tannins are extracted in low concentration; and type III, which has a pigmented forehead and tannins are extracted in high concentration (Dykes, 2019; de Morais Cardoso et al., 2017).

Sorghum phenolic compounds are divided into two classes: hydroxybenzoic and hydroxycinnamic. Hydroxybenzoic acids are directly derived from benzoic acid,

such as gallic, protocatechuic, p-hydroxybenzoic and vanillic acids. Hydroxycinnamic acids have a C6-C3 structure and include caffeic, p-coumaric, ferulic and sinapic acids. In sorghum, they can be found in free and bound forms, so that when free they are located in the pericarp, pigmented test and aleurone layer and are extracted in organic solvents. When these acids are esterified in the cell wall, a situation most prevalent in sorghum grains, they require extraction in alkaline media (Hahn, 1983; Hahn, 1984).

Most cultivars classified as type III have high molecular weight tannins and have greater antioxidant capacity in vitro and in vivo when compared to simple phenolic compounds. This antioxidant profile is responsible for the grain's anti-carcinogenic and anti-inflammatory properties. Furthermore, high molecular weight condensed tannins complex with other compounds present in the grain and form compounds reducing the digestibility of the grain and starch, and, consequently, reduce its glycemic index (Awika et al., 2009; Fu et al., 2015; Peng et al., 2020).

Sorghum has the lowest starch digestibility among cereals due to the strong association between starch granules and proteins (kaferins) and condensed tannins (Barros, Awika & Rooney, 2012; Maxson & Rooney, 1972; Mkandawire et al., 2015). Overall, most starch granules are slowly digested (30.0-66.2%) and the remainder are rapidly digestible (15.3-26.6%) or resistant (16.7-43.2%) (Mkandawire et al., 2015; Sang et al., 2008). In this context, sorghum has resistant starch and dietary fiber that act as prebiotics, which can modify the intestinal microbiota, favoring intestinal health (Chen et al., 2017; Pelpolage et al., 2019; Slavin, 2013b; de Sousa et al., 2019b; Xiao, Chen e Huang, 2017).

In this context, it is elucidated in the literature that the sorghum nutritional compounds, such as resistant starch, 3-deoxyantocyanins and condensed tannins can exert beneficial biological and physiological effects, such as weight control, reduction of caloric intake, and improvement of lipid and glucose metabolism, as well as improving intestinal health (Martinez et al., 2021b; Medina Martinez et al., 2021a; Oliveira et al., 2020; Pelpolage et al., 2019; de Sousa et al., 2016). In this sense, it is expected that the resistant starch, 3-deoxyanthocins and condensed tannins present in sorghum, enhance the antioxidant response in the brain and adipose tissue and reestablish homeostasis in the satiety control centers of *Wistar* rats fed a rich diet. in saturated fat in fructose; as well as exerting beneficial effects on intestinal health, modulating inflammatory markers and reducing anthropometric measurements in obese adults, as well as when associated with a probiotic, it is capable of leading to a

better glycemic response, increased sensation of satiety and positive modulation of health and intestinal microbiota.

4.3.2. SORGHUM BIOACTIVE COMPOUNDS

The high concentration of bioactive compounds in sorghum, with high antioxidant capacity, which can benefit human health, 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, dyslipidemia, cardiovascular diseases, cancer and hypertension (Anunciação et al., 2018; Lopes et al., 2018c; Martinez et al., 2021b; Medina Martinez et al., 2021a; Morais Cardoso, de et al., 2017; Pelpolage et al., 2019; Sousa, de et al., 2019b). In Brazil, different sorghum cultivars have been developed by the Brazilian Agricultural Research Corporation (EMBRAPA), to improve the sorghum genotype so that its consumption results in health promotion in individuals, highlighting sorghum of the BRS 305 genotype, which has high content of condensed tannins (Moraes et al., 2012) and resistant starch (Teixeira et al., 2016).

The main 3-deoxyanthocyanidins present in sorghum are luteolinidin and apigenidin. Deoxyanthocyanidins are flavonoids with high antioxidant potential and account for up to 79% of sorghum-specific flavonoids. These compounds play an important anti-carcinogenic role, increasing the activity of enzymes that facilitate the elimination of carcinogens and are also responsible for inhibiting pro-inflammatory factors, such as interleukin 1, TNFα and nitric oxide in human mononuclear cells (Sousa, de et al., 2019). Furthermore, in vitro, deoxyanthocyanidins exert antidiabetic effects (Oliveira et al., 2020).

Sorghum grains contain between 12 and 21.5% resistant starch in their chemical composition (Zhu, 2014). This percentage is due to the polymeric proanthocyanidins present in the sorghum grain, which are the main contributors in the process of formation of resistant starch in the grain. This happens due to the strong interaction between these compounds and the available starch, especially amylose, forming resistant starch (Barros, Awika & Rooney, 2014). The term resistant starch was coined by Englyst and Collaborators (1992) to describe the fraction of starch that is not hydrolyzed in the small intestine within 120 minutes and is fermented by the colonic microbiota into short-chain fatty acids. The fermentative process in the colon gives resistant starch properties like those of dietary fiber, resulting in positive

implications for colon health, such as reducing the pH of the intestinal lumen (Fernández et al., 2016; Pelpolage et al., 2019).

Like most cereals, sorghum can be subjected to different heat treatments, such as conventional cooking (wet or dry) and extrusion, and different processing influences the nutritional composition of the grains in different ways, which can also impact health. humanity (Taylor & Duodu, 2015). Regarding the effects aimed at modulating/restoring the antioxidant response in adipose tissue and in the brain, satiety control centers and intestinal health, to date there are no publications in vivo or in clinical studies that investigate the effect of offering an isolated cereal, such as sorghum, which can act as a prebiotic or associated or not with probiotics in the management of obese chronic kidney disease.

4.3.3. SORGHUM HEAT TREATMENT

Sorghum for human consumption requires prior processing and these processes can interfere with its content of bioactive compounds, especially antioxidants. Previous studies evaluated the effect of fermentation, germination and immersion in water of sorghum grains and demonstrated that there is a change in the content of phenolic compounds (Afify et al., 2012; Dicko, et al., 2005).

Cardoso et al. (2013) investigated the effects of different heat treatments on sorghum grains. The authors analyzed the effects of domestic processing with dry heat (F2-oven/grinding; F3-grinding/oven; F4-microwave oven/grinding; F5-grinding/microwave oven; F6-popped grains/grinding) and moist heat (F7-cooking in water/drying/milling) on the antioxidant profile of sorghum flour (F1-raw flour). They observed that both 3-deoxyanthocyanidins and total phenolic compounds were stable to dry heat and reduced in heat treatment with moist heat. Dry heat thermal processing increased vitamin E content and retention, although reduced carotenoid concentrations. This heat treatment allowed the antioxidant activity to remain constant or increase. The opposite effect was observed for thermal treatment with moist heat.

When investigating the content of carotenoids, tocochromanols and the variability in the profile of these compounds in sorghum grains subjected to dry cooking and extrusion, Cardoso et al. (2015a) observed that sorghum genotypes showed high variability in the profile and content of carotenoids and of tocochromanols. They also observed that different sorghum genotypes were sources of vitamins C and E, and among α-tocopherols, the predominant ones were tocochromanols. Both heat

treatments affected the content and retention of tocochromanols, such that the content, retention of tocopherols, and total tocochromanols and α-tocopherol equivalents increased after dry heating in a conventional oven. The content and retention of tocopherols, tocotrienols and, consequently, total tocochromanols and α-tocopherol equivalents decreased after extrusion.

Cardoso et al. (2015b) evaluated the effect of dry heat and extrusion of sorghum grains on the total phenolic content of the grain and observed that the effects of both processes affected the profile of sorghum phenolic compounds with variations according to the genotype, mainly due to differences in flavonoid profiles. In this investigative study, the authors reported that the effect of extrusion cooking showed that sorghum flavones and flavanones are more sensitive to extrusion cooking and dry heat in a conventional oven than 3-DXA and proanthocyanidins. Although thermal degradation was a factor, part of the changes in phenolic content can be attributed to lower extractability after processing. However, other studies demonstrated that the extrusion process increased the total phenol content in sorghum bran, as well as its antioxidant capacity (Cardoso, et al., 2017; Salazar-López et al., 2018). Furthermore, Patel et al. (2015) reported that the extrusion process can reduce the level of bioactive compounds that hinder the digestibility of cereals, thus improving their digestibility.

D'Almeida et al. (2021) investigated the effect of extrusion on sorghum genotypes BRS 330 and SC 319, with or without the addition of turmeric and observed that, although the genotypes present different amounts of condensed tannins, extrusion causes important changes in the chemical and nutritional composition of grains. In this regard, the authors pointed out that after extrusion, the SC 319 genotype showed a 64% reduction in condensed tannin content. The BRS 330 genotype showed a 52% increase in condensed tannin content. The authors also pointed out that thermal processing, depending on the cultivar, can favor not only the degradation of proanthocyanidins into flavonoids, but also induce the polymerization of part of these compounds. Regarding the total phenolic content, the authors pointed out that for the cultivar SC319 there was an increase when subjected to extrusion. The same behavior was observed for antioxidant activity. For the BRS 330 genotype, extrusion caused a reduction in total phenolic content and antioxidant activity. The authors point to the hypothesis of this distinct behavior between cultivars being the proanthocyanidin composition of the first genotype.

4.3.4. HEALTH EFFECTS OF SORGHUM CONSUMPTION

In our research group, Medina Martinéz et al. (2021a), offered BRS 305 sorghum flour, previously subjected to dry cooking, associated with a diet rich in saturated fat and fructose, to young adult male Wistar rats for ten weeks and observed a reduction in serum triglycerides, uric acid, alanine aminotransferase (ALT), level of hepatic steatosis and lipogenesis, as well as improving insulin sensitivity and glucose tolerance and increasing the concentration of PPARα protein in the liver (Medina Martinéz et al., 2021b).

In rats fed a high-fat diet, the consumption of extruded sorghum flour of the SC319 genotype, with brown pericarp, rich in dietary fiber and proanthocyanidins and 3-deoxyanthocyanidins, also improved intestinal dysbiosis by increasing Bacteroidetes and decreasing Firmicutes. Furthermore, inflammatory markers such as the transcription factor NF-κB and the resistin protein had their gene expression reduced. Consumption of sorghum flour also resulted in a reduction in oxidative stress, inhibiting the production of reactive species, contributing to an increase in antioxidant capacity. These effects were observed when sorghum flour replaced 50 and 100% of dietary fiber (de Souza et al., 2019).

In a clinical trial, the consumption of a breakfast meal containing 40 g of extruded SC 319 sorghum to adult men with overweight for eight consecutive weeks, demonstrated that consumption of this sorghum genotype was able to reduce body fat and increase glutathione peroxidase activity (Anunciação et al., 2019). In an experimental model, Llopart et al. (2017) observed that the consumption of sorghum flour (white and red), free of condensed tannins, subjected to the extrusion process, by Wistar rats promoted physiological effects in the body, whether local or systemic. Both flours exerted effects on satiety, reduced body weight, fecal pH and cecal p-glucosidase and p-glucuronidase activity. Red sorghum flour increased the secretion of immunoglobulin A, and the authors relate this result to the higher polyphenol content. White sorghum flour was able to reestablish the homeostasis of the colonic mucosa. Both flours exerted an effect on the antioxidant defense system in the colon, through erythroid-derived nuclear factor 2 (NRF2).

The consumption of sorghum extract administered by gavage (0.4 and 0.6g/kg of weight) by diabetic Wistar rats for six weeks resulted in a reduction in the concentration of triglycerides, total cholesterol and LDL-c, glucose and a reduction in the area under the glucose curve during intraperitoneal glucose tolerance tests to

normal levels. Furthermore, administration of sorghum extract at both concentrations significantly reduced the expression of phosphoenolpyruvate carboxykinase and the phosphorus-p38/p38 ratio and caused an increase in the proportion of protein kinase activated by phosphorus adenosine monophosphate (KIM and PARK, 2012).

Anunciação et al. (2018) observed in humans that ingesting drinks containing of extruded SC319 sorghum 30 minutes before consuming a glucose solution resulted in a lower postprandial glycemic response when compared to a drink without sorghum. The authors also point out that when compared to drinks without sorghum, those with sorghum reduced postprandial glycemia peaks and attribute the results to the content of tannins, resistant starch and the synergistic effect between these components, showing that preparations containing sorghum can be an effective strategy for improving glycemic control.

In this sense, Vanamala et al. (2018) reported in a systematic review that the study and increased understanding of bioactive compounds in sorghum resulted in the discovery of 3-DXAs, anti-inflammatory substances with anticarcinogenic activity. The authors also stated that sorghum extracts rich in phenolics compounds, in addition to the effects an antioxidant effect in in vitro and in vivo studies, becoming a great promise for the treatment of diseases.

In this context, this research turns relevant and important for food and nutrition science, as it demonstrates beneficial health effects promoted by this cereal, in two different genotypes, subjected to different heat treatments. The BRS 305 sorghum genotype presents a high content of resistant starch, dietary fiber and condensed tannins, compounds that can help in the management of diseases such as obesity and associated metabolic disorders, such as disturbances in the activity of markers related to satiety. These metabolic changes are common in conditions of obesity, due to the disordered increase in adipose tissue and increased food intake, demonstrating one of its beneficial effects on health.

4.4. CHAPTER 4

4.4.1. PREBIOTICS, PROBIOTICS AND SYMBIOTICS AND X CHRONIC KIDNEY DISEASE (CKD) X INTESTINAL HEALTH

Probiotics are live microorganisms that, when administered in adequate amounts, can provide health benefits to their host (Hill et al., 2014). Probiotics are developed using individual or multiple live bacterial species (such as lactobacilli and

bifidobacteria) that, after ingestion, can positively modulate the intestinal microbiota (Delzenne et al., 2011). It has been elucidated in the literature that the intestinal microbiota can play a significant role in the development of diseases such as obesity, obesity-associated inflammation and insulin resistance (Bäckhed et al., 2004; Halkjaer et al., 2016). In an experiment carried out with C57BL/6J mice for two weeks, Turnbaugh and colleagues (2006) observed that the composition of the bacterial population in the intestine affected weight regulation. In other animal models (Turnbaugh et al., 2006), associations have been found between changes in intestinal microbiota and obesity, insulin resistance and diabetes (Diamant, Blaak & Vos, de, 2011).

According to the actions exerted by the human intestinal microbiota in humans, we can recognize three main mechanisms of action of probiotics: antagonistic effects on the growth of pathogenic microorganisms and competitive adhesion to the intestinal mucosa and epithelium (antimicrobial activity), increased production of the intestinal mucus layer and reduction of intestinal permeability (barrier function) and modulation of the gastrointestinal immune system (immunomodulation) (Chang et al., 2018; Robles-Vera et al., 2018; Sanchez et al., 2017; Yadav et al., 2017).

Prebiotics include some classes of dietary carbohydrates that are resistant to degradation in the small intestine, but are metabolized by microbes in the colon, where they are fermented into short-chain fatty acids (SCFAs), gases, and other products, which directly or indirectly affect the health of the host (SCOTT and collaborator, 2013). The definition of a prebiotic was cited by Gibson and Roberfroid in 1995 as “a non-digestible food component that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon.” Based on this definition, there is a selectivity considered central to the concept of prebiotic; fibers, such as cellulose, pectins and xylans, promote the growth of various microorganisms, while prebiotics such as fructooligosaccharides and galactooligosaccharides mainly stimulate the proliferation of *Lactobacillus* and *Bifidobacterium* (Quigley, 2019).

Resistant starch is an example of a complex carbohydrate that acts as a prebiotic because it is relatively resistant to intestinal degradation by α amylose. The degree of resistance is dependent on the proportion of amylose and amylopectin in the starch molecule. Crystallinity, molecule size, structure and preparation method (e.g. cooking) also contribute to the digestibility of starch in the diet (Microbiome et al.,

2017). Depending on the type of resistant starch, the intestinal microbiota responds differently, so that there is a tendency for an increase in the Bacteroidetes/Firmicutes ratio in a diet offering type four resistant starch, and an opposite trend after ingestion of a diet containing starch. resistant type two (Upadhyaya et al., 2016; Walker, Ince, et al., 2011; Walker, Sanderson, et al., 2011).

The use of probiotics has been associated with the enrichment of the intestinal microbiota, through improvement of the immune response, restoration of intestinal permeability, and improvement of the anti-inflammatory response as a consequence of the reversal of intestinal dysbiosis, thus offering promise in promoting beneficial effects in patients with CKD (De Araújo et al., 2022).

The administration of prebiotics is associated with the prevention of increased levels of indoxyl sulfate, a compound is generally produced by colon bacteria that express tryptophanasethe. The inclusion of probiotics such as *Bifidobacterium longum* on a prebiotic administration can help reduce serum indoxyl sulfate, since its action is through a “competitive” direct/indirect inhibition/modulation mechanism. These bacteria, promoting the growth of beneficial bacteria, since the administration of probiotics could restore the permeability of the intestinal wall, reducing the absorption of indole into the bloodstream (De Mauri et al., 2022). In addition, Iwashita et al. (2018) offering a symbiotic containing glutamine, dietary fiber (guar gum) and oligosaccharide associated with the *Bifidobacterium longum* strain for elderly male Sprague-Dawley rats submitted to nephrectomy, for 8 weeks. The authors showed that the symbiotic consumption reduced serum creatinine, blood urea nitrogen and serum indoxyl sulfate, demonstrating that the use of symbiotics containing *Bifidobacterium longum* can improve kidney function and can be an adjuvant therapy to the pharmacological treatment of CKD without serious adverse events.

It has been shown that different types of probiotics, used alone or in symbiotic mixtures containing prebiotics, can promote beneficial health effects (e.g., modulation of the intestinal microbiota, decrease insulin resistance, and increased satiety) (Abevonali et al., 2019). More specifically, *Lactobacillus* species (e.g., *L. Casei Shirota* (LAB13), *L. Gasseri, L. Rhamnosus, L. Plantarum*) and *Bifidobacterium* species (e.g., *B. Infantis, B. Longum, and B. Breve B3*) have been used successfully in well-established animal models of CKD due to their low pathogenicity and low level of antibiotic resistance (Cerdó et al., 2019).

Cruz-Mora et al. (2014) offered to adult hemodialysis patients for 2 months, a symbiotic gel contained a mix of probiotics (*Lactobacillus acidophilus* and *Bifidobacterium bifidum*), containing 2.0 x 1012 colony-forming units (CFU); 2.31 g of a prebiotic fiber (inulin); 1.5 g of omega-3 fatty acids (eicosapentaenoic and docosahexaenoic acid) and vitamins (complex B, folic acid, ascorbic acid, and vitamin E). The authors observed a significant increase of *Bifidobacterium* and a reduction of *Lactobacillus*, without alter gastrointestinal symptoms. The analysis of the composition of gut microbiota considering alpha and beta diversity did not show significant difference in the bacterial groups (Cruz-Mora et al., 2014).

Lopes et al. (2018a; 2018b), were the firsts to offer a symbiotic meal containing sorghum and *Bifidobacterium longum* for CKD patients. The intervention consisted to offer a symbiotic meal containing 40 g of hybrid sorghum BRS 305, which is a cultivar rich in condensed tannins and resistant starch, previously subjected to extrusion, plus the probiotic *Bifidobacterium longum BL-G301* in concentration of 9.06 × 108 ± 5.4 × 108 CFU/100 mL, for chronic kidney patients for 7 weeks. The authors observed that there was an improvement in the inflammatory and antioxidant response, as well as a reduction in serum p-cresyl sulfate, indoxyl sulfate and the concentration of urea. It was also observed that the urea concentration was positively correlated with the serum concentration of p-cresyl sulfate and fecal pH.

Since intestinal dysbiosis plays a significant role in the development and progression of chronic kidney disease, contributing to systemic inflammation and increased concentration of uremic toxins, the use of probiotics, by providing a healthy intestinal environment, has been associated with positive effects on health of chronic kidney patients. Furthermore, the resistant starch present in sorghum can be fermented by microorganisms in the colon, increasing the production of short-chain fatty acids, reducing luminal pH, improving intestinal permeability, and when taken together they can favor the composition of the intestinal microbiota. In this sense, it is relevant to research the effects of combining sorghum BRS305 with the probiotic *Bifidobacterium longum*, since by improving intestinal health, it is possible to improve the health status of patients with chronic kidney disease.

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4. GENERAL METHODOLOGY

The thesis was conducted in 3 studies: The first one (paper 1) was performed in a *in vivo* experiment and the second (paper 2) and third (paper 3) had distinct clinical trials (Figure 1).

Our research was developed and offered BRS305 sorghum submitted to dry heat or extrusion treatment. The first paper, with experimental design, include two phases and in the first phase, metabolic disorders were induced, including insulin resistance and adiposity offering a high fat high fructose diet (HFHF) or pattern diet (AIN93M) diet for male adults *Wistar* rats during eight weeks (adapted from MARINELI et al., 2015). In the second phase the HFHF group was divided in HFHF (n=8) and HFHF + sorghum (n=8), and the sorghum amount was defined to substitute 50% of the dietary fiber recommendations to rodents. The control group continued receiving the AIN93M diet (n=8) (REEVES et al., 1993), and this phase (treatment phase) occurred by 10 weeks (Paper 1). This study was approved by Animal Use Ethics Committee of the Federal University of Viçosa (CEUA/UFV) by protocol number 38/201 (ANNEX 1).

Study 1:

Paper 1: Dry heat whole Sorghum BRS 305 flour modulate satiety and improves antioxidant response in brain of Wistar rats fed with high-fat-high-fructose diet.

The research involved animal model analyzes included quantification of condensed tannins, analysis of antioxidant capacity and content of total phenolic compounds, analysis of the 3-deoxyanthocyanins profile, analysis of food and anthocyanin consumption, brain weight, calculation of adiposity, gene expression of following markers in adipose tissue: peroxisome proliferator-activated receptor gamma (PPAR-γ), sirtuin 1 (SIRT-1), adiponectin, leptin and resistin, endocannabinoid receptor 1 (CB1), leptin, leptin receptor (LEP-r). In the brain, gene expression analysis was conducted for the enzyme superoxide dismutase (ZnSOD), nuclear factor erythroid-derived 2 (NRF2), 70 kDa heat shock protein 1 (HSP72), alpha-type peroxisome proliferator-activated receptor (PPARα). The relative expression of mRNA levels was normalized by the endogenous β-actin control for rats, using the 2-AACT method (LIVAK, 2001). Furthermore, AGE and RAGE proteins were also quantified using an ELISA kit, namely: Rat AGE ELISA kit (Bioassay Technology Laboratory®, Zheijang, Korain) and Rat RAGE ELISA kit (Bioassay Technology Laboratory®, Zheijang, Korain), respectively. Absorbance was measured by spectrophotometer (Thermo Scientific® model Multiskan GO) at a wavelength of 450 nm. To analyze the

antioxidant response in the brain, analysis of the activity of superoxide dismutase (Marklund, 1985) and catalase (AEBI, 1984) was performed. Total protein in brain homogenate was quantified by the method of Bradford (1976).

Study 2:

Paper 2: Consumption of Extruded Sorghum SC319 Improved Gut Microbiota at Genus Level and Reduced Anthropometric Markers in Men with Overweight: A Randomized Controlled Clinical Trial

The research was conducted with overweight or obesity man and was an 8-week, single-blind, controlled, randomized nutritional intervention study conducted in men with overweight with age between 18 and 40 years. This study was conducted using data from the second phase of a crossover study previously conducted by our research group. Only men were included in the study for two main reasons: the first is that males do not have as many hormonal changes as females, especially related to the menstrual period. The second reason is the fact that a previous experimental study investigating the consumption of sorghum in male *Wistar* rats fed a diet high in saturated fat (SFA), so this study can be considered a sequel. The volunteers were divided in two groups: the control group, that consumed daily the test preparations with 38g of wheat or 40g of extruded SC319 sorghum. During the intervention, they consumed the preparations in three forms: breakfast cereal with milk, dairy product, or a drink at breakfast and guided to follow a 500kcal/day caloric restriction diet. Thus, preparations were offered to both groups in the form of breakfast cereal (extruded sorghum x wheat) added with whole milk or light whole yogurt, or drink (extruded flours were mixed with skimmed milk powder, powdered juice. and sweetener). Variation between meal types (breakfast cereal/drink) was defined to increase adherence to the intervention. The amount of sorghum consumed daily was based on a usual portion of breakfast cereal (40g) and on the volume of sorghum that the subjects could ingest in a meal based on previous tests. The amount of extruded wheat (38g) was adequate the same nutritional composition of sorghum (40g). The anthropometric assessment was performed with body weight, body fat percentage, body mass index, waist circumference, waist/height ratio anthropometric measures at baseline and endpoint for both groups. Blood samples was collected for analysis of inteleucin-6, interleucin-10 and tumor necrosis factor alpha inflammatory markers at baseline and endpoint. The fecal content was collected for intestinal markers analysis at the beginning and end of the trial. Were performed analysis of short chain fatty acids (totally volatile acids,

acetic, propionic and butyric) production as well as intestinal microbiota (polymerase chain reaction and quantification of 16S RNA), to investigate gut microbiota composition using alpha diversity, beta diversity and predictional function (KEGG and LeFSE) analysis. The study was approved by the Human Research Ethics Committee of the Federal University of Viçosa, Brazil (CAAE: 13630513.0.0000.5153) (ANNEX 2).
Study 3:

Paper 3: A Symbiotic Meal Containing Extruded Sorghum and Probiotic (*Bifidobacterium longum*) Ameliorated Intestinal Health Markers in Individuals with Chronic Kidney Disease: A Secondary Analysis of a Subsample from a Previous Randomized and Controlled Clinical Trial.

The research was conducted with chronic kidney patients with age between 18 and 65 years in the hemodialysis sector of Hospital São João Batista, Viçosa-MG. This is a controlled, randomized and single-blind clinical trial. The participants were characterized in terms of clinical and sociodemographic aspects and were then randomly divided into two groups - control group (CG) and symbiotic group (SG). The intervention took place over 7 weeks, respecting the blood collection routine of the hemodialysis service. The study protocol was approved by the Human Research Ethics Committee of the Federal University of Viçosa, Brazil (protocol number 701.796/2014) (ANNEX 3).

On hemodialysis days, the participants of control group received a food kit containing pasteurized milk - LP (100 mL) and extruded corn flakes (40g) and the intervention group received a kit with the probiotic dairy drink - BLP (100 mL), with the *Bifidobacterium longum* strain (4 x 10^8 CFU/100 mL) and extruded sorghum flakes (40g). The volunteers underwent an anthropometric assessment, including weight, height and body mass index at beginning and end of the study. The food intake analysis was performed using three 24-hour dietary recall, considering one day of hemodialysis, one interdialytic day and one weekend. The Dietpro® nutrition software system (version 5i) was used to assess the intake of nutrients. The intestinal symptoms (present constipation, nausea, heartburn, bloating, intestinal gas, diarrhea or belching) were accessed by DIXSON Questionnaire and Bristol Scale. Blood samples were collected at baseline and endpoint to analyze uremic marker that included p-cresyl sulfate, indoxyl sulfate and acid-3-indole acetic. Feces for analysis of short chain fatty acids (acetic, propionic and butyric) as well as intestinal microbiota composition were collected at the beginning and end of the trial.

For study 1 (paper 1), the data normality was verified using Shapiro-Wilk normality test. The data that did not showed normal distribution were transformed into Log^{10} for parametric statistical analysis. The data from this research were submitted to Analysis of Variance (ANOVA) followed by post-hoc Newman Keuls at 5% probability. In addition, was performed Pearson's correlation analysis to check the correlation between variables.

On study 2 (paper 2), for body composition (body weight, body fat percentage, body mass index, waist circumference, waist/height ratio), inflammatory markers (inteleucin-6, interleukin-10 and tumor necrosis factor alpha), organic acids (total volatile acids, propionic, butyric and acetic), fecal pH, and reverse transcriptase reaction (PCR), the statistical analysis was performed using SPSS 20.0 software. The normality of the data was assessed by Shapiro-Wilk test. The average of each variable at the beginning and end of the intervention were compared using paired t test or Wilcoxon. The difference between the averages of the variables for each group at the beginning and end of the intervention periods were compared using the Student t test or Mann-Whitney. Averages and times were submitted to ANOVA followed by Newman-Keuls post-hoc test or Kruskall-Wallis followed by Dunn's post-hoc test. Cohen's d effect size was calculated from the difference between the means of the groups, divided by the mean of their SD. The magnitude of the effect was quantified as null (Cohen's $d < 0.19$), small (Cohen's $d = 0.2$ to 0.49), medium (Cohen's $d = 0.5$ to 0.79), large (Cohen's $d = 0.8$ to 1.29), and very large (Cohen's $d > 1.30$).

For the analysis of microbiota sequencing data on studies 2 and 3, the data were initially subjected to the Kolmogorov-Smirnov normality test. The Chao, Shannon and Simpson indices were used to estimate alpha diversity. To evaluate the grouping of samples, Principal Coordinate Analysis (PCoA) was performed, based on the Bray-Curtis dissimilarity index and similarity test for non-parametric data (ANOSIM, permutation number = 1000) using the Past software (HAMMER et al., 2001). Functional prediction analysis was conducted using PiCRUST 2.0. The data were corrected using the FDR (false discovery rate) criterion in the STAMP software. Statistically significant p values associated with microbial clades and functions identified by LEfSe were corrected by Benjamin FDR correction.

From study 3 (paper 3), the data set was tested for normality by the Kolmogorov-Smirnov test, and parametric data were submitted to ANOVA followed by Tukey post-hoc, for multiple comparison. Non-parametric and independent data were submitted to

the Kruskal-Wallis test followed by the Mann-Whitney test, for multiple comparisons. T tests were applied to compared baseline and endpoint results of each group.All statistical analyzes of this research were performed using GraphPad version 9.0 software. The significance level established was 5% (α=0.05).

5. THESYS PUBLICATION

The initial review and the experiments performed in the present thesis were published between 2021 and 2022 as 3 original papers (Table 1).

Table 1. Description of papers published in the thesis.

Paper type	Paper title	Journal/ Impactor factor	Year of Publication
Original paper 1	Dry heat whole Sorghum BRS 305 flour modulate satiety and improves antioxidant response in brain of Wistar rats fed with high-fat high-fructose diet	Food Research International / 7.425	Published in 2023
Original paper 2	Consumption of Extruded Sorghum SC319 Improved Gut Microbiota at Level Genus and Reduced Anthropometric Markers in Men with Overweight: A Randomized Controlled Clinical Trial	Nutrients / 6.706	Published in 2023
Original paper 3	A Symbiotic Meal Containing Extruded Sorghum and Probiotic (Bifidobacterium longum) Ameliorated Intestinal Health Markers in Individuals with Chronic Kidney Disease: A Secondary Analysis of a Subsample from a Previous Randomized and Controlled Clinical Trial	Nutrients / 6.706	Published in 2024

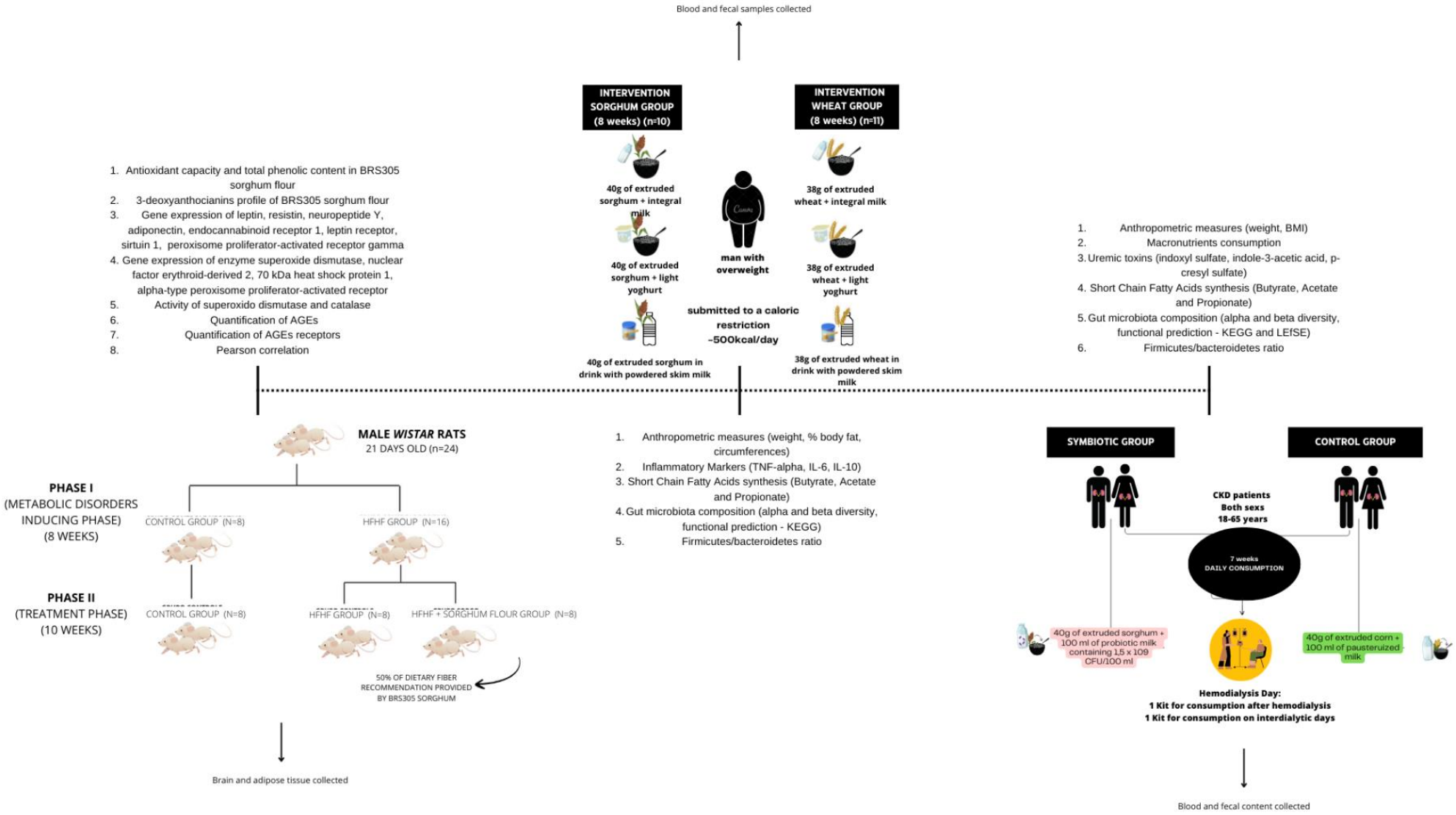

Figure 1. Flowchart of the general methodology of the thesis.

6. ORIGINAL RESEARCH RESULTS

6.1. PAPER 1

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Dry heat whole Sorghum BRS 305 flour modulate satiety and improves antioxidant response in brain of *Wistar* rats fed with high-fat high-fructose diet

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High-fat high-fructose diet

ABSTRACT

Sorghum BRS 305 (*Sorghum bicolor* L. Moench) is a cereal with high tannins and anthocyanins content and keep better the resistant starch when submitted to dry heat treatment. Our objective was to investigate the effects of BRS 305 dry heat treatment whole sorghum flour on satiety and antioxidant response in brain and adipose tissue of *Wistar* rats fed with a high fat high fructose diet (HFHF). Male *Wistar* rats were divided in two groups: control (n = 8) and HFHF (n = 16) for eight weeks. After, animals of HFHF group were divided: HFHF (n = 8) and HFHF + BRS 305 sorghum whole flour (n = 8), for 10 weeks. Sorghum consumption reduced gene expression of leptin, resistin, and endocannabinoid receptor 1 type (CB1) in adipose and brain tissues compared to HFHF group. In brain, sorghum consumption also promotes reduction in neuropeptide Y (NPY) gene expression. BRS305 sorghum consumption improved gene expression of sirtuin-1 (SIRT1) in adipose tissue, and in the brain increased

1. INTRODUCTION

The western dietary pattern diet, rich in saturated fats, sugars and foods poor in dietary fiber, vitamins and minerals are directly related to development of chronic diseases, such as obesity, chronic kidney disease, diabetes mellitus and arterial hypertension, especially when associated with a sedentary lifestyle (Christie et al., 2020; Khoshbin & Camilleri, 2020; Marventano et al., 2018; Vandevijvere et al., 2015). Further, it has been appointed that excessive weight gain and adiposity is associated with a reduction in feeding control by an interoceptive hunger and satiety contextual stimuli leading to a enhance in food consumption (Christie et al., 2020; Christie et al., 2020).

Resistin and leptin are hormones related to satiety control, which are responsible to influence the food intake since the leptin can reduce the food intake and resistin can reduce the glucose uptake by cells. These hormones can be controlled by many factors, including the receptors in the arcuate nucleus of the brain, such as type 1 endocannabinoid receptors (Di Patrizio, 2021; Feinle-Bisset, 2014). In addition, the consumption of a diet rich in fat and/or fructose is associated with leptin resistance and hyperactivation of type 1 endocannabinoid receptors, that in turn, is associated with the increase on food intake, driving to metabolic changes, obesity (Drori et al., 2020; Silvestri & Di Marzo, 2013), systemic oxidative damage, and inflammation (Saiyasit et al., 2020).

In addition to effects in satiety centers and markers, the western diet leads to a high lipid deposition, which is associated with high levels of reactive oxygen species (ROS), such as superoxide anions, hydroxyl radicals, and hydrogen peroxide (H_2O_2) (Li et al., 2021; Maurizi et al., 2018) that can affect all tissues of the organism. The brain is an organ highly vulnerable to oxidation due to presence of large amounts of unsaturated fatty acids that can suffer lipid peroxidation by ROS (Batandier et al., 2020; Derosa et al., 2018; Langley et al., 2020; Shal et al., 2018). Furthermore, central nervous system is adversely affected by high levels of circulating free fatty acids, advanced glycation end products (AGEs), and high blood levels of triglycerides or cholesterol (Langley et al., 2020; Li et al., 2021). These fatty acids can interact with many transcription factors, such as peroxisome proliferator-activated receptor alpha (PPARα), which acts by activating lipid regulatory proteins (Polley et al., 2019).

Previous studies of our research group have shown that the consumption of the HFHF diet leads to changes in oxidative stress as well as on glucose metabolism, causing

hyperglycemia and insulin resistance (Martinez et al., 2021a; Martinez et al., 2021b). Then, the consumption of sources of antioxidant and anti-inflammatory compounds is alternative to maintain the brain health (Ali et al., 2018; Henriques et al., 2020; Si et al., 2021). In this context, sorghum consumption can be an alternative due to be a source of energy, carbohydrates, proteins, and bioactive compounds, mainly flavonoids and condensed tannins. For sorghum, the dry treatment heat keeps resistant starch concentration (Teixeira et al., 2016), due to its ability to increase satiety feelings. Further, it together with kaferins concentration in BRS 305 whole sorghum flour can promoting satiety and restoring the physiological activity of receptors and proteins that act as satiety regulators (Barros et al., 2012).

Heat treatment of sorghum grains is necessary for human consumption. Thermal treatment with dry heat allows 3-deoxyanthocyanidins and total phenolic compounds to remain stable (Bianco-Gomes et al., 2022; Cardoso et al., 2014). Thus, the aim of this study is to investigate bioactive properties of dry heat BRS 305 whole sorghum flour on satiety, adipogenesis and antioxidant response in brain and adipose tissue of *Wistar* rats fed with a high fat and high fructose diet (HFHF). We hypothesized that whole BRS 305 sorghum rich in resistant starch, tannin and anthocyanins added to HFHF diet may decrease leptin resistance, metabolic disorders, the hyperactivation of type 1 endocannabinoid receptor, in addition to improve the antioxidant capacity, and metabolic disorders promoted by AGEs, *in vivo*.

2. METHODS AND MATERIALS

2.1. Materials

BRS 305 sorghum grains is a hybrid type and have a brown pericarp, high tannin content, and resistant starch. Grains harvest in 08/2015 were provided by Embrapa Milho e Sorgo (Sete Lagoas, MG, Brazil) (-19.466672726578615ºS, -44.17357630467641ºW). The grains were selected, sieved to remove impurities, and submitted to dry heat treatment using an oven with air circulation at 105ºC for 30 minutes (Moraes, De *et al.*, 2006). The grains were ground in a knife mill (Brabender, Duisburg, Germany) with a 1.0 mm stainless steel sieve mash (Medina Martinez et al., 2021) and this flour were kept at -20ºC until used.

2.2. Dry heat BRS 305 sorghum flour chemical composition

The chemical composition of sorghum flour obtained from grains under dry heat treatment for protein, ash, total dietary fiber, insoluble dietary fiber, soluble dietary fiber, resistant starch, condensed tannins, total phenolics compounds and antioxidant capacity is showed on table 2**.**

2.2.1. Sorghum chemical composition of macronutrients

BRS305 sorghum flour obtained from grains under dry heat treatment contains 9.09% of moisture, 66.61% of total carbohydrate, 12.97% of proteins, 5.01% of lipids and 1.13% of ash (Martinez et al., 2020).

2.2.2. BRS 305 sorghum dietary fiber content

The total dietary fiber content of BRS 305 sorghum flour were perfomed according to AOAC Method 985.29 (AOAC, 2019). To simulate the digestion step, duplicate samples of BRS 305 sorghum flour were incubated with pancreatic α-amylase for 30 minutes at 95°C in pH = 6.0 to starch hydrolase. After, we changed the pH to 7.5, added the protease and incubated the sorghum samples at 60ºC for 30 minutes. At least, we changed the pH to 4.5, added the amyloglucosidase and maintained the sorghum samples incubated at 60ºC for 30 minutes. In all these steps the samples were mixed vigorously to maintain continuous suspension. For the measurement of IDF, the digestate is filtered and the IDF is determined gravimetrically after correction for protein and ash in the residue. For the measurement of the soluble fiber, ethanol is added to the filtrate of the insoluble fiber and the precipitated of soluble fiber is captured by filtration and determined gravimetrically after correction for protein and ash. Total dietary fiber is measured adding insoluble and soluble fiber.

2.2.3. BRS 305 sorghum flour resistant starch content

The analysis of resistant starch was conducted following AOAC Method 2009.01 in flour after dry heat treatment of sorghum grains. involved assessing its content (RS) by subjecting it to simulated digestion with pancreatic α-amylase and amyloglucosidase. For this, a commercially available kit (Resistant Star Assay Kit AACC 32–40, Megazyme) was utilized, following the manufacturer's guidelines. Thus, 0.1g of sample were put, in duplicate, in falcon tubes and were added 4ml of pancreatic α-amylase containing amilogycosidase. The falcon tubes were sealed with parafilm, vortexed and incubated in a water bath at 37ºC, aligned in the direction of movement, under agitation level 3, for 16h. When removed from the water bath, 4ml of 99% ethanol was added and the samples were vigorously stirred on a vortex. Then, the sample was centrifuged at 3000prm for 10 minutes and then the supernatant was discarded. Then, the sample was resuspended twice using 50% ethanol. 2ml of 2M KOH was added to dissolve the undigested starch, and the samples were placed in a shaker homogenizer for 20 minutes to resuspend the pellet. After this time, 8ml of sodium acetate solution (pH=3.8) and 0.1ml of amyloglucosidase were added. After homogenization, the tubes were placed in a water bath at 50°C for 30 minutes, with vortexing every 10 minutes. At the end of this step, the samples were centrifuged for 10 min at 1500rpm. 0.1ml of each duplicate was transferred to test tubes, and 0.1ml of glucose was added. For the blank, 0.1ml of phosphate buffer was added. Finally, 3ml of GOPOD solution was added to each tube, incubated at 50ºC for 20 minutes, and the reading was performed in a Multiskan Go® spectrophotometer at 510nm.

2.2.4. Condensed tannins

The first step was the extract production. For this, 0.5g of dry heat sorghum flour were added to a 25ml of solution containing 1% of HCL and 99% of mannitol and maintained under agitation for 16 hours. After this time, the samples were centrifugated for 10min at 1008G. Condensed tannins in sorghum flour were analyzed using the vanillin/HCl reaction method, according to Maxson and Rooney (1972) and Price, Scoyoc and Butler (1978). 1 mL of the previously prepared extract was pipetted into test tubes and 2.5 mL of 1% vanillin solution in methanol and 2.5 mL of 8% HCL solution in methanol were added. The tubes were kept at rest for 20 min and the absorbances read at 500 nm.

The blank was made using 1 mL of the extract and five milliliters of 4% HCL in methanol. An analytical catechin curve was constructed where the compound was diluted in methanol. Aliquots of 0 mL, 0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL and 1 mL were taken from the concentrated solution and the volume adjusted to 1 mL in tubes using methanol and added to each tube. 2.5 mL of 1% vanillin solution in methanol and 2.5 mL of 8% HCL solution in methanol. Absorbances were read at 500 nm and results expressed in milligram equivalent of catechin per gram of sample (mg EC/g of sample).

2.2.5. Total phenolics compounds

2.2.5.1. Extract production

To prepare the extract, 1.0g of the sample was weighed in a falcon tube and suspended in an extracting solution (4ml of 50% methanol, 4ml of 70% acetone and 2ml of water) (10mL). The tubes were placed on an automatic shaker for 16 hours for extraction. Subsequently, the solution was centrifuged for 10 minutes at 3000g. The supernatant was transferred to another tube and stored under refrigeration until analysis. This extract was used to determine total phenolic concentrations and antioxidant activity.

2.2.5.2. Total Phenolics Analysis

The total phenolic compounds of dry heat sorghum were determined using the Folin-Ciocalteau reagent (SINGLETON et al., 1999). For analysis, 500 µL of extract prepared for the determination of antioxidant activity were added to 500 µL of 20% Folin-Ciocalteau solution and 500 µL of 7.5% sodium carbonate solution. Then, the solution was vortexed and allowed to stand for 30 min at room temperature (25°C).

The absorbance reading was performed in a spectrophotometer (Thermoscientific, Evolution 606, USA) at 765 nm. Quantification was performed using an analytical curve obtained by reading the absorbance of solutions with different concentrations of gallic acid. Results were expressed in milligrams of gallic acid equivalents per gram of sample (mg GAE/g).

2.2.6. Antioxidant activity analysis

The antioxidant activity of dry heat sorghum was determined by the ability to scavenge the free radical DPPH (2,2-diphenyl-1-picryl-hydrazyl), according to the methodology described by Bloor (2001). Initially, the absorbance of the previously prepared DPPH solution (0.1 mM) was read. This absorbance was recorded to standardize the control solution. 200 µL of the extract previously prepared how described in topic 2.2.6.1. and diluted (1:5 ratio), were pipetted into tubes and 1.5 mL of the DPPH solution (0.1 mM) was added. The tubes were shaken and then left to rest for 30 min in the dark. The solution used to make the flour extract was used as a blank. After resting, the reading of the samples and the blank was carried out in a spectrophotometer at 517 nm. The results were expressed in % SRL (free radical scavenging).

2.3. BRS305 Sorghum flour 3-deoxyanthocyanins profile

Sorghum 3-deoxyanthocyanins were determined by high performance liquid chromatography (HPLC) (Yang *et al.*, 2012; Yang, Browning e Awika, 2009), with modifications. Samples were extracted with acidified methanol (1% HCl), centrifuged and an aliquot of the extract was injected into high performance liquid chromatography (HPLC) and anthocyanins (luteolinidine, apigeninidine and methoxylated derivatives) was detected at a wavelength of 480 nm. Results were expressed in micrograms of compound (µg)/gram of sample on a dry basis.

2.4. Experimental design and diets

According with sample calculation, eight animals for group were required (Fontelles et al., 2010.; Theodoro et al., 2021). This study was approved by Animal Use Ethics Committee of the Federal University of Viçosa (CEUA/UFV) by protocol number 38/2019 and the animals were obtained from Central Bioterium of Biological and Health of UFV, Brazil. All experimental procedures with animals were carried out under ethical principles in animal experimentation.

Animals were randomized by body weight into two groups: high-fat high-fructose diet (HFHF) (n=16) (31% of saturated fat and 20% of fructose), to induce metabolic disorders (Medina Martinez *et al.*, 2021a; Theodoro *et al.*, 2021) and control group fed

with AIN-93M diet (Reeves et al., 1993) (n=8), by eight weeks. After this period, they were divided in two groups: HFHF (n=8) and HFHF added by sorghum BRS 305 whole flour (n=8) by 10 weeks. Animals of control group (AIN-93M) received the same diet since the beginning of the experiment. The amount of sorghum was enough to replace 50% of total dietary fiber content of diet, corresponding to 195.4 g/kg of diet (Table 1). This amount of sorghum flour provided nutrients that replaced 100% of corn starch, 22.5% of soybean oil, and 19.8% of protein, so that diet was isoproteic, isocaloric, and isoglycidic. At the end of experimental procedures (18^{th} weeks), animals were anesthetized with Isoflurane® (Isoforine, Cristália) and euthanized using cardiac punction. The brain and adipose tissue (epididymal, visceral, and retroperitoneal) were collected, weighed, immediately frozen with liquid nitrogen, and stored at -80°C for subsequent analysis.

Table 1. Experimental diets composition.

	Experimental diets		
Ingredients (g/kg)	**AIN93-M**	**HFHF**	**HFHF + Sorghum****
Albumin*	136.60	136.60	108.80
Maltodextrin	155.00	45.00	46.50
Corn Starch	463.30	134.66	-
Sucrose	100.00	28.64	31.00
Soybean Oil	40.00	40.00	31.10
Sorghum Whole Flour	-	-	195.40
Lard	-	310.00	310.00
Fructose	-	200.00	200.00
Cellulose	55.80	55.80	27.90
Mineral Mix	35.00	35.00	35.00
Vitamin Mix	10.00	10.00	10.00
L-cystine	1.80	1.80	1.80
Cholin Bitartarate	2.50	2.50	2.50
Macronutrients			
Protein (%)	11.98	11.95	11.94
Carbohydrate (%)	73.22	42.27	40.57
Lipids (%)	3.98	34.81	35.00
Caloric Density (kcal/g)	3.78	5.33	5.25
Anthocyanins (ng/mg)			
Total anthocyanins	-	-	6.14
Luteolindin chloride	-	-	2.15
Apigeninidine chloride	-	-	0.19
5-Metoxi luteolindin	-	-	3.00
5-Metoxi apigeninidine	-	-	0.79

*Albumin based on 88% protein content. AIN-93M: diet formulated for maintenance of adult rodents.; HFHF: diet high in saturated fat and fructose; HFHF + Sorghum Flour: HFHF added with extruded sorghum flour. ** Chemical composition of sorghum flour obtained from grains under dry heat treatment in g/100g: total carbohydrates: 66.61g; lipids: 5.01g; protein: 12.97g; ash: 1.13g; moisture: 9.09g (Martinez et al., 2020), available carbohydrates: 30.21g; resistant starch: 20.90g total dietary fiber: 15.50g; insoluble dietary fiber: 15.19g; soluble dietary fiber: 0.30g.

2.5. Food consumption and biometric measurements

Weight gain was measured following the equation: (final weight – initial weight). Food consumption was measured weekly, and the adiposity was calculated by the ratio of the percentage of fat and the body weight multiplied by 100 (Pereira et al., 2012). Lee index was calculated by the formula: $\sqrt[3]{body\ weight} \div$ naso-anal length x 1000 (Novelli *et al.*, 2007).

2.6. Gene expression related to satiety and antioxidant response in the adipose and brain tissues

The gene expression was analyzed by the RealTime PCR System (RT-qPCR). Briefly, the adipose tissue (200 mg) was crushed in liquid nitrogen and mRNA was extracted using the mirVanaTM miRNA Isolation Kit (mirVanaTM miRNA Isolation Kit, Ambion, Life Technolgies) according to the manufacturer's protocol. A total of 200 mg of brain were macerated in liquid nitrogen and homogenized in TRIzol reagent. The extracted mRNA was quantified in the spectrophotometer Multiskan™ GO (ThermoFisher Scientifics; Waltham, MA, EUA) and 2 µg of mRNA was used to synthesize 1.5 µg of complementary DNA (cDNA), using an M-MLV reverse transcription kit (Invitrogen Corp., Grand Island, NY). For detection, the Master Mix SYBR Green Fluorescence PCR was used and sequences of sense and antisense oligonucleotides (Supplementary Materials - Table 1). The relative expressions of mRNA levels were normalized by endogenous control β-actin.

In adipose tissue were analyzed the following mRNA protein: peroxisome proliferator-activated receptor gamma (PPAR-γ), sirtuin 1 (SIRT-1), adiponectin, leptin and resistin. In brain were analyzed: endocannabinoid receptor 1 (CB1), leptin, leptin receptor (LEP-r), enzyme superoxide dismutase (ZnSOD), erythroid-derived nuclear factor 2 (NRF2), heat shock protein 72 (HSP72), peroxisomal proliferator-activated receptor alpha (PPARα).

Supplementary Table 1. Sequence of oligonucleotides used for RT-qPCR related to satiety and oxidative stress in adipose tissue and brain.

Gene	Sense (5'- 3')	Antisense (5'- 3')
	Adipose Tissue	
Adiponectin	AATCCTGCCCAGTCATGAAG	CATCTCCTGGGTCACCCTTA
SIRT-1	ACAGTGAGAAAATGCTGGC	GCCACTGTCACTGTTACTGC
Leptin	CCAGGATGACACCAAAACCCTC	ATCCAGGCTCTCTGGCTTCTGC
Resistin	CTACATTGCTGGTCAGTCTCC	GCTGTCCAGTCTATGCTTCC
PPARγ	CATTTCTGCTCCACACTATGAA	CGGGAAGGACTTTATGTATGAG
CB1	ATATTCTCTGGAAGGCTCACAGCC	AGCATACTGCAGAATGCAAACACC
LEP	CCAGGATGACACCAAAACCCTC	ATCCAGGCTCTCTGGCTTCTGC
	Brain	
CB1	ATATTCTCTGGAAGGCTCACAGCC	AGCATACTGCAGAATGCAAACACC
LEP-r	GAGAGGCTGCTGAAATCGTC	GACTCCTGAGCCATCCAGTC
ZnSOD	GAGCAGAAGGCAAGCGGTGAA	CCACATTGCCCAGGTCTC
NRF2	CACATCCAGACAGACACCAGT	CTACAAATGGGAATGTCTCTGC
HSP72	AGGCCAACAAGATCACCATC	TAGGACTCGAGCGCATTCTT
PPARα	ATCTGCTTCAAGTGGGGAGA	CCTGCCTTCCCCTGTGAACT
β-actin	GTC GTA CCA CTG GCA TTG TG	TCA GCT GTG GTG GTG AA

All oligonucleotides will be designed using the Primer 3 Plus program and obtained from Sigma Aldrich Brasil Ltda. CB1: Endocannabinoid receptor 1; LEP: leptin; LEP-r: leptin receptor; ZnSOD: superoxide dismutase; NRF2: erythroid-derived nuclear factor 2; HSP72: 70 kDa 1 heat shock protein 1; PPARα: receptor activated by alpha-type peroxisome proliferators.

2.7. Antioxidant Capacity

To prepare the homogenate, 200 mg of macerated brain were weighed and mixed with 800 µL of phosphate buffer (50 mM) and 1 mM EDTA (pH 7.4) to produce a 5% homogenate (w/v). Total proteins were quantified using the Bradford reagent (Bradford, 1976). The antioxidant enzyme superoxide dismutase (SOD) activity was analyzed by determining the inhibition of the autooxidation of the pyrogallol. The analysis was carried out on a spectrophotometer (Multiskan GO, Thermo Scientific, Ratastie, Finland) at 570 nm, and the results were expressed as units of SOD activity per milligram of protein (Marklund, 1985). Enzymatic activity of catalase was calculated by the decomposition of hydrogen peroxide according to Lambert Beer's law and was recorded in micromoles per milliliter of the sample (Aebi, 1984). At 0, 30, and 60 seconds after the reaction was initiated, the absorbance at 240 nm was determined on a spectrophotometer (T70 + UV/VIS Spectrometer, Taylors, USA).

2.8. *In silico* analysis

The interactions among anthocyanins identified in sorghum flour with markers of inflammation and satiety were evaluated by molecular docking. The 3D crystal structures of PPARα (PDB: 3VI8), CB1 (PDB: 5u09), and leptin (PDB: 3v60) were obtained in Protein Data Bank (PBD) website (http://www.rcsb.org/pdb/home/home.do). The 2D structure of anthocyanins 5-Methoxy Luteolidin, 7-Methoxy Apigenidin, Apigenidin Chloride, and Luteolidin Chloride were obtained from the PubChem Compound database (https://pubchem.ncbi.nlm.nih.gov/) and their 3D were obtained using MarvinSketch Program.

Flexible torsions, charges and grid size were assigned using AutoDock Tools and docking calculations were performed by AutoDock Vina (Trott e Olson, 2009). The anthocyanin that had the lowest binding energy (highest binding affinity) with each marker was selected as a representative image to be visualized in the program Discovery Studio version 2016 Client (Dassault Systèmes BIOVIA, San Diego, CA, USA).

2.9. AGE and RAGE quantification

To determine advanced glycation product (AGE) and advanced glycation product receptor (RAGE) concentration, adipose tissue and brain samples were homogenized in 50 mM phosphate buffer with EDTA. AGEs and RAGE was assessed by immunoassay using ELISA kit (Bioassay Technology Laboratory®, Zheijang, Korain). Absorbance was measured by spectrophotometry (Thermo Scientific® model Multiskan GO, Ratastie, Finland) at a wavelength of 450 nm. Protein concentration in samples was calculated using corresponding standard curve and were normalized by protein present in sample (Bradford, 1976).

2.10. Statistical Analyses

Data normality was verified using Shapiro-Wilk test. The data that did not present a normal distribution were transformed into Log^{10} for parametric statistical analysis. All the data were submitted to Analysis of Variance (ANOVA) followed by *post-hoc* Newman Keuls. Alpha value was determined 5%. Correlation analysis was conducted by Pearson's test. The analysis was performed in GraphPad Prism version 9.0.

3. RESULTS

3.1. Chemical composition of dry heat sorghum BRS305 flour

The chemical composition of dry heat sorghum BRS305 flour for protein, ash, total dietary fiber, insoluble dietary fiber, soluble dietary fiber, resistant starch, condensed tannins, total phenolics compounds and antioxidant capacity is showed on table 2.

Table 2. Dietary fiber, resistant starch, condensed tannins, antioxidant capacity and total phenolics compounds of BRS305 sorghum flour obtained from grains under dry heat treatment.

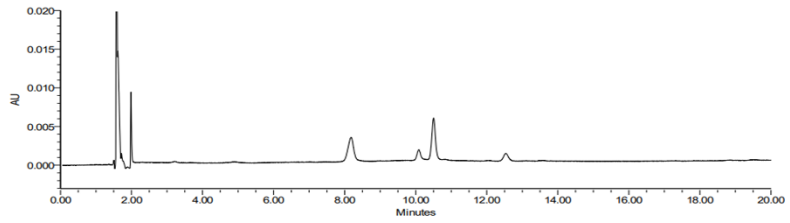
Chemical composition (g/100g)	Sorghum flour*
Total Dietary Fiber	15.50 ± 0.12
Insoluble Dietary Fiber	15.19 ± 0.10
Soluble Dietary Fiber	0.30 ± 0.01
Resistant Starch	20.90 ± 1.18
Condensed Tannins	73.83 ± 14.85
Antioxidant Capacity (uM Trolox/g)	1573.31 ± 22.42
Total Phenolic Compounds (mg GAE/g)	24.03 ± 3.21

* Values are shown by means and standard deviation.

3.2. Anthocyanin's profile

The anthocyanin profile analysis (Fig 1A) revealed that the BRS 305 hybrid sorghum flour showed predominantly luteolidin chloride (11.01 ± 0.07µg/g) and 5-methoxy luteolidin (15.40 ± 0.43µg/g) (Figure 1B).

A



Peak Results

	Name	RT
1	Luteolinidin Chloride	8.183
2	Apigeninidin Chloride	10.090
3		10.504
4	7-Metoxi Apigeninidin	12.536
5	5,7-Dimetoxi Luteolinidin	14.100
6	5,7-Dimetoxi Apigeninidin	16.900

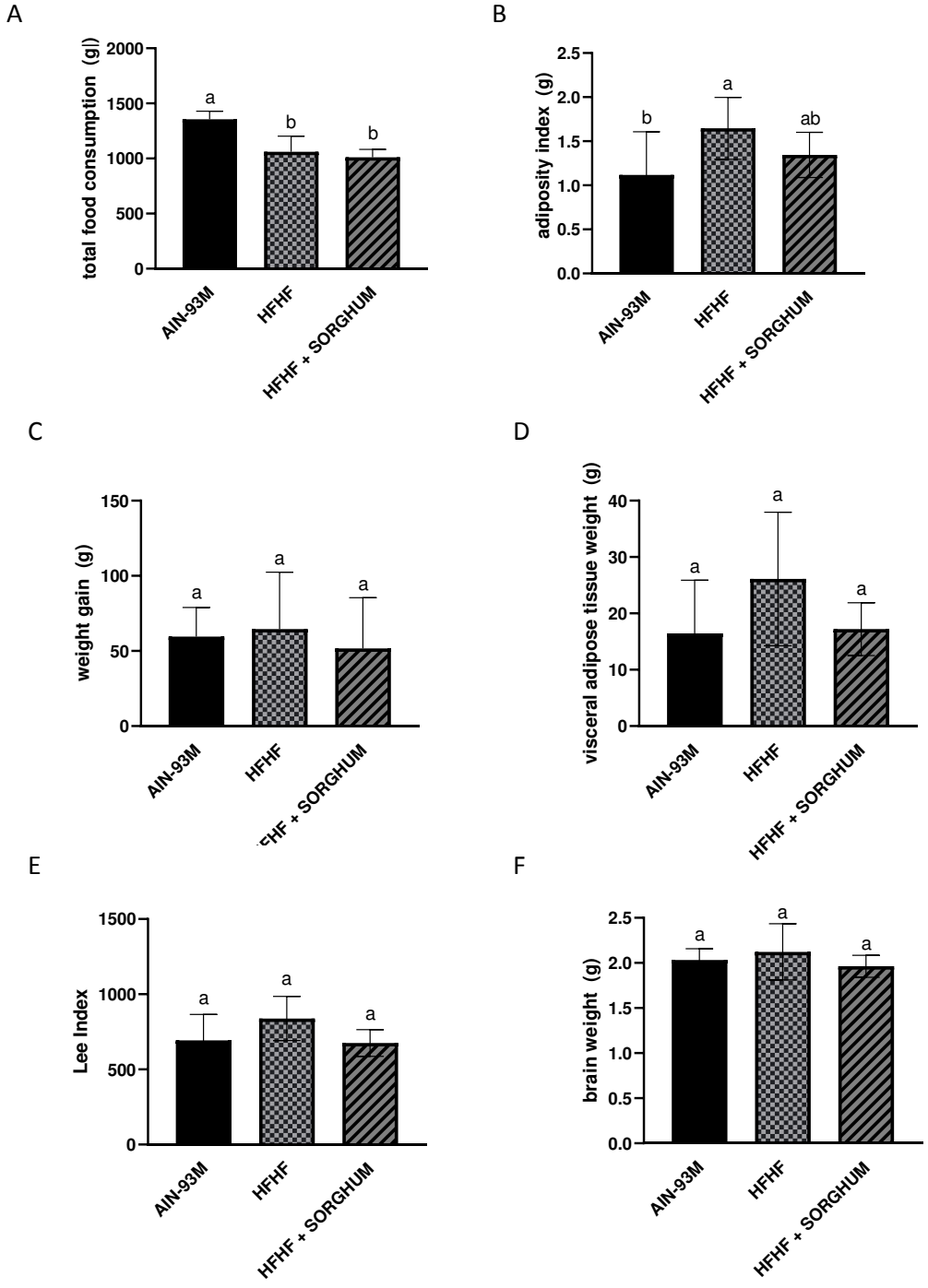
B

Anthocyanins	µg/g
Total anthocyanins	31.42 ± 0.32
Luteolidin chloride	11.01 ± 0.07
Apigeninidine chloride	0.97 ± 0.11
5-methoxy luteolidin	15.40 ± 0.43
7-methoxy apigeninidine	4.65 ± 0.11

Fig. 1. Anthocyanin's profile of sorghum BRS 305 by HPLC analysis. The main 3-deoxyanthocyanins presents in sorghum BRS305 whole flour are luteolinidin chloride and 5-methoxy apigeninidine. (A) anthocyanin profile chromatogram (B) Anthocyanin types of contents in sorghum BRS 305 flour.

3.3. Effects of BRS 305 hybrid sorghum flour on biometric measures and food consumption

Experimental groups that received HFHF diet presented lower total food consumption compared with AIN-93M group (Supplementary Materials - Fig 1A). The sorghum-fed animals did not differ from the animals fed with HFHF or AIN-93M in terms of adiposity index (Supplementary Materials - Fig 1B). The consumption of sorghum flour in HFHF diet did not change the weight gain (Supplementary Materials - Fig 1C), visceral adipose tissue weight (Supplementary Materials - Fig 1D), lee index (Supplementary Materials - Fig 1E) and brain weight (Supplementary Materials - Fig 1F) compared to AIN-93M and HFHF groups. Comparing weekly food consumption, experimental groups that received HFHF diet showed a lower food consumption since the first treatment week (Supplementary Materials – Fig 2A and 2B). The body weight did not differ between groups in all treatment week (Supplementary Materials – Fig. 3A and 3B).



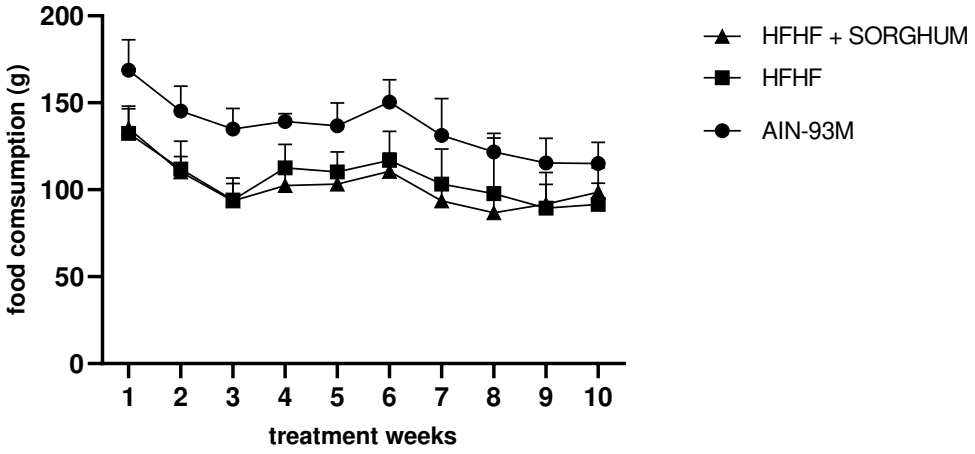
Supplementary Fig 1. Total food consumption and biometric measures of experimental groups. A: total food consumption. B: adiposity index. C: weight gain. D: visceral adipose tissue weight. E: lee index. F: brain weight. AIN-93M: animals fed with AIN-93M diet; HFHF: animals fed with high-fat high-fructose diet; HFHF + Sorghum: animals fed with

high-fat high-fructose diet added with sorghum flour. Data were submitted to ANOVA One-Way followed by Newman-Keuls post-hoc considering α=5%.

A

Treatment week	Experimental group food consumption		
	AIN-93M	HFHF	HFHF + Sorghum
1	168.69 ± 17.61[a]	132.44 ± 15.62[b]	135.20 ± 11.37[b]
2	145.30 ± 14.29[a]	111.87 ± 16.09[b]	110.27 ± 8.85[b]
3	134.92 ± 11.79[a]	94.11 ± 12.60[b]	93.55 ± 10.10[b]
4	139.20 ± 4.47[a]	112.60 ± 13.54[b]	102.38 ± 11.51[b]
5	136.78 ± 13.07[a]	110.23 ± 11.46[b]	103.18 ± 8.72[b]
6	150.40 ± 12.79[a]	116.95 ± 16.69[b]	110.64 ± 10.41[b]
7	131.20 ± 21.16[a]	103.31 ± 20.16[b]	93.64 ± 12.88[b]
8	121.69 ± 10.71[a]	97.72 ± 32.00[b]	86.76 ± 14.85[b]
9	115.35 ± 14.21[a]	89.40 ± 20.48[b]	91.81 ± 11.29[b]
10	115.05 ± 12.25[a]	91.58 ± 12.13[b]	98.62 ± 13.88[b]

B

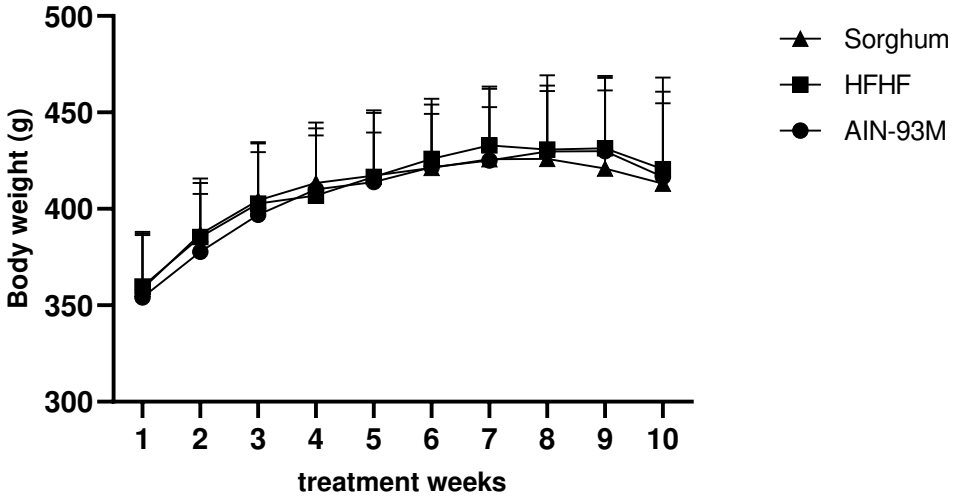


Supplementary Fig 2. Weekly food consumption of experimental groups (n=8). (A) Table showing means and stardard deviation for food consumption by each treatment week. (B) Graph of weekly food consumption for each treatment week. AIN-93M: animals fed with AIN-93M diet; HFHF: animals fed with high-fat high-fructose diet; HFHF + Sorghum: animals fed with high-fat high-fructose diet added with sorghum flour. Data were submitted to ANOVA One-Way followed by Newman-Keuls post-hoc considering α=5%.

A

Treatment week	Experimental group body weight		
	AIN-93M	HFHF	HFHF + Sorghum
1	354.06 ± 32.21[a]	359.73 ± 26.69[a]	358.50 ± 29.58[a]
2	377.79 ± 29.97[a]	385.45 ± 27.98[a]	386.85 ± 28.83[a]
3	396.84 ± 32.64[a]	402.73 ± 31.25[a]	404.48 ± 30.14[a]
4	410.02 ± 31.68[a]	406.93 ± 31.20[a]	413.39 ± 31.43[a]
5	413.93 ± 25.62[a]	416.50 ± 33.23[a]	417.19 ± 33.88[a]
6	421.60 ± 27.72[a]	425.96 ± 31.12[a]	421.22 ± 32.92[a]
7	425.07 ± 27.75[a]	432.84 ± 30.56[a]	425.73 ± 36.50[a]
8	429.69 ± 31.40[a]	430.78 ± 33.21[a]	425.86 ± 43.36[a]
9	429.86 ± 38.03[a]	431.47 ± 37.38[a]	420.95 ± 40.52[a]
10	416.54 ± 44.22[a]	420.51 ± 47.50[a]	413.16 ± 41.52[a]

B



Supplementary Fig 3. Weekly body weight of experimental groups (n=8). (A) Table showing means and stardard deviation for body weight by each treatment week. (B) Graph of body weight for each treatment week. AIN-93M: animals fed with AIN-93M diet; HFHF: animals fed with high-fat high-fructose diet; HFHF + Sorghum: animals fed with high-fat high-fructose diet added with sorghum flour. Data were submitted to ANOVA One-Way followed by Newman-Keuls post-hoc considering α=5%.

3.4. Effects of BRS 305 hybrid sorghum flour in gene expression of markers and centers of satiety

The consumption of sorghum flour (HFHF + sorghum flour) did not alter adiponectin gene expression (Fig 2A), compared to other groups (HFHF and AIN93-M). However, sorghum was effective to decrease the markers related to satiety centers, including resistin (Fig 2B), and leptin (Fig 2C) in adipose tissue compared to control groups (AIN-93M and HFHF), and CB1 (Fig 2D) relative to HFHF group. In brain, sorghum consumption associated with HFHF diet decreased the gene expression of leptin receptor (Fig 2E), NPY (Fig 2F), and endocannabinoid receptor 1 (Fig 2G) relative to HFHF and AIN-93M groups.

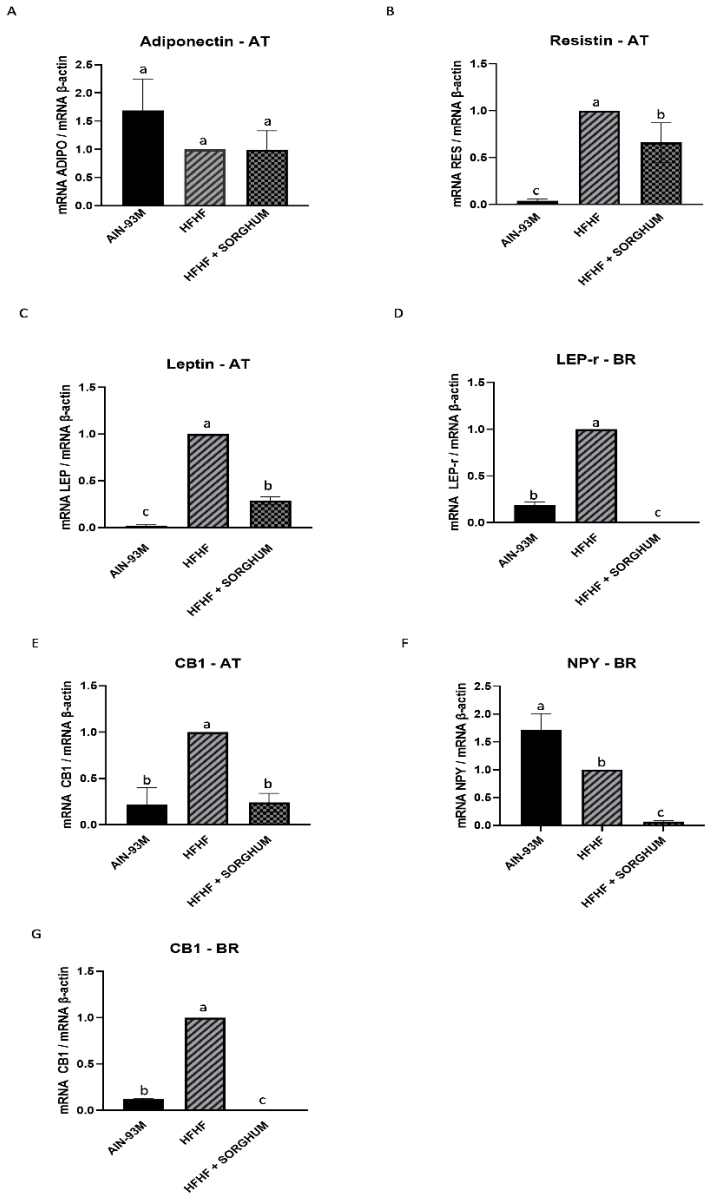


Fig. 2. Consumption, adiposity and satiety markers gene expression in adipose tissue and brain. (A) adiponectin gene expression in adipose tissue. (B) gene expression of resistin in adipose tissue. (C) gene expression of leptin in adipose tissue (D) gene expression of leptin receptor in brain. (E) gene expression of CB1 in adipose tissue. (F) gene expression of NPY in brain. (G) gene expression of CB1 in brain. AT: adipose tissue; BR: brain; AIN-93 M: animals fed with AIN-93 M diet; HFHF: animals fed with high-fat high-fructose diet; HFHF + Sorghum: animals fed with high-fat high-fructose diet added with sorghum flour. Data were submitted to ANOVA One-Way followed by Newman-Keuls post-hoc considering α =5%. Different letters in each figure appointed statistical difference.

3.5. Effects of BRS 305 hybrid sorghum flour on antioxidant response in adipose and brain tissues

In the adipose tissue, sorghum flour promoted similar values of PPARγ gene expression compared to HFHF group and lower than AIN-93M group (Fig 3A). The SIRT1 gene expression in HFHF associated with sorghum flour group was higher than HFHF group, become similar to control group (AIN-93M) (Fig 3B). In the brain, PPARα gene expression increased compared to HFHF and AIN-93M groups (Fig 3C). Superoxide dismutase gene expression did not change among experimental groups (Fig 3D), but superoxide dismutase activity was enhanced in sorghum group (HFHF + sorghum flour) compared to HFHF group (Fig 3E). Further, the catalase activity increased in the sorghum group compared to AIN 93-M and HFHF groups (Fig 3F). For markers of antioxidant response, sorghum flour consumption increased HSP72 (Fig 3G) and NRF2 (Fig 3H) gene expression compared to AIN93-M and HFHF groups.

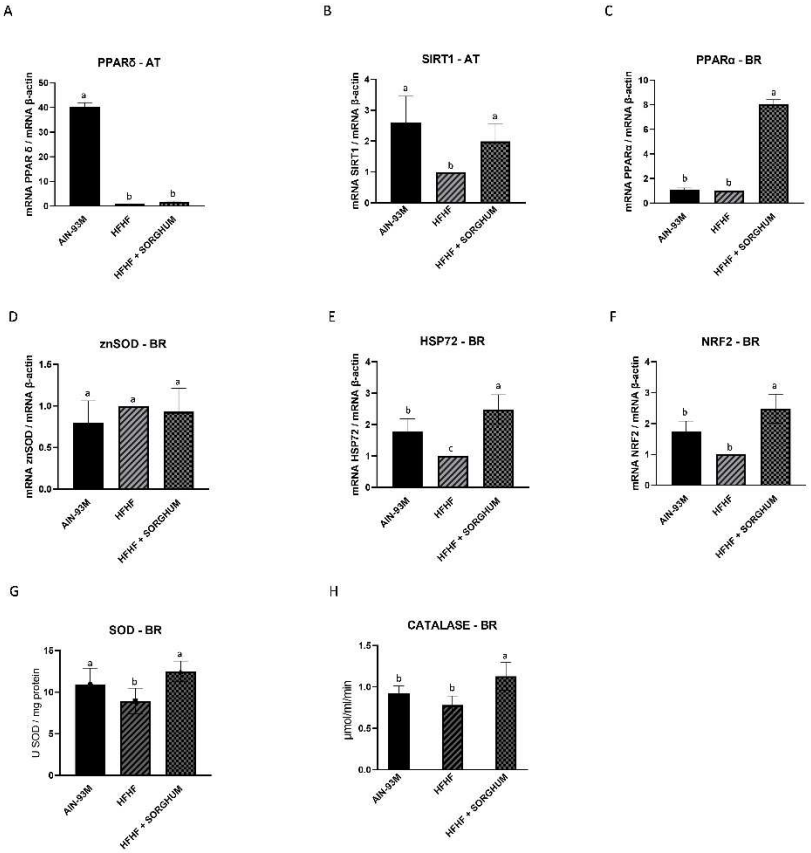


Fig. 3. Antioxidant response in adipose tissue (A-B) and brain (C–H). (A) peroxisome proliferator-activated receptors gamma (PPARγ) gene expression. (B) sirtuin-1 (SIRT1) gene expression. (C) peroxisome proliferator-activated receptors alpha (PPARα) gene expression. (D) superoxido dismutase (ZnSOD) gene expression. (E) heat shock protein 72 (HSP72) gene expression. (F) erythroid-derived nuclear factor 2 (Nrf2) gene expression. (G) superoxido dismutase (SOD) activity. (H) catalase activity. AT: adipose tissue; BR: brain. AIN-93 M: animals fed with AIN-93 M diet; HFHF: animals fed with high-fat high-fructose diet; HFHF + Sorghum: animals fed with high-fat high-fructose diet added with sorghum flour. Data were submitted to ANOVA One-Way followed by Newman-Keuls post-hoc considering α = 5%. Different letters in each figure appointed statistical difference.

3.6. Molecular docking

The *in silico* analysis of the interaction between anthocyanins from sorghum and inflammation and satiety markers showed that every compounds had interaction with PPARα, CB1, and leptin receptor. All interactions showed low binding energy by estimated free energy (EFE), which means that connections occurring spontaneously (Supplementary Materials - Table 2).

Supplementary Table 2. Estimated free energy binding (EFE) and chemical interactions among the anthocyanins presents in sorghum flour.

Anthocyanins	PPARα		CB1		Leptin receptor	
	EFE	Ligand interaction	EFE	Ligand interaction	EFE	Ligand interaction
5-Methoxy Luteolidin	**-8.5**	PHE A: 351; MET A: 355; ILE A: 354; GLU A: 269; CYS A: 276; HIS A: 440; TYR A: 464; SER A: 280.	-8.0	MET A: 384; CYS A: 386; PHE A: 268; VAL A: 196; MET A: 103	-6.4	LEU A: 538; PRO A: 537; ASP A: 532; ARG A: 615; ASN A: 567; LEU A: 568
7-Methoxy Apigenidin	-8.3	SER A: 323; TYR A: 214; MET A: 220; MET A: 320; ASN A: 219	**-8.7**	LEU A: 387; CYS A: 386; VAL A: 196; MET A: 103; PHE A: 102.	-6.5	ASP A: 532; ARG A: 615; VAL A: 535; LEU A: 442; PRO A: 537; LEU A: 568
Apigenidin Chloride	-8.2	TYR A: 214; SER A: 323; LYS A: 222; TYR A: 334; MET A: 320; MET A: 220; THR A: 279; THR A: 283.	-8.5	MET A: 103; CYS A: 386; VAL A: 196; SER A: 383	**-6.6**	ARG A: 615; LEU A: 442; VAL A: 535; PRO A: 537; LEU A: 538; CYS A: 613
Luteolidin Chloride	-8.0	LYS A: 222; TYR A: 214; SER A: 323; MET A: 320; MET A: 220; ASN A: 219	-8.6	SER A: 390; VAL A: 196; LEU A: 387; CYS A: 386; MET A: 103; PHE A: 102.	-6.3	ASP A: 532; VAL A: 535; LEU A: 442; PRO A: 537

EFE: Estimated free energy. Docking calculation were carried out using AutoDock Vina. Negative values mean spontaneous reaction. Bold values represent the strongest interactions.

The anthocyanins, 5-methoxy luteolidin, 7-methoxy apigenidin, and apigenidin chloride showed highest interaction with PPARα (EFE −8.5) (Fig.4A), CB1 (EFE −8.7) (Fig. 4B), and leptin receptor (EFE −6.6) (Fig. 4C), respectively.

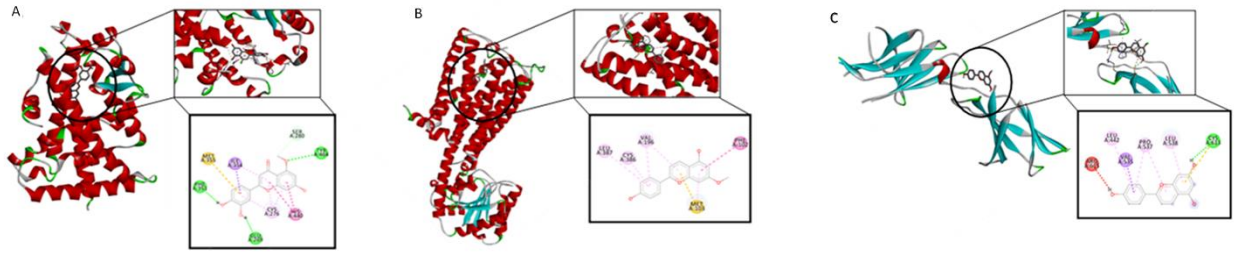


Fig. 4. The in silico interaction of the anthocyanins from sorghum with markers of energy homeostasis and satiety and anthocyanins profile. (A) Interaction between PPARα and 5-Methoxy Luteolidin; (B) Interaction between CB1 and 7-Methoxy Apigenidin; (C) Interaction between leptin receptor and Apigenidin Chloride. 3-deoxyanthocyanins presents in sorghum BRS305 whole flour have strong interactions with markers of satiety and energy homeostasis. Datas analyzed by AutoDock Vina® and visualized using Discovery Studio 2016 Client®.

3.7. AGE and RAGE quantification

Sorghum consumption in a HFHF group did not reduce an AGEs protein quantification in adipose (Fig 5A) and brain (Fig 5B) tissues compared to the HFHF group; however, it become similar to control group (AIN-93M). In adipose tissue, the consumption of the HFHF diet with or without sorghum increased RAGE receptors protein quantification when compared to the control group (Fig 5C). However, in the brain, no differences were observed among experimental groups for RAGE receptors protein quantification (Fig 5D).

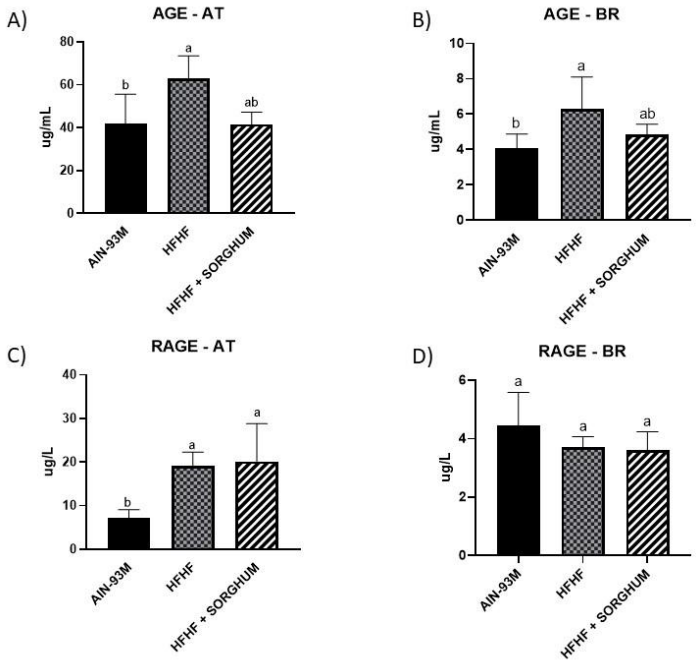


Fig. 5. AGE and RAGE quantification in adipose and brain tissue of animals treated with sorghum BRS305 whole flour. (A) advanced glycation end products (AGEs) concentrations in adipose tissue. (B) advanced glycation end products (AGEs) concentrations in brain. (C) advanced glycation end products receptor (RAGEs) in adipose tissue. (D) advanced glycation end products receptor (RAGEs) in brain. AGE value was expressed in μg/mL and RAGE value was expressed in μg/L. AT: adipose tissue; BR: brain. AIN-93 M: animals fed with AIN-93 M diet; HFHF: animals fed with high-fat high-fructose diet; HFHF + Sorghum: animals fed with high-fat high-fructose diet added with sorghum flour. Data were submitted to ANOVA One-Way followed by Newman-Keuls post-hoc considering α = 5%. Different letters in each figure appointed statistical difference.

3.8. Pearson's correlation analysis

The anthocyanin consumption presented positive correlation with the activation of the antioxidant response (NRF2, HSP72, CAT, and SOD activity) and negative correlation with CB1, leptin receptors, and NPY hormone (Figure 6). Furthermore, it was observed a negative correlation between the quantification of RAGE in the brain and adiponectin and negative correlation with resistin. RAGE in adipose tissue showed a positive correlation

with CB1 and leptin receptor in brain, and with resistin and CB1 in adipose tissue. Leptin receptor was correlated negatively with gene expression of HSP72, Nrf2, PPARα, and with activity of SOD and CAT. AGE concentration was positively correlated in adipose and brain tissues, with the activation of CB1 and leptin gene expression receptors in the brain, as well as with the gene expression of leptin and resistin in adipose tissue (Figure 6B).

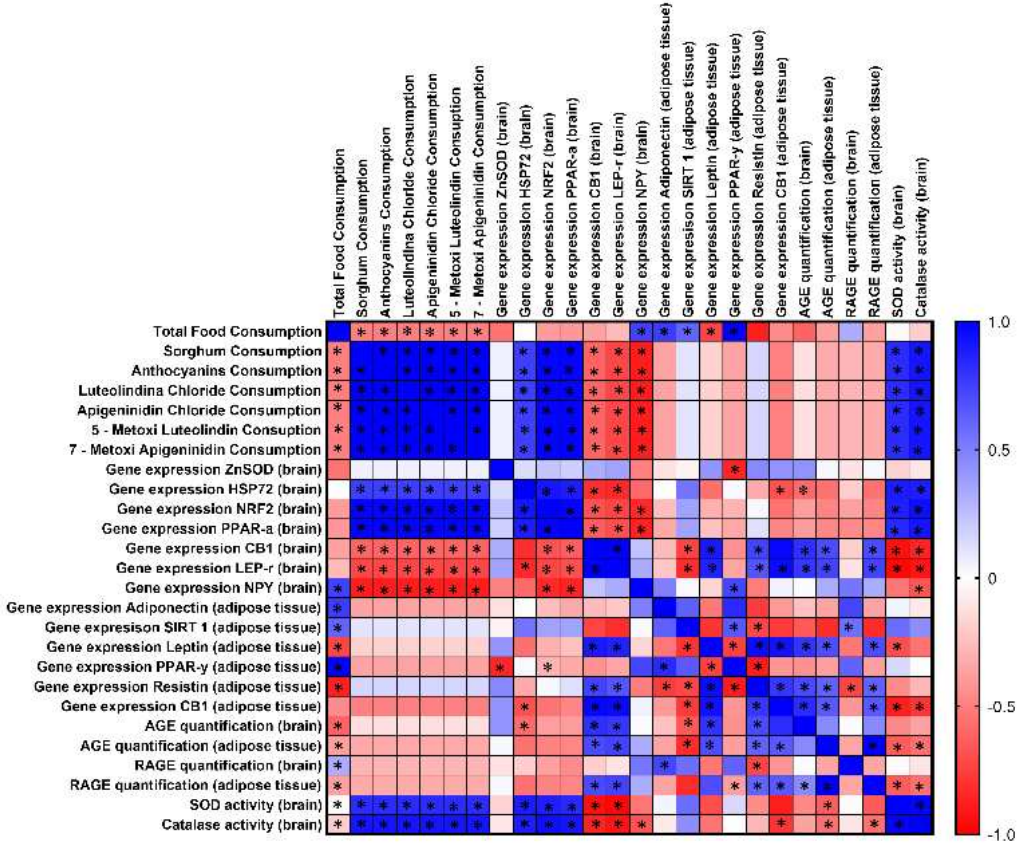


Fig. 6. Heatmap of Pearson's correlation among variables. ZnSOD: superoxide dismutase activated by zinc. HSP72: heat shock protein 72. Nrf2: erythroid-derived nuclear factor 2 in brain. PPARα: peroxisome proliferator-activated receptors alpha. CB1: endocannabinoid receptor 1. LEP-r: leptin receptors. NPY: neuropeptide Y. SIRT1: sirtuin. PPAR-γ: peroxisome proliferator-activated receptors gama. AGE: advanced glycation products. RAGE: advanced glycation products receptors. SOD: superoxide dismutase. Data were submitted by Pearson's correlation, considering α = 5%. Each * in squares corresponding to statistical difference.

4. DISCUSSION

The HFHF diet consumption promote metabolic disorders, such as leptin resistance associated to a higher leptin gene expression, besides to promote the hyperactivation of leptin receptor in brain and type 1 endocannabinoid receptors and reduction of antioxidant capacity in brain and adipose tissue. However, the consumption of BRS305 whole sorghum flour associated with HFHF restored the normal activity of leptin and resistin genes expression, leptin receptor activity as well as type 1 endocanabinoid receptors in brain and adipose tissue. Further, the antioxidant response in brain increased the gene expression of NRF2, HSP72 and PPARα, and the activity of SOD and CAT. Then, our hypothesis that sorghum BRS305 consumption can reestablish physiological activity of pathways related to satiety and antioxidant response in brain and adipose tissue of rats fed with HFHF diet was confirmed. These effects can be attributed to the sorghum composition. Since the grain was submitted to dry heat treatment (105ºC/30 min), it preserved the 3-deoxyanthocyanins (luteolin and apigenin), dietary fiber, resistant starch, condensed tannins, and increased total antioxidant capacity (Cardoso *et al*., 2013; Cardoso *et al*., 2015; Martinez *et al*., 2020). Animals fed with HFHF diets reduced food consumption compared to AIN-93M group. Fat proportion (31%) in the HFHF diets can promote high feeling of satiety in relation to AIN-93M diet, which is rich in carbohydrates (72%) (Yeung, 2020). The adiposity promoted by consumption of HFHF diet was related to leptin resistance, since limited leptin access in the central nervous system causes leptin uncoupling with the receptor. Then, the elevated level of circulating leptin can lead to hyperactivation of its hypothalamic receptor as observed in other studies (Tri *et al*., 2020; Tu *et al*., 2019). Furthermore, the HFHF diet reduced the antioxidant capacity which helped to suppress leptin signaling in the hypothalamus, favoring the development of leptin resistance, and consequently increasing resistin gene expression. The consumption of BRS305 whole sorghum flour associated with HFHF diet restored antioxidant capacity as observed by Li *et al*., (2021), which offered a bioactive compound of millet grains (vitexin, 10-30 mg/kg BW) to C57 BL/6 mice fed with high fat diet. In addition, in our study, sorghum restored the leptin gene expression in adipose tissue and activity of receptors related to leptin metabolism, as leptin receptor in brain and endocannabinoid receptor type 1 (CB1) in adipose tissue and brain. These effects can be

attributed to bioactive compounds in the sorghum, since molecular docking analysis showed a strong estimated free energy binding between sorghum 3-DXAs and leptin and CB1, restoring the physiological activity of these satiety center mechanisms. Furthermore, leptin adequate levels associated with a reduction of resistin production and activity improved the functionality of mechanisms related to satiety (Popovic *et al*., 2014). In addition, according with molecular docking analysis 3-DXAs presented strong estimated free energy binding with PPARα, a transcription factor associated with energy homeostasis (Preidis *et al*., 2017).

The addition of sorghum flour to the HFHF diet regulated the leptin gene expression in adipose tissue, which was upregulates by HFHF diet in adipose tissue. The resistant starch and dietary fiber impacts leptin levels specially to promote low glycemic index, meaning it does not cause a rapid rise in blood glucose levels after a meal. This can help stabilize blood glucose and insulin levels, because high blood glucose levels and insulin resistance can contribute to leptin resistance, so the ability of resistant starch to regulate blood glucose may indirectly influence leptin levels (Keenan *et al*., 2015). As observed in our study, in other studies, resistant starch from rice (Wan *et al*., 2020) and banana flours (Rosado *et al*., 2021) also regulated leptin gene expression. Moreover, the increase in AGE concentrations in the adipose tissue and in the brain was directly correlated with leptin and resistin levels as well as the hyperactivation of leptin and type 1 endocannabinoid receptors, relating to the hyperleptinemia caused by the HFHF diet consumption. In this context, the reduction in antioxidant capacity caused by the consumption of this diet may contribute to the endogenous formation of AGEs. However, the addition of BRS 305 whole sorghum flour to the HFHF diet did not promote differences in the concentrations of AGEs. It is known that anthocyanins may play a competitive inhibitory role on AGEs, as they can bind to RAGE, in order to prevent the interaction of AGEs with the functional side of their receptor (Fatchiyah *et al*., 2015; Khan *et al*., 2021), which may explain the results found for RAGE in brain and adipose tissues. Furthermore, RAGE was inversely associated with leptin levels. The inhibition of leptin action can increase RAGE expression in pancreatic beta cells and lead to lower insulin secretion (Garay-Sevilla *et al*., 2021). This corroborates with our findings, since the levels of

circulating leptin were reduced in the adipose tissue of animals fed with HFHF diet associated with whole sorghum flour.

Condensed tannins are compounds known to associate with proteins on hydroxyl groups, carbonyl groups, or aromatic rings, reducing their digestibility (Slabbert, 1999). In our study, the total consumption of condensed tannins was 120.97 mg, corresponding to 1.73 mg of per day during treatment phase. The reduction of carbohydrate digestibility impacts directly on the glucose metabolism, observed in our research group, after sorghum BRS 305 consumption (Martinez *et al*., 2021a). These bioactive compounds were appointed to interact with α-amylase and glucoamylase (Barrett *et al*., 2013). However, the authors appointed those different tannins can interact in varying degrees and strength with these enzymes. These interactions can digest carbohydrates and protein reduce the gastric empty and affects the enteroendocrine system, altering the production of peptide hormones that control digestive processes such as ghrelin, cholecystokinin (CCK), glucagon-like-peptide-1 (GLP-1) and neuropeptide Y (NPY), consequently, reducing the food intake (Serrano et al., 2016). The reversion of disorders on glucose metabolism, such as insulin resistance impacts the leptin resistance condition, reducing their production and normalizing their receptor activity in brain (Butiaeva *et al*., 2021).

The consumption of the HFHF diet decreased PPARγ gene expression in adipose tissue (Kao et al., 2020), and the addition of whole sorghum flour to HFHF diet did not improve this parameter. The HFHF diet rich in saturated fat probably reduced the polyunsaturated fatty acids intake in the animals, which are the endogenous ligands of PPAR-γ (El-Ashmawy *et al*., 2018), decreasing PPAR-γ gene expression in our study.

The downregulation in antioxidant response induced by HFHF diet, such as SOD activity, and SIRT-1, HSP72, PPARγ gene expressions indicated dissociation of the Keap1-Nrf2 complex and translocation of Nrf2 to the nucleus to activate the expression of antioxidant enzymes (Batandier *et al*., 2020). Sorghum flour increased SOD and catalase activity as well HSP72, PPARα, Nrf2 gene expression in brain and SIRT-1 gene expression in adipose tissue. These effects may be associated with increased the antioxidant response, since it is an activator of the transcription factor Nrf2 (Ma, 2013). Furthermore, the increase in the gene expression of the HSP72 protein may evidence the

neuroprotective role of BRS 305 whole sorghum flour consumption, considering that one of its main functions is to facilitate the repair of DNA damage caused by the accumulation of reactive oxygen species (Devi *et al*., 2012; Dwivedi & Jena, 2020).

Anthocyanins are antioxidants compounds with anti-inflammatory (Cremonini *et al*., 2022), anticarcinogenic (Mostafa *et al*., 2023), and cardioprotective effects (Adriouch *et al*., 2018). Then, the 3-DXAs present in sorghum BRS 305, mainly luteolidin chloride and 5-methoxy luteolidin can enhance the antioxidant capacity in the HFHF diet. This fact can be evidenced by increase in catalase and superoxide dismutase activities. In addition, these compounds can be directly correlated with an increase in PPARα gene expression. *In silico* analyzes showed that the 3-DXAS present in the BRS 305 whole sorghum flour directly bind to type 1 endocannabinoid receptors, leptin receptors and PPARα in brain. The interactions between these bioactive compounds and the biological markers *in silico* demonstrate an important role in the maintenance/restoration of mechanisms related to the satiety and the antioxidant response.

The sorghum flour in high fat high fructose was added to replace 50% of the dietary fiber for rodent recommendation, which means 2.5g/100g of experimental diet (Reeves *et al*., 1993). To supply 50% of dietary fiber recommendation for human (IOM, 2002), it is necessary the consumption of approximate 98 g of sorghum flour/day. This amount of flour is equivalent to two thirds of cup and can be consumed in preparation as bread, cake, biscuit and pasta.

5. CONCLUSION

The consumption of BRS 305 whole sorghum flour was effective in regulate leptin and leptin and CB1 receptors gene expression that act in the centers of brain satiety in rats fed with a HFHF diet. Furthermore, sorghum flour consumption was able to increase the markers of antioxidant response, demonstrating an important interaction between anthocyanins present in sorghum and brain leptin receptors. Then, the improvement in brain antioxidant response shows a neuroprotective role of this cereal in metabolic disorders induced by adherence to the western dietary pattern. Further studies are needed to investigate the modulatory potential of anthocyanins in the mechanisms of satiety regulation.

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Conflict of Interest Statement: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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6.2. PAPER 2

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Article

Consumption of Extruded Sorghum SC319 Improved Gut Microbiota at Genus Level and Reduced Anthropometric Markers in Men with Overweight: A Randomized Controlled Clinical Trial

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Abstract: Background: Sorghum is a cereal source of energy, carbohydrates, resistant starch, proanthocyanidins, and 3-deoxyanthocyanins; it promotes satiety by slowing digestion and benefits intestinal health. Objective: This study investigated the effects of extruded sorghum SC319 consumption on intestinal health, weight loss, and inflammatory markers in men with overweight. Methods: This was a randomized, controlled, single-blind clinical trial. Twenty-one men were randomly allocated into one of two groups: the sorghum group (test), which received 40 g of extruded SC319 whole

1. Introduction

Obesity is a disease related to complex interactions among genetic, socioeconomic, cultural, and environmental influences. It is a disease with multifactorial causes [1]. Obesity is frequently associated with the dysregulation of lipid, glucose, and cholesterol metabolism, in addition to increased oxidative stress and the establishment of low-grade chronic inflammation, which are risk factors for developing non-communicable chronic diseases [2,3]. It is estimated that the number of people with obesity is about to double by 2030, affecting one billion people worldwide [4].

The inflammatory environment caused by lipid accumulation results in a non-specificactivation of the immune system, contributing to a large extent to the development of alterations in intestinal behavior, altering the functionality of enterocytes and other structures, and favoring the development of intestinal dysbiosis [5, 6]. Intestinal health involves biological, mechanical, and structural changes that affect the intestinal environment. Thus, the main parameters related to intestinal health are the gut barrier, nutrient digestion and absorption, gut microbiome, fecal pH, SCFA production, mucus layer, barrier function, and mucosal immune responses [7,8]. The consequences of dysbiosis include the reduction in bacteria that synthesize short-chain fatty acids, the reduction in the activity of other bacteria and mucus production, and the increase in intestinal permeability [9]. Thus, these effects can increase the endotoxins in the circulation, activating the immune and inflammatory response [10,11].

Studies have shown that the intestinal microbiota can influence adiposity and weight gain [12], and weight gain can lead to changes in the intestinal microbiota composition [13]. In turn, weight loss interventions have been shown to induce changes in the gut microbiota. Emerging evidence suggests that the gut microbiota and its metabolites may play a pivotal role in mediating the effects of an energy restriction diet [14]. In addition, prebiotics acts as dietary fibers, serve as fuel for beneficial gut bacteria, and can also positively influence gut microbiota composition [15].

Sorghum grains (Sorghum bicolor (L.) Moench) are a source of resistant starch, energy, carbohydrates, proteins, and bioactive compounds, especially proanthocyanidins and3-deoxyanthocyanins [16 – 18]. The SC319 sorghum genotype utilized in the present study is a genotype with high antioxidant capacity due to its chemical composition, which

presents bioactive compounds such as phenolic acids, flavonoids, tannins, 3-deoxyanthocyanidins, and vitamin E [19– 21]. Previous research of our group demonstrated that this sorghum genotype exhibits favorable palatability and consumer acceptance [21], and its consumption led to a notable reduction in the glycemic response of subsequent meals among healthy adults [22]. Studies in an experimental model have shown the benefits of consuming this grain on metabolic markers, including lipid and glucose metabolism [23–25]. In addition to the prebiotic effects of the dietary fiber present in whole grains such as sorghum, the SC319 sorghum genotype presents bioactive compounds such as proanthocyanidins and 3-deoxyanthocyanins, which may modulate the mucosal immune responses, inflammation, and the intestinal microbiota [26 –28], thus promoting the intestinal health of individuals.

Hence, it is essential to clarify the effect of extruded sorghum in the markers linked to the intestinal health of men with overweight, associating the consumption of this cereal with an energy restriction diet. This study aimed to investigate the potential beneficial effects promoted by extruded SC319 sorghum consumption on weight loss, inflammatory markers, and intestinal health, including fecal pH, SCFA production, target species, and gut microbiota composition in overweight men. As it is a genotype rich in tannins, this type of sorghum can favor weight and body fat loss and modulate the intestinal microbiota due to the reduced digestibility of macronutrients such as carbohydrates and proteins.

2. Materials and Methods

2.1. Raw Materials and Processing

Whole-grain sorghum (SC319 genotype) was grown in Nova Porteirinha, MG, Brazil, by Embrapa Milho e Sorgo. The grains were harvested in September 2013. They were milled into flour using a disc mill (Perten Instruments, Huddinge, Sweden) at position 2 . The sorghum flour was combined with 10% fine granulated sugar (sucrose) and 0.5% iodized salt (NaCl). The mixture was processed using a twin-screw extruder (Clextral, Firminy, France) with a screw speed of 600 rpm and temperature ranging from 30 to 140 ◦C. The extruder had a screw diameter of 25 mm and a length of 1000 mm, resulting in an L/D ratio of 40. The die had four round openings measuring 2.0 mm in diameter and 9 mm in length. A gravimetric feeder (Schenck Process, Darmstadt, Germany) delivered the formulation to the extruder, while distilled water was added to adjust the moisture content

to 12%. The extruded sorghum breakfast cereal was stored in polyethylene bags at 10 ± 2 ◦C.

Similarly, whole-grain wheat flour from SL Alimentos in Mauá da Serra, PR, Brazil, was processed with the addition of 10% sucrose and 0.5% iodized salt. The wheat flour mixture was extruded using a twin-screw extruder (Clextral, Firminy, France) at a screw speed of 200 rpm and temperature ranging from 50 to 143 ◦C. The processing conditions were comparable to those used for sorghum. The resulting whole-grain wheat breakfast cereal was also stored in polyethylene bags at 10 ± 2 ◦C until it was ready for consumption.

2.2. Trial Design

This was an 8-week, single-blind, controlled, randomized nutritional intervention study conducted in men with overweight. This study was conducted using data from the second phase of a crossover study previously conducted by our research group [29]. Samples related to intestinal health were collected only in the second phase of the study, pointing to the need for carrying out this investigative study. The previous study was a crossover, randomized, controlled, single-blind clinical trial, lasting 16 weeks and with 4 weeks washout period to eliminate residual effects of the first intervention period. The

participants included in the study were randomly allocated in a 1:1 ratio to receive extruded SC319 whole sorghum or extruded whole wheat (Figure 1).

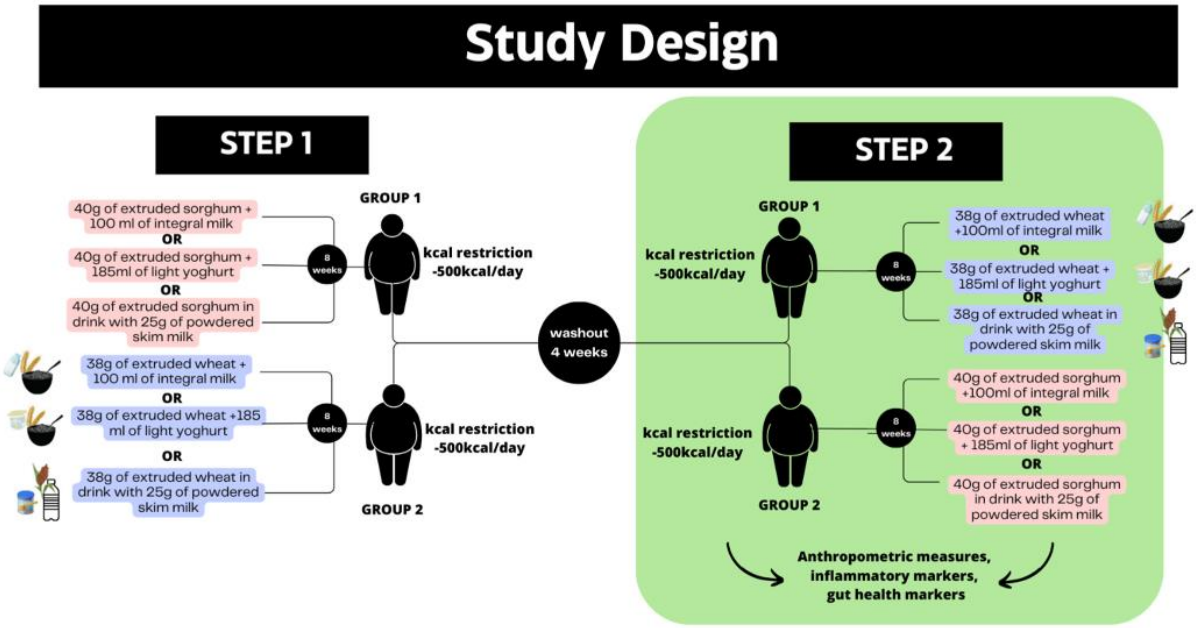


Figure 1. Study design and interventions. Data from the second phase of a crossover study were used in this study.

The study was approved by the Human Research Ethics Committee of the Federal University of Viçosa, Brazil (CAAE: 13630513.0.0000.5153). All participants were informed about the objectives of the study and provided written informed consent.

2.3. Participants

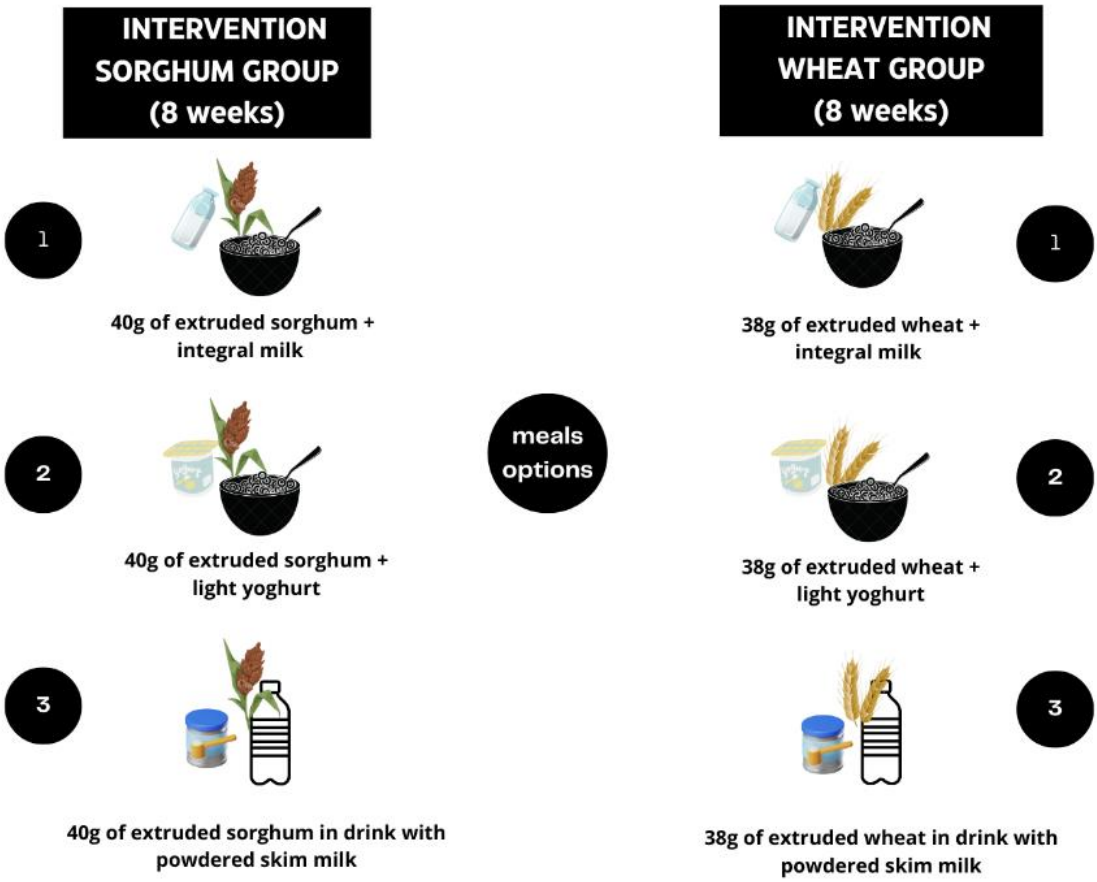
Volunteers were recruited in Viçosa-MG, Brazil, through advertisements on social networks, pamphlets, and posters. An email address and a telephone number were made available to individuals who were interested in participating in the study. In the first contact, the objectives and conditions of the study were informed to the potential volunteers. Then, screening was conducted for those who chose to participate in the study. During screening, a selection form was completed and, when they met the eligibility criteria, respecting the inclusion and non-inclusion criteria, a nutritional assessment was performed.

Eligibility criteria were male; age 18–40 years; body mass index (BMI) 27.0–34.9 kg/m^2; waist circumference ≥ 90 cm; fasting capillary blood glucose 70–99 mg/dL; capillary cholesterol < 240 mg/dL; capillary triglycerides < 150 mg/dL; and absence of acute and chronic diseases other than obesity and food allergies. Non-inclusion criteria were individuals with eating disorders (lactose or gluten intolerances), the use of medications known to affect appetite, glycaemia, and energy or lipid metabolism; alcohol consumers and/or smokers; use of dietary fiber supplement; and recent changes in body weight (<5 kg in the past three months). Exclusion criteria were no consumption of the test food for more than six days (consecutive or not) and use of antibiotics during the intervention. Only men were included in the study for two main reasons: the first is that males do not have as many hormonal changes as females, especially related to the menstrual period, considering that the woman's reproductive status linked to the ovarian cycle is imperative while examining disparities between sexes in terms of health and vulnerability to diseases, scrutinizing the impacts of drugs, and exploring behavior. In addition, men suffer fewer changes in hormones associated with body composition compared to women, which meant that, in this case, male subjects were recruited for the study. The second reason is the fact that a previous experimental study investigated the consumption of sorghum in male Wistar rats fed a diet high in saturated fat (SFA) to induce overweight, since the consumption of this type of diet is associated with the development of obesity, so this study can be considered a sequel [23, 24]. The individuals included in this study did not have diabetes, disorders in lipid metabolism, and/or arterial hypertension. The intervention time of 8 weeks was calculated by estimating a weight loss of 4 kg in two months, providing a weight loss of 3 to 5% of body weight, which is considered a beneficial effect for overweight and obese individuals [30].

2.4. Interventions and Test Meals

Volunteers attended the laboratory daily to consume the test preparations (breakfast cereal with milk or dairy product, or a drink) at breakfast and guided to follow a 500 kcal/day caloric restriction diet. On the weekends, the volunteers consumed the meals at home. The volunteers were instructed to consume the entire amount of test food provided. Thus, meals were offered to both groups in the form of breakfast cereal (extruded sorghum × wheat) added with whole milk or light whole yogurt, or a drink

(extruded flours were mixed with skimmed milk powder, powdered juice, and sweetener) (Supplementary Figure S1). Variation between meal types (breakfast cereal/drink) was defined to increase adherence to the intervention. The amount of sorghum consumed daily was based on a usual portion of breakfast cereal (40g) and on the volume of sorghum that the subjects could ingest in a meal based on previous tests [22]. The amount of extruded wheat (38 g) was adequate compared to the same nutritional composition of sorghum (40 g). Further, the meals had the same amount of milk or dairy products; however, for each meal the macronutrient content was different due to the ingredient composition. However, in each extruded sorghum or wheat meal a similar content of calories, macronutrients, and dietary fiber was offered [21]. All volunteers consumed milk, yogurt, or drink the same number of times. A dietitian professional calculated the caloric restriction (500 kcal/day) for both groups to achieve weight loss of 2 kg/month. At the beginning of the intervention, the individuals replied to a food frequency questionnaire (FFQ) to estimate the caloric consumption before the intervention. Then, they received a food prescription according to their individual energy and nutrient requirements, restricted to 500 kcal/day. The volunteers received a substitutive list of food, by food groups. During the intervention period, the adherence to the diet prescription was assessed using the food record of 3 non-consecutive days, including a weekend. They were guided to maintain the physical activity level. The dietary prescription was based on Dietary Reference Intakes (DRIs) [31], and the volunteers received a replacement food list organized by food groups.



Supplementary Figure 1. Details of the meals consumed throughout the study.

2.5. Outcomes

The primary outcome of this study was the effect on anthropometric measurements such as body weight, waist circumference, sagittal abdominal diameter, waist-to-height ratio, and body fat percentage. The second outcome was the effect of the interventions on intestinal health, including short-chain fatty acid synthesis, fecal pH, gut microbiota composition, and inflammatory markers, such as interleukin 6, interleukin 10, and tumor necrosis factor-α.

2.6. Randomization, Allocation, and Sample Power

Participants were randomized using a random sequence, according to the corresponding numbers received prior to the intervention, using a Microsoft Excel 365 software spreadsheet for distribution between groups. Allocation concealment occurred so that investigators or research participants did not know whether the next eligible participant would receive a treatment or control intervention. This was masked until such

time as the intervention was initiated. A power of 94.54% was obtained considering the mean difference in body fat percentage between the groups (effect size = 1.64), bilateral α of 5%, and sample size of the groups. Calculations were performed using the GPower software version 3.1.9.7.

2.7. Assessment of Anthropometry and Body Composition Markers

Anthropometric and body composition evaluations were performed by a single trained researcher. Body weight was assessed using an electronic platform scale (Model 2096 PP, Toledo, Brazil), with a capacity of 150 kg and precision of 50 g. Height was measured using a stadiometer (Alturexata®, São Paulo, Brazil) fixed to the wall [32]. BMI (kg/m2) was computed and classified according to the WHO (2000). Waist circumference (WC) was measured to the nearest 0.1 cm with a flexible band at the midpoint between the last rib and the iliac crest (World Health Organization, 2000). WC ≥ 90 cm was adopted as a criterion to classify abdominal obesity. Sagittal abdominal diameter (SAD) was measured with a portable sliding beam abdominal caliper (Holtain Kahn Abdominal Caliper®, Holtain Ltd., Dyfed, Wales, UK) at the midpoint between the iliac crests. Waist-to-height ratio (WHtR) was calculated as WC divided by height (cm). Body composition was assessed by dual-energy X-ray absorptiometry (DXA) (GE Healthcare, Lunar Prodigy Advance), and the results were expressed as total body fat (%). Body fat % higher than 25% was used to classify individuals with overweight [33].

2.8. Inflammatory Markers

All participants underwent overnight fasting, and blood samples were collected at baseline and endpoint. Enzyme-linked immunosorbent assay (ELISA) was utilized to analyze the levels of inflammatory markers, including interleukin 6, interleukin 10, and tumor necrosis factor-α. The Milliplex Map Human Cytokine/Chemokine Magnetic Bead kit (HCYTOMAG-code 60K, Millipore, Darmstadt, Germany) was employed for the accurate measurement of these markers in the blood samples.

2.9. Fecal Samples

At baseline and endpoint (8 weeks after) of the intervention, study participants were instructed to provide a fecal sample as close to the collection time as possible. If immediate processing was not feasible, the samples were refrigerated at 4 ◦C for a maximum of 12 h. Participants transported the fecal samples to the laboratory in polystyrene containers along with ice cubes to ensure temperature preservation. Upon arrival, the samples were weighed and transferred into micro tubes, then subsequently stored at −80 ◦C until analysis.

2.10. Fecal pH

The fecal pH level was assessed using a digital pH meter T-1000 (Tekna, São Paulo, Brazil). To perform the measurement, one gram of feces was transferred into a 15 mL falcon-type tube, and then 10 mL of ultrapure water was introduced. The mixture was thoroughly homogenized by vortexing, and the pH reading was obtained using a digital pH meter.

2.11. Organic Acid Analysis

To extract and identify organic acids from fecal samples, 500 mg of feces was weighed in duplicate and stored at −80 ◦C until further analysis. The frozen feces were thawed at room temperature (23 ± 2 ◦C) and homogenized with 1 mL of ultrapure water. Subsequently, the samples underwent centrifugation at 12,000× g for 10 min at 4 ◦C, using a Himac CT 15RE centrifuge (Hitachi, Tokyo, Japan). After centrifugation, the resulting supernatants were processed following the procedure described by Siegfried et al. (1984) [34].

High-performance liquid chromatography (HPLC) coupled with a refractive index (RI) Shodex RI-101 was employed to determine the levels of organic acids (acetic, succinic, formic, propionic, valeric, isovaleric, isobutyric, and butyric acid). The HPLC system used was Dionex Ultimate 3000 Dual detector HPLC (Dionex Corporation, Sunnyvale, CA, USA). A Bio-Rad HPX-87H column (300 mm × 4.6 mm) equipped with a Bio-Rad Cation H guard column was utilized under a column temperature of 45 ◦C, and the injection volume for each sample was 20 µL. The mobile phase for the chromatography was a mixture of concentrated sulfuric acid, EDTA, and ultrapure water,

flowing at a rate of 0.7 mL/min. To generate the standard curve for quantification, the following organic acids were used at specific concentrations: acetic, succinic, formic, propionic, valeric, isovaleric, isobutyric, and butyric acid. The concentrations for the acids were set at 10 mmol/L, except for isovaleric acid at 5 mmol/L and acetic acid at 20 mmol/L.

2.12. Fecal Sample DNA Extraction

The extraction of DNA from fecal samples was performed using the QIAamp Fast DNA Stool Mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's guidelines. Each extraction involved 200 ± 20 mg of feces as the starting material. Following purification, the isolated DNA was preserved at −80 ◦C until it was ready for subsequent analysis.

2.13. Quantitative Real-Time Polymerase Chain Reaction (qPCR) Analysis of Gut Microbiota DNA Concentration

In this study, the researchers aimed to quantify the concentration of gut microbiota DNA through quantitative real-time polymerase chain reaction (qPCR) analysis. The DNA concentration was determined by measuring the absorbance at 260 nm (A260), while its purity was assessed by calculating the A260/A280 ratio using a Multiskan ™ 1500 spectrophotometer (Thermo Fisher Scientifics; Waltham, MA, USA). For PCR analysis, group-specific primers (Supplementary Table S1) targeting the 16S rRNA gene were used. These primers were obtained from Alpha DNA e Diagnósticos Moleculares LTDA (Goiânia, GO, Brazil). The PCR reactions were performed using a CFX96 Touch ™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with the Primer Express software version 3.0.

Each well of the microtiter plate contained a reaction mixture consisting of QuantiNova SYBR® Green PCR Kit (Qiagen, Hilden, Germany), forward and reverse primers at con- centrations of 300 nM, and nuclease-free water, totaling 23 µL. To this mixture, 2.0 µL of each sample or standard was added, followed by brief centrifugation using a Labnet MPS1000 model, and then the plate was subjected to PCR analysis. The PCR amplification conditions included an initial denaturation of the DNA at 95 ◦C for 10 min, followed by 40 cycles of denaturation at 95 ◦C for 10s, primer annealing at the

optimized temperature for 20 s, and extension at 72 ◦C for 15s. After amplification, a melting curve analysis was performed to distinguish the targeted PCR product from non-specific products. To determine the bacterial concentration in each sample, Ct (cycle threshold) values were obtained and compared to standard curves generated from serial dilutions of DNA isolated from pure cultures of different reference strains. These strains, including Bacteroides ovatus ATCC 8483, Escherichia coli ATCC 11775, and Lactobacillus delbrueckii UFV H2b20 CCT 3744, were obtained from the American Type Culture Collection (ATCC) and the Tropical Cultures Collection. The standard curves exhibited a linear relationship between cell numbers and Ct values (r2 = 0.99–0.96), allowing for accurate quantification of bacterial concentrations in the samples.

Supplementary Table 1. PCR primers sequences used.

Group	Primer sequences	Standard genomic DNA	References
Total Bacteria	F-GCAGGCCTAACACATGCAAGTC R-CTGCTGCCTCCCGTAGGAGT	*Escherichia coli*	(Castillo et al., 2006)
Firmicutes	F-ATGTGGTTTAATTCGAAGCA R- AGCTGACGACAACCATGCAC	*Lactobacillus delbrueckii*	(Guo et al., 2008)
Bacteroidetes	F- CATGTGGTTTAATTCGATGAT R- AGCTGACGACAACCATGCAG	*Bacteroides ovatus*	(Guo et al., 2008)
Proteobacteria	F-CATGACGTTACCCGCAGAAGAAG R- CTCTACGAGACTCAAGCTTGC	*Escherichia coli*	(Friswell et al., 2010)

Oligonucleotides used as primers (F: forward; R: reverse) for quantification of 16S rDNA genes.

2.14. Analysis of Gut Microbiota

The sequencing of variable regions of the 16S rRNA gene of members of the Bacteria domains (V3–V4) was carried out by the company Argonne National Laboratory® (Lemont, IL, USA) using the MiSeq platform (Illumina, San Diego, CA, USA). Data processing and analysis were performed using the Mothur v.1.40.0 program [35]. The sequences were aligned using the SILVA v.132 16S rRNA gene reference database [36]. The taxonomic classification was carried out using the same database mentioned above. The operational taxonomic unit (OTU) was grouped with a 97% similarity cutoff. For alpha

diversity analysis, the indices Chao1, Shannon, and Simpson were applied. Beta diversity was assessed by Principal Coordinate Analysis (PCoA) based on the Bray–Curtis dissimilarity index and a similarity test for non-parametric data (ANOSIM, permutation number = 1000) [37].

Metagenome functional predictive analysis was carried out using PICRUSt2 software version 2.3.0. Normalized OTU abundance was identified, and the assigned functional traits were predicted based on reference genomes using the Kyoto Encyclopedia of Genes and Genomes (KEGG). The most abundant metabolic processes and significant fold-change differences in functional pathways between groups adopting an unpaired t-test (to analyze sorghum at two points of data collection) or paired t-test (for beginning and endpoint group analysis) (α = 95%) using STAMP software version 2.1.3. were plotted.

2.15. Statistical Analysis

For body composition, inflammatory markers, organic acids, fecal pH, and PCR, statistical analysis was performed using SPSS 20.0 software. The normality of the data was assessed by Shapiro–Wilk test. The average of each variable at the beginning and end of the intervention was compared using paired t-test or Wilcoxon test. The difference between the averages of the variables at the beginning and end of the intervention periods were compared using Student's t-test or the Mann–Whitney test. Averages and times were submitted to ANOVA followed by Newman–Keuls post hoc test or Kruskal–Wallis followed by Dunn's post hoc test. Cohen's d effect size was calculated from the difference between the means of the groups, divided by the mean of their SD. The magnitude of the effect was quantified as null (Cohen's d < 0.19), small (Cohen's d = 0.2 to 0.49), medium (Cohen's d = 0.5 to 0.79), large (Cohen's d = 0.8 to 1.29), and very large (Cohen's d > 1.30) [38].

For analysis related to gut microbiota composition the normality of the data was assessed by Kolmogorov–Smirnov test. The α diversity index and Firmicutes/Bacteroidetes ratio statistical analysis was performed using GraphPad version 9.0. Principal Coordinate Analysis (PCoA) based on the Bray–Curtis dissimilarity index was accessed using Past software version 4.0.5. Metagenome functional predictive

analysis was carried out using PICRUSt2 software version 2.3.0. Differences between averages were analyzed using STAMP software version 2.1.3. White's non-parametric t-test was performed followed by Benjamin FDR correction. Statistically significant p values associated with microbial clades and functions identified by LEfSe were corrected by Benjamin FDR correction. The level of significance in two-tailed tests was set at 5%.

3. Results

Thirty-six overweight men were recruited to the study. However, 12 not meeting inclusion criteria and 24 participants were included in study. Three participants left the study for personal reasons and 21 individuals finished the study (Fig 2). The average age of participants in the second phase (n=21) was 25.6 ± 4.6 years. At baseline participants of both groups did not present differences in the anthropometric, biochemical, and food consumption markers.

CONSORT FLOW DIAGRAM

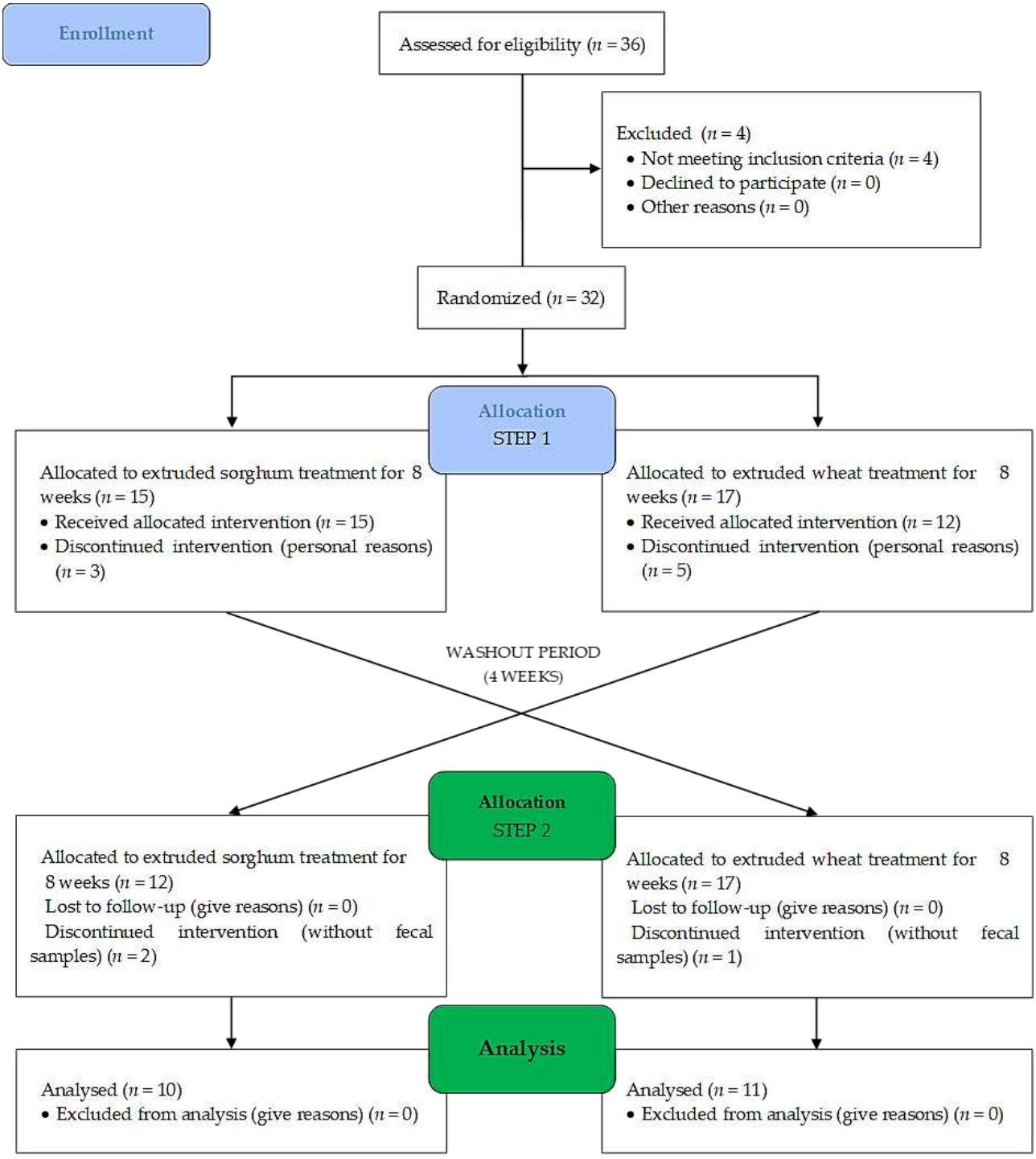


Figure 2. Study Design of intervention protocol. Were utilized in this study just data from time 2 of intervention.

3.1. Anthropometric measures and inflammatory markers

A significant weight loss (-1.25 ± 0.84 kg, p = 0.0015), and waist circumference (-2.33 ± 1.74 cm, p = 0.012), sagittal abdominal diameter (-0.70 ± 0.67 cm, p = 0.034), waist-to-height ratio (-0.011 ± 0.012, p = 0.047), and body fat percentage (-2.97 ± 1.91%, p = 0.013) reduction were observed after sorghum consumption for eight weeks compared to baseline (Table 1). Body fat percentage reduction was higher in the sorghum group when compared to the wheat group (-2.97 ± 1.91% vs -0.16 ± 1.47%, p = 0.005) with a very large effect size (Cohen's d = 1.67). Inflammatory markers unchanged after sorghum consumption. However, wheat consumption increased IL-6 levels (Table 1).

3.2. SCFAs synthesis and gut microbiota PCR

We did not observe significant changes in fecal concentrations of acetic, propionic, and butyric acids after sorghum or wheat consumption for 8 weeks (Table 2). Despite this, a numeric increase in butyric acid was observed in sorghum group compared to control with a small effect size (Cohen's = 0.35). In addition, fecal pH and intestinal microbiota composition did not change after 8 weeks of intervention groups (Table 2).

Table 1. Anthropometric variables and inflammatory markers serum concentrations of the participants at baseline and end-point by treatment.

Variables	Sorghum group (n=12)		Wheat group (n=10)		Δ p value[1]	Cohen's d (95% CI)
	Baseline	Endpoint	Baseline	Endpoint		
Weight (kg)	84.37 ± 7.09[a]	83.12 ± 6.96[b]	91.83 ± 11.49[a]	90.68 ± 11.10[a]	0.919	-0.06 (-0.9;0.78)
BMI (kg/m²)	28.16 ± 0.96[a]	27.14 ± 0.98[b]	28.83 ± 2.10[a]	28.49 ± 2.38[a]	0.907	-0.16 (-1.00;0.68)
WC (cm)	94.46 ± 3.47[a]	92.18 ± 3.84[b]	100.60 ± 6.10[a]	99.59 ± 6.79[a]	0.169	-0.66 (-1.52;0.20)
SAD (cm)	20.83 ± 1.20[a]	20.13 ± 0.99[b]	22.64 ± 1.98[a]	22.11 ± 1.46[a]	0.673	-0.10 (-0.94;0.74)
WHtR	0.54 ± 0.04[a]	0.53 ± 0.01[b]	0.57 ± 0.04[a]	0.56 ± 0.05[a]	0.346	-0.37 (-1.21;0.48)
BF (%)	29.15 ± 4.53[a]	26.18 ± 4.97[b]	31.77 ± 6.68[a]	31.61 ± 6.91[a]	0.005	-1.67 (-2.64;-0.7)
Inflammatory Markers	**Baseline**	**Endpoint**	**Baseline**	**Endpoint**	**Δ p value[1]**	**Cohen's d (95% CI)**
IL-6 (pg/mL)	0.87 ± 0.19[a]	1.15 ± 0.03[a]	0.77 ± 0.28[a]	1.01 ± 0.18[b]	0.703	-0.21 (-1.05;0.64)
IL-10 (pg/mL)	1.50 ± 1.01[a]	1.73 ± 0.88[a]	0.96 ± 0.49[a]	1.17 ± 0.62[a]	0.848	-0.09 (-0.93;0.75)
TNFα (pg/mL)	7.54 ± 5.10[a]	9.02 ± 5.34[a]	6.41 ± 2.60[a]	7.02 ± 2.74[a]	0.274	0.58 (-0.28;1.44)

n = 11 participants in each group. Data are expressed as mean ± standard deviation. BMI: body mass index, WC: waist circumference, SAD: sagittal abdominal diameter, WHtR: waist-to-height ratio, BF: body fat percentage, IL-6: interleucin 6, IL-10: Interleucin 10; TNFα: tumor necrosis fator alpha. Different letters at the same line for each group means $p<0.05$ from paired t test or for Wilcoxon matched-pairs signed-rank test, as statistical within group differences (baseline vs. endpoint).

[1] $p < 0.05$ from Student's t test or for Mann–Whitney test, as statistical significance between diet differences (sorghum vs. wheat).

Table 2. Effect of sorghum and wheat consumption on concentration of volatile fatty acids (VFA) of overweight subjects and percentages of target species in samples relative to total bacteria content.

Variables	Sorghum group		Wheat group		Δ p value between groups ²	Cohen's d (95% CI)
	Baseline (n=10)	Endpoint (n=10)	Baseline (n=11)	Endpoint (n=11)		
Total VFAs[1]	28.35 ± 11.83[a]	31.82 ± 11.78[a]	29.65 ± 10.41[a]	27.42 ± 12.87[a]	0.436	0.06 (-0.78;0.9)
Acetic acid	14.73 ± 1.96[a]	14.76 ± 3.7[a]	14.09 ± 3.52[a]	14.56 ± 5.40[a]	0.809	0.20 (-0.65;1.04)
Propionic acid	6.32 ± 2.7[a]	6.05 ± 3.12[a]	7.14 ± 3.99[a]	6.18. ± 2.90[a]	0.641	-0.06 (-0.9;0.78)
Butiric acid	4.90 ± 2.51[a]	5.46 ± 3.77[a]	5.89 ± 2.99[a]	5.01 ± 2.90[a]	0.456	0.35 (-0.5;1.19)
Fecal pH	6.73 ± 0.37[a]	6.68 ± 0.55[a]	6.85 ± 0.45[a]	6.96 ± 0.53[a]	0.254	-
Target taxon	**Baseline (n=10)**	**Endpoint (n=10)**	**Baseline (n=11)**	**Endpoint (n=11)**	**Δ p value between groups**[1]	
Bacteroidetes	108.89 ± 40.00[a]	106.51 ± 24.66[a]	73.96 ± 51.99[a]	106.39 ± 53.13[a]	0.115	-
Proteobacteria	0.19 ± 0.25[a]	0.20 ± 0.21[a]	1.30 ± 1.67[a]	3.56 ± 11.00[a]	0.075	-
Firmicutes	4.16 ± 1.53[a]	6.29 ± 4.54[a]	10.74 ± 11.30[a]	4.74 ± 2.97[a]	0.250	-

[1] Total VFA (mmol/l), acetic acid, propionic acid, butyric acid, isobutyric acid, formic acid, succinic acid, valeric acid and isovaleric acid (mol/100 mol). Different letters at the same line for each group means $p<0.05$ from paired t test or for Wilcoxon matched-pairs signed-rank test, as statistical within group differences (baseline vs. endpoint). ² $p < 0.05$ from Student's t test or for Mann–Whitney test, as statistical significance between diet differences (sorghum vs. wheat).

3.3. Gut microbiota analysis

Sequencing 16S rRNA gene from stool samples generated 1,383,378 raw sequences. After filtering and cleaning the sequences, 1,001,142 good quality sequences were obtained. The Good's coverage obtained in samples was > 99%, indicating good sequencing coverage. Raw filtered reads and normalized reads counts per group are showed in Supplementary Table 2.

Supplementary Table 2. Sequencing data at baseline and at the end of 8 weeks of interventions, according to each group.

Treatment		Good's coverage	Raw Sequences	After filtering and cleaning		After normalization	
			Reads	Reads	OTUs	Reads	OTUs
WG	B	0.997 ± 0.001	34755 ± 11648	25255 ± 8518	283 ± 60	12783 ± 39	315 ± 101
	E	0.997 ± 0.001	32591 ± 13435	23709 ± 9609	326 ± 74	12792 ± 19	290 ± 93
SG	B	0.997 ± 0.001	29315 ± 5997	21249 ± 3868	341 ± 116	12792 ± 22	274 ± 45
	E	0.997 ± 0.001	34942 ± 6300	25004 ± 3966	314 ± 115	12779 ± 33	292 ± 79

Values presented in mean ± standard deviation. WG: wheat group; SG: sorghum group; B: baseline; E: endpoint.

The α-diversity, microbial richness, and diversity were not different for Chao1 (Fig. 3A), Simpson (Fig. 3B) and Shannon (Fig. 3C) indexes, at baseline and endpoint intra- and inter- groups. The β-diversity intragroup by Principal coordinate analysis (PCoA) to sorghum group represented approximately 59.36% (Fig. 3D), and to wheat group represented approximately 61.30% of the dissimilarity in bacterial species composition (Fig. 3E). The β-diversity intergroup by PCoA, at baseline, represented ap-proximately 56.52% (Fig. 3F) and at endpoint represented 59.16% of dissimilarity in bacterial species composition (Fig. 3G). The clustering of the bacterial community did not present differences intergroup at phyla, classes, orders, families, and genera levels. Furthermore, in intragroup sorghum was observed a reduction of Clostridi-um_sensu_strictu1, Dorea and Odoribacter (Fig 4D-F) and an increase of CAG-873 and Turicibacter and (Fig. 4G and Fig.4H) at endpoint.

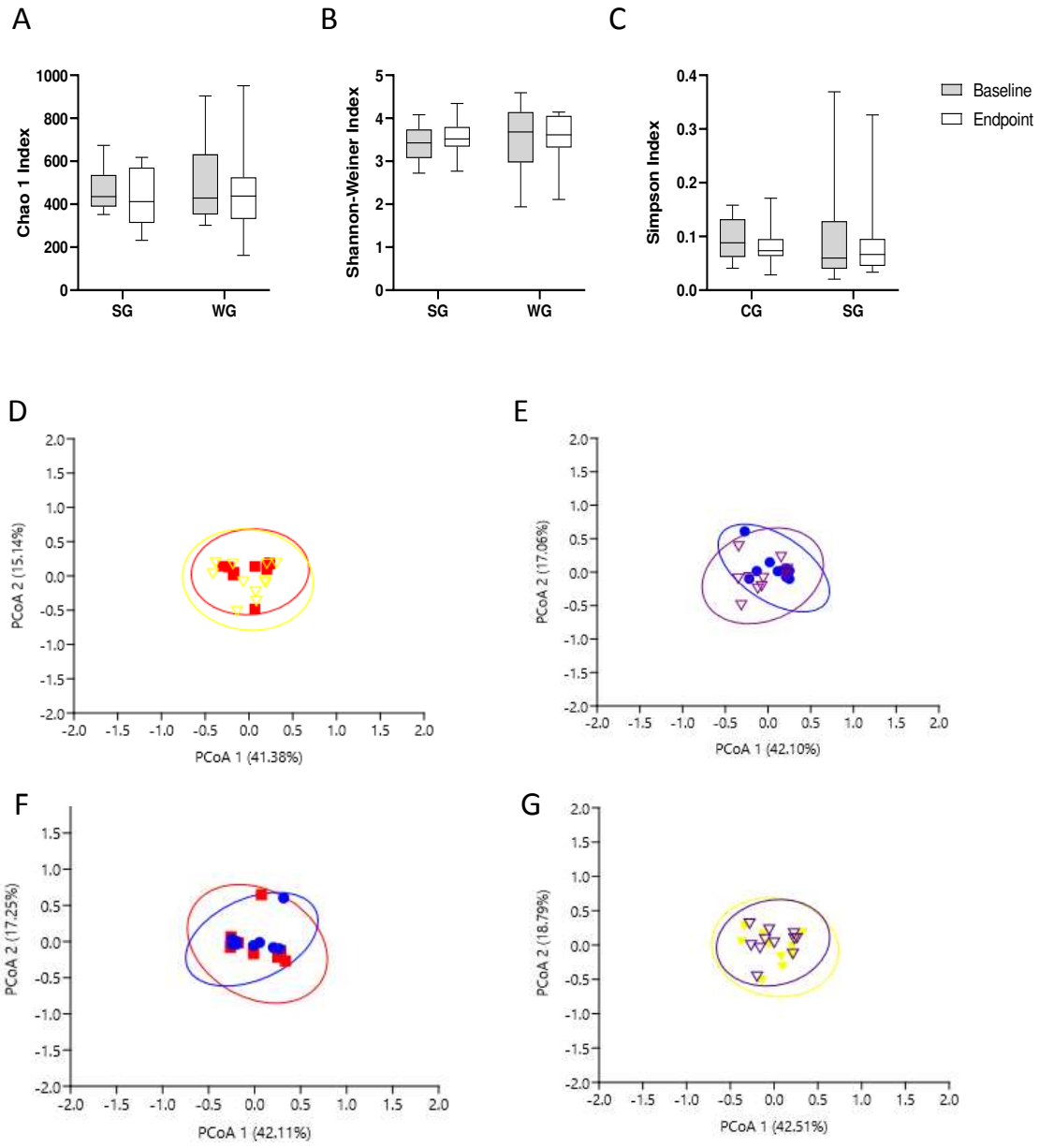
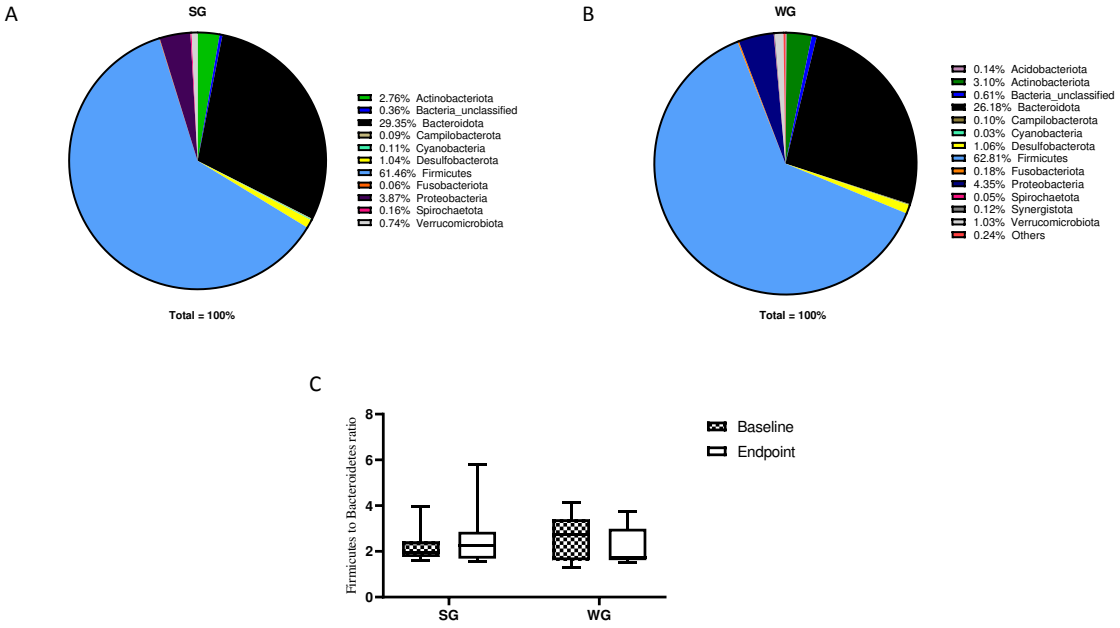


Figure 3. Alpha diversity index at baseline and enpoint after 8 weeks intervention with a test meals containing 40g of extruded sorghum by day estimated by (A) Chao, (B) Shannon-Weiner and (C) Simpson. Statistical analysis was perfomed using paired t-test or Wilcoxon test (sorghum and wheat groups baseline vs endpoint), or unpaired t-test or Wilcoxon test (sorghum x wheat group at baseline and endpoint). Significance was established $p < 0.05$. Analysis were performed using Graphpad version 9.0. SG: sorghum group; WG: wheat group. Beta diversity was estimated by Principal Co-ordinate Analysis (PCoA) based on Bray-Curtis similarity distance of cecal microbial communities in man

with obesity. (D) sorghum intragroup; (E) wheat intragroup; (F) wheat and sorghum inter-groups at baseline; (G) wheat and sorghum intergroups at endpoint. Permutational multivariate analysis of variance (PERMANOVA) were conducted in software STAMP version 2.0.2 considering α=5%.

Interventions groups presented 21 phyla, 35 classes, 77 orders, 134 families, 302 genera. All groups had 8 predominant phylum, some like Firmicutes (sorghum group: 61.45 ± 4.92%; wheat group: 62.81 ± 7.55%), followed by Bacteroidetes (sorghum group: 29.35 ± 4.67%; wheat group: 26.17 ± 6.92%), Proteobacteria (sorghum group: 3.87 ± 1.09%; wheat group: 4.34 ± 2.18%), Actinobacteria (sorghum group: 2.75 ± 1.18%; wheat group: 3.10 ± 1.02%) and Desulfobacterium (sorghum group: 1.04 ± 0.47%; wheat group: 1.06 ± 0.42%) (Supplementary Fig. 2A and 2B). At intra- and inter-groups firmicutes/bacteroidetes ratio was similar ($p > 0.05$) (Supplementary Fig. 2C).



Supplementary Figure 2. Relative abundance at phylum level at the end of intervention with consumption of extruded sorghum SC319 during 8 weeks in the cecal microbiota. (A) Bacterial composition at phylum level of sorghum group (n=10); (B) Bacterial composition at phylum level of wheat group (n=11); (C) Firmicutes to Bacteroidetes ratio at baseline and endpoint of sorghum and wheat groups. Data of cecal microbiota were analyzed by Dunn's test with FDR and Bonferroni corrections in software STAMP version 2.0.2, considering a significance of $p < 0.05$. Firmicutes to Bacteroidetes

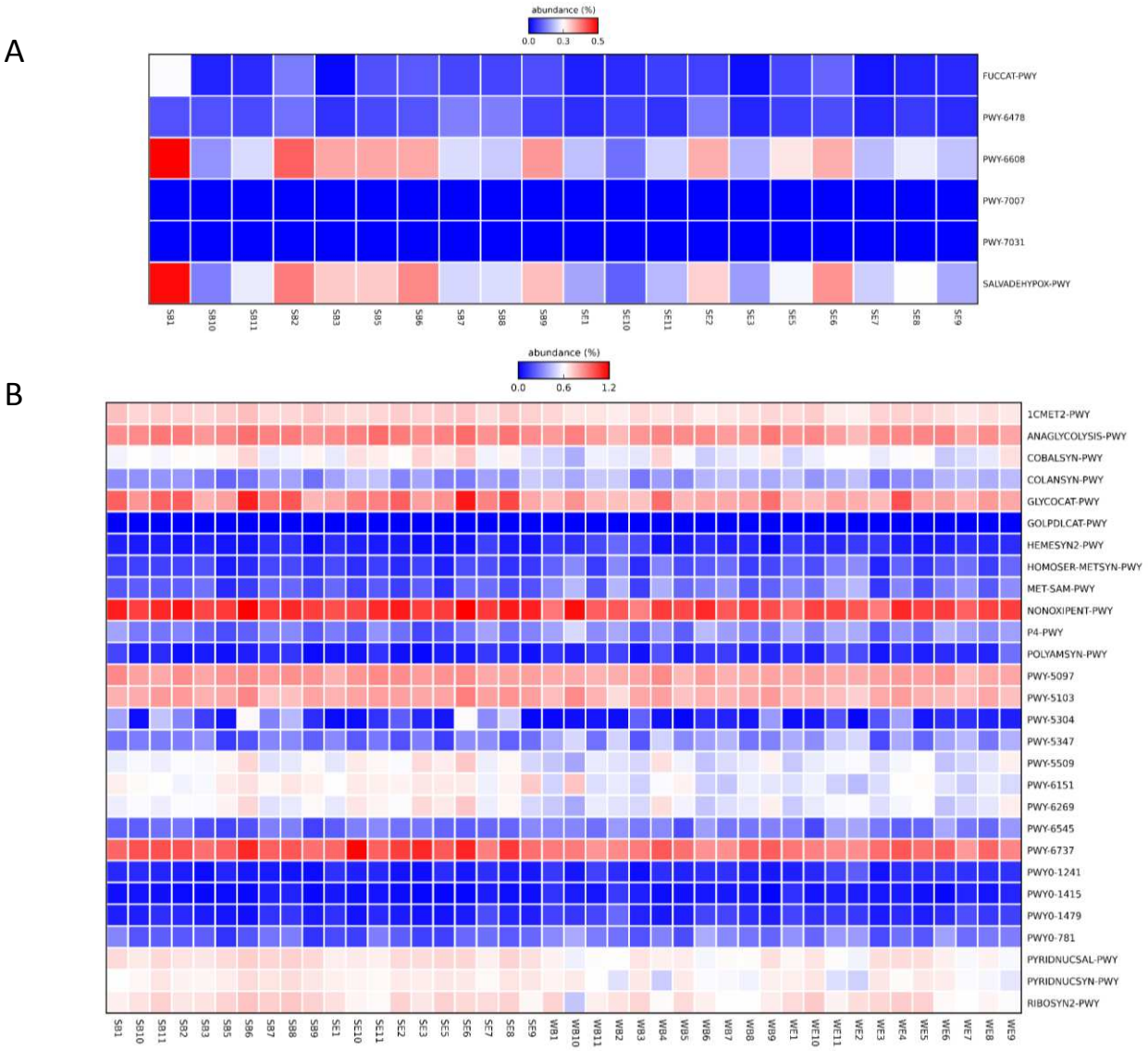
ratio was analyzed using paired t-test or Wilcoxon test (sorghum and wheat groups baseline vs endpoint), or unpaired t-test or Wilcoxon test (sorghum x wheat group at baseline and endpoint). Significance was established $p < 0.05$. Analyzes were performed using Graphpad version 9.0. SG: sorghum group; WG: wheat group.

3.4. Functional prediction analysis (KEGG analysis)

According with KEGG metabolic pathway analysis at intragroup, extruded sorghum SC319 consumption increased methyl ketone biosynthesis (p=0.04), protein N-glycosylation (bacterial) (p=0.01) while decreased GDP-D-glycero-α-D-manno-heptose biosynthesis (p=0.01), adenosine nucleo-tides degradation II (p=0.02), guanosine nucleotides degradation III (p=0.02), and fu-cose degradation (p=0.04) metabolic pathways (Supplementary Fig. 3A).

Comparing extruded sorghum and wheat groups metabolic pathways by KEGG intergroup analysis at endpoint, sorghum consumption increased S-adenosyl-L-methionine cycle I (p<0.001), NAD salvage pathway I (p<0.001), N10-formyl-tetrahydrofolate biosynthesis (p<0.001), starch degradation V (p<0.001) (Fig 4A), pentose phosphate pathway (non-oxidative branch) (p=0.003), L-lysine bio-synthesis VI (p=0.005), glycolysis III (from glucose) (p=0.006) (Fig 4B), glycogen degradation I (bacterial) (p=0.007) (Fig 4C), colonic acid building blocks biosynthesis (p=0.012), NAD biosynthesis I (from aspartate) (p=0.013), adenosylcobalamin biosyn-thesis from cobyrinate a,c-diamide I (p=0.017), adenosylcobalamin salvage from co-binamide II (p=0.021), adenosylcobalamin salvage from cobinamide I (p=0.038), su-perpathway of sulfur oxidation (Acidianus ambivalens) (p=0.043), L-isoleucine bio-synthesis III (p=0.045), flavin biosynthesis I (bacteria and plants) (p=0.047), and re-duced L-methionine biosynthesis I (p=0.003), superpathway of S-adenosyl-L-methionine biosynthesis (p=0.003), superpathway of L-methionine bio-synthesis (transsulfuration) (p=0.004), aspartate superpathway (p=0.006), ADP-L-glycero-β-D-manno-heptose biosynthesis (p=0.012), tRNA processing (p=0.016), pyrimidine deoxyribonucleotides de novo biosynthesis III (p=0.021), super-pathway of L-lysine, L-threonine and L-methionine biosynthesis I (p=0.023), super-pathway of heme biosynthesis from uroporphyrinogen-III (p=0.024), heme biosynthesis II (anaerobic) (p=0.034), superpathway of glycerol

degradation to 1,3-propanediol (p=0.047), and superpathway of polyamine biosynthesis I (p=0.048) metabolic path-ways compared to wheat consumption (Suplemmentary Fig. 3B).



Supplementary Figure 3. Microbial metabolic pathways in stool of man with obesity. (A) Microbial metabolic pathways in stool of man with obesity that received a meal containing 40g of extruded sorghum for 8 weeks at baseline and endpoint weeks by White's parametric t test. (B) Microbial metabolic pathways in stool of man with obesity that received a meal containing 38g of extruded wheat for 8 weeks at baseline and endpoint weeks by White's parametric t test. All metabolic pathways that showed differences were appointed in the figures. Statistical analyzes were performed in STAMP software

considering α=0.05. SB: sorghum group at baseline; SE: sorghum group at endpoint; WB: wheat group at baseline; WE: wheat group at endpoint.

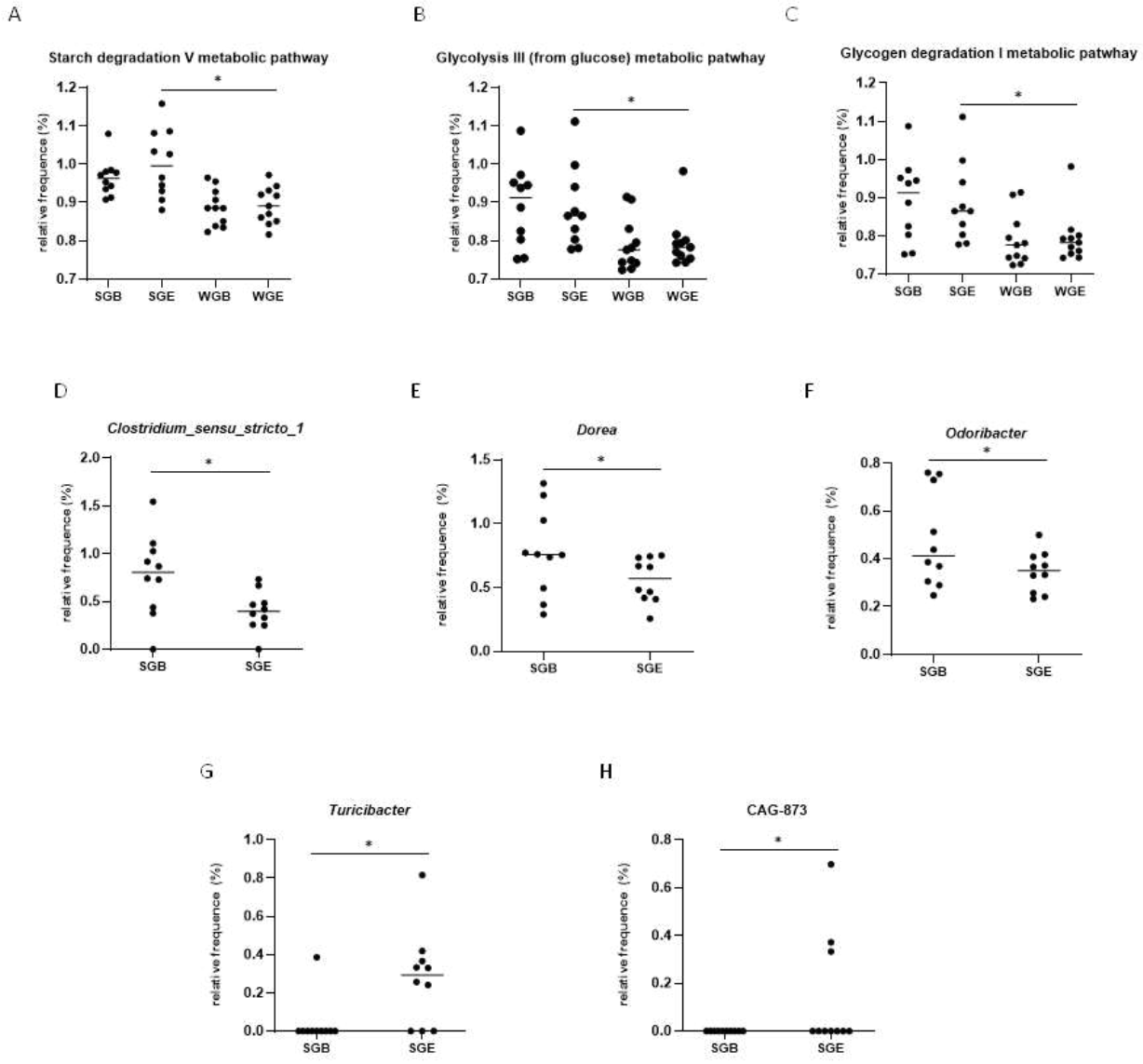
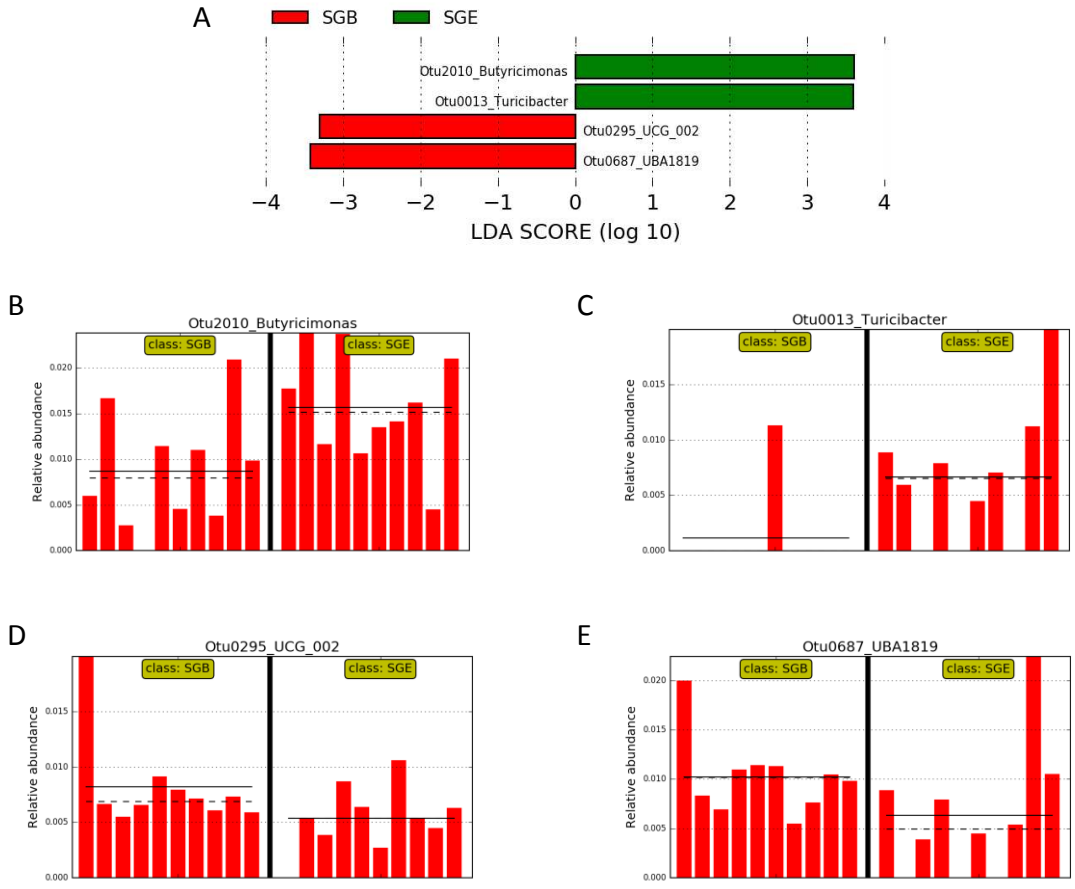


Figure 4. Microbial metabolic pathways in stool of man with obesity. (A) starch degradation V metabolic pathway intergroup, (B) Glycolysis III (from glucose) metabolic pathway intergroup and (C) Glycogen degradation I metabolic pathway intergroup in stool of overweight man at endpoint by White's parametric t test by White's parametric t test. (D) Clostridium_sensu_stricto_1 sorghum intragroup relative abundance. (E) Dorea sorghum intragroup relative abundance. (F) Odoribacter sorghum intragroup relative abundance. (G) Turicibacter sorghum intragroup relative abundance. (H) CAG-873

sorghum intragroup relative abundance. Statistical analysis were performed in STAMP software considering α=0.05) SGB: sorghum group at baseline; SGE: sorghum group at endpoint; WGB: wheat group at baseline; WGE: wheat group at endpoint.

3.5. LEfSE analysis

All OTUs were analyzed by Linear Discriminant Analysis Effect Size (LEfSe) to identify dominant cecal microbiota and intestinal biomarkers using taxonomy (Supplementary Fig. 4A). As a result, 4 dominant OTUs with effect size >3 was identified. The sorghum and wheat groups did not show a higher number of dominant taxa in in-tergroup at baseline and endpoint. For intragroup, sorghum showed a higher size ef-fect on the dominant community to *Butyrucunibas* (Supplementary Fig. 4B) and *Turicibacter* (Supplementary Fig. 4C) at endpoint, and at baseline to UCG_002 (Supple-mentary Fig. 4D) and UBA1819 (Supplementary Fig. 4E). This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.



Supplementary Figure 4. Histogram of LEfSe method to compute linear discriminant analysis (LDA) scores of differences in dominant microorganisms between baseline and endpoint of sorghum group. SGB: sorghum group at baseline; SGE: sorghum group at endpoint. Significant differences were considered with $p<0.05$.

4. Discussion

This study investigated the effects of consumption of extruded SC319 whole sor-ghum or extruded whole wheat associated with a daily 500kcal diet energy-restriction, on modulation of intestinal health with a focus on gut microbiota, short-chain fatty acids production and fecal pH, and on weight loss and inflammation markers, by 8 weeks. Extruded sorghum and whole wheat did not show differences in α and β- di-versity indexes, short-chain fatty acids synthesis, and fecal pH. However, sorghum consumption promoted alterations at the genus level compared to baseline and endpoint, reducing Clostridium_sensu_stricto 1, Dorea, and Odoribacter, and increasing CAG-873 and

Turicibacter. The sorghum group showed a higher weight loss relative to the whole wheat group. Probably these effects are due to sorghum's chemical composi-tion in 3-deoxyanthocyanins, resistant starch and, proanthocyanidins [18].

Extruded whole wheat and sorghum have a similar concentration of total dietary fiber promoting similar intake of dietary fiber when were consumed by 8 weeks, which resulted in similar effects on fecal pH, short chain fatty acid synthesis, and microbial diversity. However, sorghum SC319 promoted a weight loss intragroup, probably due it's the unique phenolic compounds, 3-deoxyanthocianins, plus proanthocyanins which complex with starch, reducing their digestibility [39], and turn it them available to be fermented by the intestinal microbiota, favoring weight loss. These effects were confirmed in our study by KEGG analysis, which sorghum group activated starch and glycogen degradation pathways compared to wheat.

The extruded SC319 sorghum consumption demonstrated a tendency to reduce proteobacteria phylum, which most of them are pathogenic. At genus level, Clostridium sensu stricto 1, Dorea and Odoribacter were reduced at endpoint. Clostridium sensu stricto 1 is a cluster of Clostridium species that includes commensal and pathogenic species. Members of this cluster exhibit a consistent capacity to synthesis butyrate [40], which may did not alter butyrate synthesis in the sorghum group. Dorea is a microorganism that is positively associated with prediabetes and glucose concentrations [41]. Alt-hough in our study the individuals did not present change in glucose metabolism, the reduction of Dorea can be considered a protective effect for hyperglycemia, insulin re-sistance and other disturbances in glucose metabolism, resulting in diabetes mellitus. Odoribacter was correlated to expression of inflammatory cytokines such as TNFα and IFNγ [42]. However, in our study no difference was observed in TNFα serum concen-trations. Furthermore, extruded SC319 sorghum consumption increased Turicibacter and CAG_873 at genus level. A study showed that Turicibacter is correlated with pro-inflammatory cytokines IL-1 [43], and other pointed in overall immune activation [44], however we did not observed difference on inflammation markers.

We observed that overweight male individuals intaking 40g/day of extruded whole sorghum SC319, rich in 3-deoxyanthocyanidins and proanthocyanidins, by 8 weeks, did not change the anti-inflammatory markers. Previous study with these indi-viduals also

showed absence of effects on the antioxidant response [30]. On the other hand, the overweight males' individuals intaking 38g/day of extruded whole wheat increased IL-6 concentrations. Probably the wheat allergenicity promoted this effect because the sensitization at gluten can moderate the antigen-specific inflammatory markers such as IL-6 [45]. Further, we did not observe modulation in microbiota composition, which did not change SCFAs at inter and intragroups in wheat group.

The intake of extruded sorghum is an alternative to wheat consumption, which can improve the life quality of populations, specially to individual with celiac disease. Thus, sorghum is a good strategy to substitute wheat intake, since it did not have al-lergen compounds, and when combined with other ingredients can be used in the pro-duction of beverage, pasta, bread, cakes, biscuits, among other bakery products. The special technofunctional and biofunctional properties of kafirins such as non-allergenic and slow digestibility by mammalian proteases amplifying applications of sorghum flour in food production [46]. Furthermore, the phenolic compounds pre-sent in sorghum grains may exert other beneficial effects on the body, such as improv-ing glucose metabolism and adipogenesis [47], and non-communicable chronic diseas-es [23,48].

Although no effects were observed on the composition of the intestinal microbiota in extruded whole sorghum and wheat groups, sorghum can be an alternative for wheat, since it it's non-allergenic cereal and demonstrated enhance the weight loss and showed similar results to wheat on SCFAs synthesis and fecal pH.

The strength of the present study is to associate two interventions, namely the use of extruded whole sorghum, a prebiotic, with caloric restriction diet, investigating sys-temic effects, on body composition, inflammatory markers and intestinal health, en-couraging consumption of this cereal in the human population. The limitations of this study can be: the small number of volunteers in each group; the unusual consumption of sorghum for the most part of worldwide population, besides be considered a whole ingredient for Brazilian Official Institutions; and the time of intervention, eight weeks couldn't be a sufficient time to find effects on intestinal health; gut microbiota compo-sition; and the data collection, just in the second phase of a crossover study, once one the weight loss was more effective in the first weeks with a caloric restricted diet [49]. Further, next study needs considerate the increase of sorghum intake because cereals are a major component of

the diet, and we can use it to prepare others food product. Thus, our study revealed that the daily consumption of 40g extruded sorghum SC319 for 8 weeks enhances weight loss probably due the phenolic compounds, 3-deoxyanthocianins, plus proanthocyanidins. Furthermore, extruded whole sorghum has a similar effect to extruded whole wheat on intestinal health, with no differences for intestinal microbiota, SCFA synthesis and fecal pH.

5. Conclusions

In Brazilian man with overweight, SC319 extruded sorghum was able intergroup to reduce the body fat percentage, without difference to SCFAs synthesis, fecal pH, α and β diversity, and inflammatory markers. Besides this, the extruded SC319 con-sumption for 8 weeks improved metabolic pathways related to carbohydrate metabo-lism compared to extruded wheat consumption. Sorghum consumption intragroup improved weight loss and decreased anthropometric measures and relative abundance of harmful microorganism at genus level.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1. Details of the meals consumed throughout the study; Figure S2. Relative abundance at phylum level at the end of intervention with consumption of extruded sorghum SC319 during 8 weeks in the cecal microbiota. (A) Bacterial composition at phylum level of sorghum group (n=10); (B) Bacterial composition at phylum level of wheat group (n=11); (C) Fir-micutes to Bacteroidetes ratio at baseline and endpoint of sorghum and wheat groups. Data of cecal microbiota were analyzed by Dunn's test with FDR and Bonferroni corrections in software STAMP version 2.0.2, considering a significance of $p < 0.05$. Firmicutes to Bacteroidetes ratio was analyzed using paired t-test or Wilcoxon test (sorghum and wheat groups baseline vs endpoint), or unpaired t-test or Wilcoxon test (sorghum x wheat group at baseline and endpoint). Significance was estab-lished $p < 0.05$. Analyzes were performed using Graphpad version 9.0. SG: sorghum group; WG: wheat group.; Figure S3. Microbial metabolic pathways in stool of man with obesity. (A) Microbial metabolic pathways in stool of man with obesity that received a meal containing 40g of extruded sorghum for 8 weeks at baseline and endpoint weeks by White's parametric t test. (B) Microbial metabolic pathways in stool of man with obesity

that received a meal containing 38g of extruded wheat for 8 weeks at baseline and endpoint weeks by White's parametric t test. All metabolic pathways that showed differences were appointed in the figures. Statistical analyzes were per-formed in STAMP software considering α=0.05. SB: sorghum group at baseline; SE: sorghum group at endpoint; WB: wheat group at baseline; WE: wheat group at endpoint.; Figure S4. Histogram of LEfSe method to compute linear discriminant analysis (LDA) scores of differences in dominant microorganisms between baseline and endpoint of sorghum group. SGB: sorghum group at baseline; SGE: sorghum group at endpoint. Significant differences were considered with $p<0.05$.; Table S1. PCR primers sequences used.; Table 2. Sequencing data at baseline and at the end of 8 weeks of interventions, according to each group.

Author Contributions: Haira Guedes Lúcio: Conceptualization; Data curation; Investigation; Formal analysis; Roles/Writing original draft; Software. Pâmela Cristina Anunciação: Conceptu-alization; Data curation; Investigation; Formal analysis. Barbara Pereira da Silva: Conceptualiza-tion; Data curation; Investigation; Methodology; Project administration. Alessandra da Silva: Data curation; Investigation; Methodology. Carlos Wanderlei Piler de Carvalho: Supply of Raw Material, Resources; Methodology; Funding acquisition. Valéria Aparecida Vieira Queiroz: Material, Re-sources; Methodology; Funding acquisition. Helena Maria Pinheiro Sant'Ana: Data curation; In-vestigation; Methodology; Validation; Writing review and editing. Hércia Stampini Duarte Mar-tino: Conceptualization; Data curation; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Roles/Writing original draft; Writing review & editing.

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Institutional Review Board Statement: The study was conducted in accordance with the Decla-ration of Helsinki, and approved by by the Human Research Ethics Committee of the Federal University of Viçosa, Brazil (CAAE: 13630513.0.0000.5153) in October, 13, 2014.

Informed Consent Statement: All participants were informed about the objectives of the study and provided written informed consent.

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6.3. PAPER 3

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Article

A Symbiotic Meal Containing Extruded Sorghum and Probiotic (*Bifidobacterium longum*) Ameliorated Intestinal Health Markers in Individuals with Chronic Kidney Disease: A Secondary Analysis of a Subsample from a Previous Randomized and Controlled Clinical Trial

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1. Introduction

Chronic kidney disease (CKD) is a clinical syndrome secondary to definitive alteration in the function and/or structure of the kidneys. It is characterized by its irreversibility and slow and progressive evolution, with a high risk of complications and mortality [1]. In 2017, CKD prevalence was between 9.1% and 13.4% in the worldwide population [2]. In addition, CKD is associated with a higher risk of cardiovascular disease, severity, and death [3].

Evidence suggests that CKD causes intestinal dysbiosis, with alterations in gut microbiota composition and intestinal functionality. These effects break the intestinal epithelial barrier and increase intestinal permeability, production, and entry of endotoxins, which favors systemic inflammation. The systemic inflammation promoted by CKD has been associated with a reduction in the populations of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* and an increase in potentially pathogenic bacteria such as *Escherichia coli* and *Clostridium* spp., microorganisms capable of producing toxins and harmful substances such as p-cresol and indoxyl sulfate, which directly interfere with intestinal health, favoring intestinal dysbiosis, increasing intestinal permeability, and reducing SCFA synthesis [4,5].

In addition, other factors such as the reduction in *Lactobacillus* and *Bifidobacterium*, as well as the low intake of dietary fiber, can also reduce the synthesis of SCFAs, favoring intestinal dysbiosis. The intestinal dysbiosis in CKD includes increased species of the genera Enterobacteriaceae and Pseudomonadaceae of the phylum Proteobacteria, *Bacteroidaceae*, and *Clostridiaceae* and decreased species of *Lactobacillacea*, *Prevotellaceae*, and *Bifidobacteriaceae* [4–6]. Surprisingly, acting as a vicious cycle, the species that expand in CKD are generally able to induce local and systemic inflammation directly and indirectly [6]. In this context, intestinal dysbiosis is associated with the progression of CKD, including proteinuric renal disorders and associated morbidities, including inflammation, hypertension, and diabetes [6,7].

The consumption of whole grains and cereals promotes healthy intestinal microbiota phenotypes, thus increasing their richness and diversity, as well as the production of short-chain fatty acids (SCFAs) [8]. The BRS 305 sorghum genotype is rich in dietary fiber, polyphenols, condensed tannin, and resistant starch compared to other genotypes

of the grain, such as red and white sorghum. Lopes et al. (2018), who used the same genotype and heat treatment for sorghum, revealed that extruded sorghum breakfast cereal was composed of 8.84% of total dietary fiber, of which 8.78% was insoluble fiber and 0.07% was soluble fiber, 71.04% of carbohydrates, 11.26% of proteins, 0.41% of lipids, 1.03% of resistant starch, 1.87% of ash, and 6.57% of moisture. The authors also observed that this extruded sorghum showed 340.33 $mg \cdot 100^{-1}$ g of phosphorus, 0.33 $mg \cdot 100^{-1}$ g of copper, 1.93 $mg \cdot 100^{-1}$ g of zinc, 1.45 $mg \cdot 100^{-1}$ g of magnesium, 102.00 $mg \cdot 100^{-1}$ g of calcium, 1.45 $mg \cdot 100^{-1}$ g of manganese, 5.59 $mg \cdot 100^{-1}$ g of iron, and 353.00 $mg \cdot 100^{-1}$ g of potassium. In terms of phenolic compounds, the extruded sorghum contains 1.10 ± 0.02 mg of gallic acid equivalent/g of sample of phenolic compounds and 0.71 ± 0.08 catechin equivalent/g of sample of condensed tannins (proanthocyanidins). The antioxidant activity observed in extruded sorghum was 4.68 ± 0.01 µmol trolox/g, the main 3-deoxyanthocyanins present in this sorghum were luteolinidin and 5-methoxyluteolinidin, and the authors detected traces of apigeninidin and 7-methoxyapigeninidin [16].

These compounds are associated with intestinal modulation, as they are non-digestible carbohydrates fermented by gut microorganisms, which increases SCFA synthesis [9]. The beneficial effect of sorghum BRS 305 consumption on health has already been demonstrated. In rodent models, the BRS 305 sorghum whole flour modulated the gut microbiota composition, the abundance of SCFA-producing bacteria, and intestinal morphology [10]. In CKD patients, a symbiotic meal containing BRS 305 extruded sorghum reduced uremic toxins, fecal pH, and urea concentration [11].

On the other hand, studies have demonstrated that probiotic supplementation, such as with *Bifidobacterium longum*, isolated or associated with other microorganisms, led to positive changes in the intestinal microbiota, as well as gastrointestinal symptoms, such as increased frequency of bowel movements in healthy subjects [12] or in subjects with persistent gastrointestinal symptoms, such as lactose intolerance [13]. In CKD patients, the administration of a symbiotic meal containing *Bifidobacterium longum* and *Lactobacillus acidophilus* alongside 60 mg of fructooligosaccharides (FOSs) for 60 days improved constipation symptoms and constipation-related quality of life [14]. Another study pointed out that the offer of a low-protein diet (0.6 g/kg/body weight) associated with a probiotic containing *Bifidobacterium longum* 5×10^9 CFU/mL and *Lactobacillus reuteri* 1

× 10^9 CFU/mL for 60 days reduced blood urea nitrogen and microbiota toxins, including indoxyl sulfate and lipoprotein-associated phospholipase A2 [15].

Thus, the present study aimed to investigate the effects of the consumption of a symbiotic meal containing extruded sorghum BRS 305 and *Bifidobacterium longum* 108CFU/100 mL strain on uremic toxin serum levels, SCFA production, and the gut microbiota composition of CKD patients. We hypothesized that this symbiotic meal may improve gut microbiota diversity, gastrointestinal symptoms, and SCFA production, in addition to reducing the blood level of uremic toxins in CKD patients.

2. Materials and Methods

2.1. Study Design

This is a controlled, randomized, single-blind clinical trial, conducted for 7 weeks, with CKD patients submitted to hemodialysis for at least 3 months. This study uses data from a subsample of a randomized, controlled, single-blind clinical trial previously conducted by our research group [11,16], since some volunteers donated stool samples for data analysis. The analysis of the intestinal microbiota and its association with markers of intestinal health have not been previously explored, which explains the need for this new investigation study. The participants included in the study were randomly allocated in a 1:1 ratio to receive a symbiotic meal containing extruded BRS 305 whole sorghum plus a probiotic milk containing *Bifidobacterium longum* 2.5 × 10^6 CFU/mL or pasteurized milk plus extruded corn (Figure 1).

This study was conducted according to the guidelines in the Declaration of Helsinki, and all procedures involving human subjects/patients were approved by the Human Research Ethics Committee of the Federal University of Vicosa, MG, Brazil (protocol number 701.796/2014). It was registered at www.ensaiosclinicos.gov.br under ID number RBR-2d9ny6. Written informed consent was obtained from all subjects/patients.

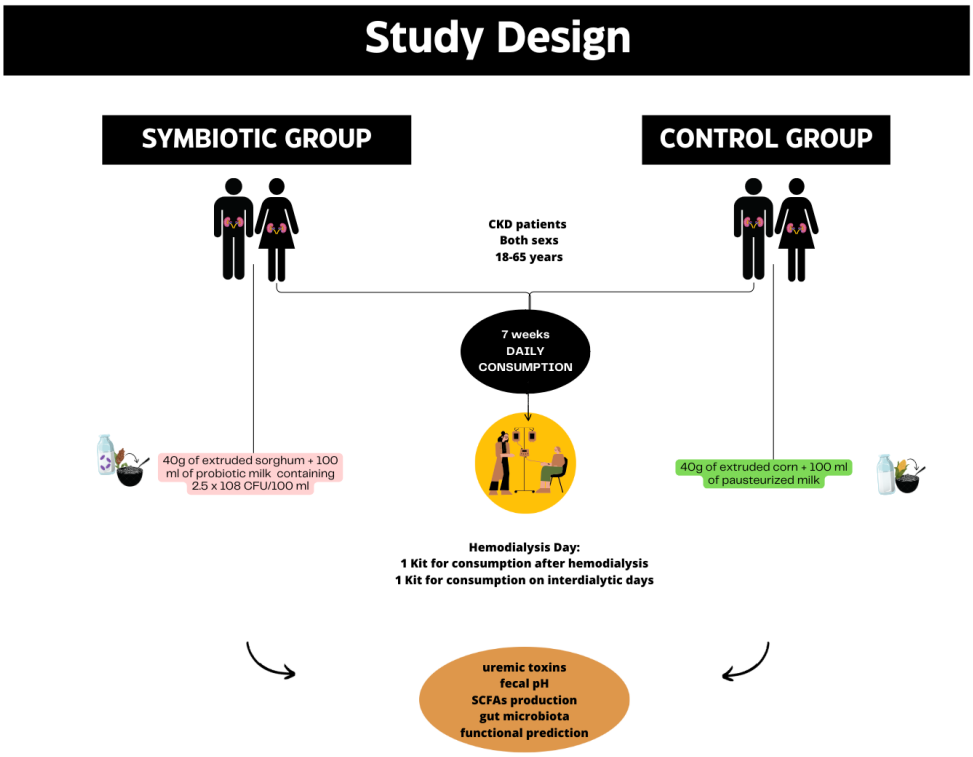

Figure 1. Study design of research.

2.2. Participants

The participants were recruited at the Hemodialysis Sector of Hospital São João Batista, located in Viçosa, Minas Gerais, Brazil, between March and June 2015. The first step to recruit volunteers was a meeting with the medical team from the hospital nephrology sector. Then, a conversation with each patient on hemodialysis was conducted to explain to them that a meal containing sorghum and milk would be offered daily for 7 weeks. The patients who agreed to participate in the study were screened to investigate whether they met the eligibility criteria and did not meet any of the non-inclusion criteria. Those who were fit to participate in the study signed the free and informed consent form and were included in the study. Further information about this step is available in Lopes et al. (2018) [16].

Our eligibility criteria were patients of both sexes with CKD who were at least 18 years of age who had been submitted to hemodialysis sessions three times a week in the Nephrology Sector of the Hospital São João Batista, Viçosa, Brazil, for at least three months. The non-inclusion criteria were the presence of auditory deficiency, autoimmune

diseases, hepatitis B and C virus infection, implanted catheters, hemodynamic instability, and lactose intolerance or discomfort when consuming milk (Figure 2). The exclusion criteria were the use of antibiotics during the intervention and non-consumption of the symbiotic meal for more than five days (consecutive or not). Participants were characterized by sociodemographic and clinical aspects before the intervention period. The collection of information on sociodemographic and clinical aspects was obtained from medical records and through questions asked in direct interviews, collecting information such as time of disease, associated morbidities, food consumption, measurement of weight and height, and calculation of BMI. They received the meals for a period of 7 weeks, respecting the routine of blood collection at the hemodialysis service.

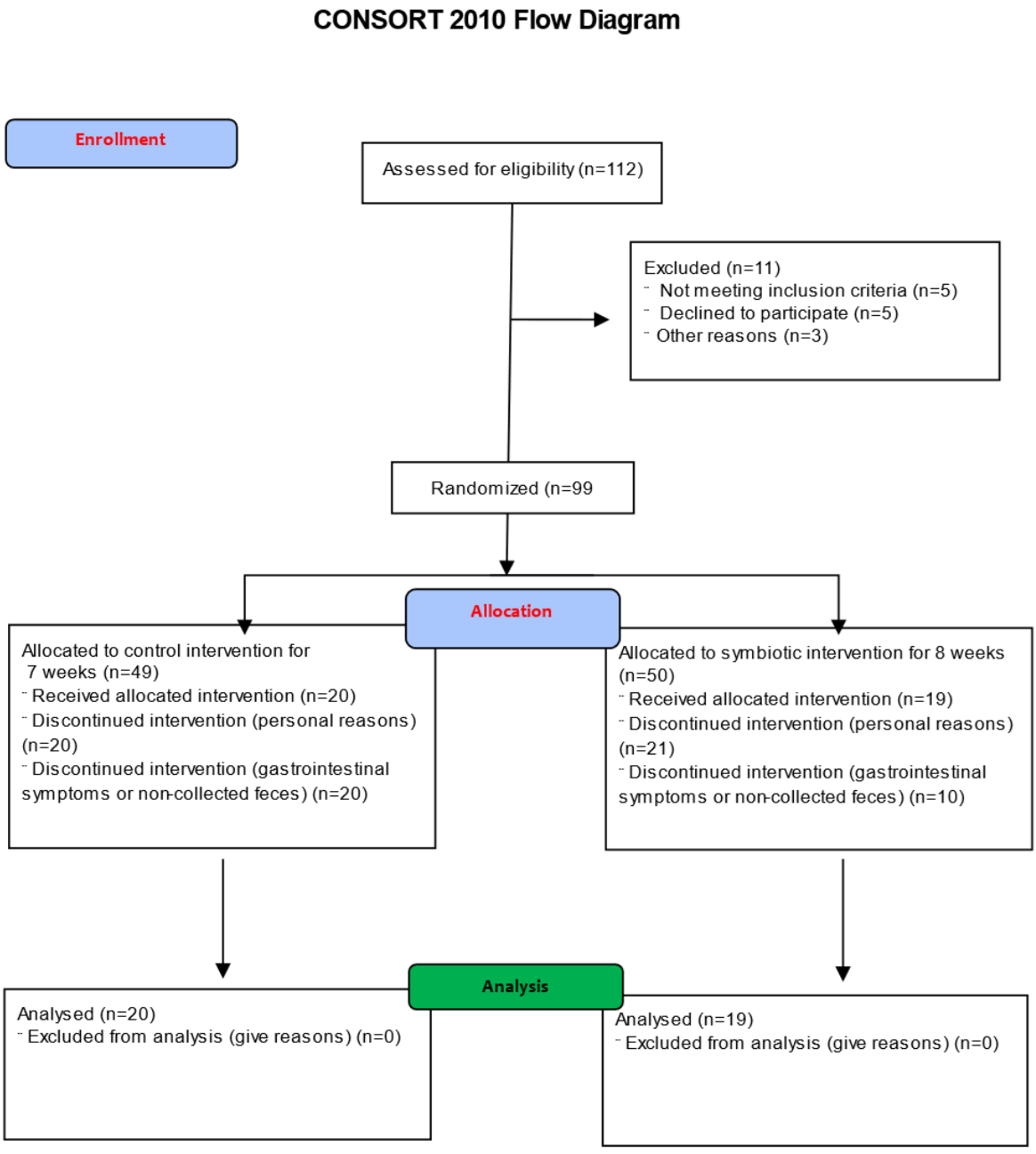

Figure 2. Consort flow diagram of study design of intervention protocol.

2.3. Randomization, Allocation, and Sample power

The sequence of allocation and attribution of participants in the two groups was randomly and blindly performed. Therefore, after the randomization, the participants were allocated into two intervention groups—the control group (CG) and the symbiotic group

(SG). The randomization was performed by drawing lots, using paper, in which one individual was drawn at random for the CG and another individual for the SG. The sample size required (n = 19/group) was based on the comparison of the means of serum urea levels, considering that it is a relevant variable in renal patients on dialysis. The present study presented 96.68% statistical power (α = 0.05) to detect a 22.9 reduction in serum urea levels, considering the baseline data of our subjects [17].

2.4. Raw Material and Meal Preparation

The sorghum grains were provided by Embrapa Milho e Sorgo, Sete Lagoas, MG, Brazil (−19.466672726578615° S, −44.17357630467641° W). The BRS 305 sorghum hybrid is rich in tannins and resistant starch. The grains used in this study were cultivated between April and July 2014, while the corn grains were obtained from the 2013/2014 crop. After harvest, the grains were packed in plastic bags and sent to Embrapa Agroindústria de Alimentos, Rio de Janeiro, Brazil, for the extrusion process. The chemical composition of extruded sorghum and corn was determined by the AOAC methodology (Table S2) [16,18].

Supplementary Table 2. Nutritional composition of sorghum and corn used in test meals.

Variables (g/100g)	Extruded Sorghum		Extruded Corn	
	Mean	**SD**	**Mean**	**SD**
Quantity	6.57	0.28	6.29	0.31
Ash	1.87	0.40	1.66	0.26
Lipids	0.41	0.13	0.81	0.91
Protein	11.26	1.04	12.66	0.81
Total Dietary Fiber	8.84	0.12	7.28	0.870
Soluble Fiber	0.07	0.55	0.87	0.35
Insoluble Fiber	8.78	1.70	6.41	0.90
Resistant Starch	1.03	0.00	0.15	0.02
Carbohydrates	71.04	0.74	71.30	0.39
Phenolic Compounds (mg galic acid equivalent/g sample)	1.10	0.02	0.81	0.01
Condensed Tannins (catechin equivalent/g sample)	0.71	0.08[a]	0.00[b]	0.00
Total Energy Value (kcal·100 g–1)	332.91[b]	1.67	343.13[a]	5.16

Values expressed in mean ± Standart deviation (SD). * Values expressed in dry matter. ** Mean of three replicates. Same letters on the line do not differ by t test for independent samples at 5% probability.

2.5. Interventions

All study participants were instructed to follow the usual pattern of diet, physical activity, and lifestyle. Their food intake and intestinal symptoms, as well as marker uremic and inflammatory symptoms, were assessed at the beginning and end of the intervention. Their clinical data, including dialysis time, were collected through the Metabolic Questionnaire adapted from Dixon [19], with multiple-choice questions, to identify the occurrence

of gastrointestinal symptoms. In addition, the Bristol scale [20] was applied to verify stool consistency, and the 24 h food recall was used to assess the pattern of food consumption.

The intervention consisted of two groups that received two dairy meals. The dairy meals used in the study were 100 mL of pasteurized milk plus extruded corn, which was supplied to the CG, and 100 mL of pasteurized milk with the probiotic bacteria *Bifidobacterium longum* (Granotec do Brazil S.A) 2.5×10^6 CFU/mL plus extruded sorghum added, supplied to the SG (Supplementary Figure S1). The beverages were produced weekly at the dairy plant of the Federal University of Viçosa. Milk pasteurized with probiotics was inoculated with a direct vat set (DVS)-type culture from Granotec do Brazil S.A. to present a minimum concentration of viable cells of *Bifidobacterium longum* of 10^8UFC/portion (100 mL of milk) [11]. The drinks were packaged in plastic bottles with an aluminum seal and labeled with the following information: date of manufacture, expiration date, and instructions for conservation and consumption. Storage was carried out under refrigeration at 4 ± 2 °C for up to 8 days to preserve the product.

Embrapa Agroindústria de Alimentos	**RESULTADO DE ANÁLISE**	Página 1 de 1 Número R015615

DADOS DO CLIENTE

Solicitante:	Eduardo Henrique Miranda Walter
Plano de Ação/Atividade:	02.11.07.020.00.04
Nome do Material:	Leite UFV - último
Número da Requisição:	0065/2015
Data de Entrada:	12/02/2015
Data da Análise:	23/02/2015
Código CRA:	1500759

RESULTADOS OBTIDOS

Nome da Análise	Identificação da Amostra
	Leite 1500759
Enumeração de Bifidobacterium spp (UFC/g)*	$1,9 \times 10^8$
Coliformes a 45º C (UFC/mL)	<3
Coliformes a 35º C (UFC /mL)	>1100
Salmonella sp. (ausência em 25mL)	Ausência
Contagem Padrão em Placas de Aeróbios mesófilas * (UFC/g)	>$2,5 \times 10^5$ estimado

" Valores estimados referem-se a contagens abaixo ou acima dos limites estabelecidos pela metodologia. Os limites estabelecidos são: * entre 25 e 250 UFC/g."

OBSERVAÇÕES

- Referência completa do método utilizado: *Compedium of Methods for the Microbiological Examination of foods* (2001).
- O Resultado da Análise refere-se exclusivamente à amostra ensaiada, sendo o solicitante responsável pela amostragem e coleta do material.
- Este Resultado de Análise só pode ser reproduzido por completo e com autorização deste laboratório.
- Prazo máximo para pedido de contra prova de análise é de 30 (trinta) dias, a partir da data de emissão do Resultado de Análise.

Rio de Janeiro, 09 de março de 2015.

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Janine Passos Lima da Silva
Responsável Técnica
Laboratório de Microbiologia
"Dispensa assinatura quando consultado eletronicamente"

Empresa Brasileira de Pesquisa Agropecuária – Ministério da Agricultura, Pecuária e Abastecimento
Av. das Américas, 29.501 – Guaratiba – 23020-470 – Rio de Janeiro, RJ – Telefone (0xx21) 3622-9600 – Fax (0xx21) 3622-9713
Homepage: https://www.embrapa.br/agroindustria-de-alimentos - correio eletrônico: ctaa.cra@embrapa.br

Supplementary Figure 1. **Analysis report on the cellular prediction of the probiotic drink containing *Bifidobacterium longum*.** The Embrapa laboratory, where the analysis carried, has an analysis accredited by ISO 17:025.

The participants in the control group received a food kit containing pasteurized milk- MP (100 mL) and extruded corn flakes (40 g). The intervention group received a kit with probiotic dairy drink (PDD) (100 mL) containing the *Bifidobacterium longum* (4×10^8 CFU/100 mL) strain and extruded sorghum flakes (40 g) (Table S1). The number of extruded cereals offered daily to the volunteers was based on a usual portion of breakfast cereal (40 g) [21]. Two food kits were given to the patients during hemodialysis. One of them should be consumed in the third hour of hemodialysis and the other on the interdialytic day. Patients that could not consume the products in the nephrology sector were instructed to take them home and consume them together on the same day. During hemodialysis, the participants answered a questionnaire about the consumption of the offered meals and the occurrence of adverse effects to assess their adherence to the study protocol and possible complications during the study.

Supplementary table 1. Nutritional composition of the control and test meals by 100g/portion.

Compounds (g/100g)	Drink Type				
	Extruded Sorghum Meal		Extruded Corn Moisture Meal		
	Mean	SD	Mean	SD	p value
Carbohydrate	33.42	0.29	33.52	0.15	0.61
Lipids	3.36	0.05	3.53	0.04	0.01
Protein	7.90	0.42	8.56	0.33	0.14
Total Dietary Fiber	3.54	0.45	2.91	0.35	0.04
Phenolic Compounds (mg galic acid equivalent/g sample)	44.20	0.84	32.60	0.36	<0.001
Condensed Tannins (catechin equivalent/g sample)	72.78	12.92	0.00	0.00	-
Total Energy Value (kcal/100g)	196.16	0.67	200.25	2.06	0.03

Values expressed in mean ± standard deviation (SD). * Values expressed in dry matter. ** Mean of three replicates. The data were submitted to unpaired t test at 5% probability, in Graphpad prism version 9.0.

2.6. Outcomes

Their feces were collected at the baseline and endpoint of the intervention for the analysis of their gut microbiota and short-chain fatty acids. Stool samples were collected by the participants in sterile bottles and kept at a temperature of −18° until the moment of hemodialysis. The participants transported the containers to the nephrology sector in Styrofoam packaging with ice cubes to maintain the temperature. The samples collected during hemodialysis were aliquoted and stored at −80 °C. The anthropometric measurements and collection of feces and blood samples were carried out in the beginning and at the end of the experiment.

The present study primarily detected the effects of the interventions on gut microbiota composition and markers related to intestinal health, such as gastrointestinal symptoms, short-chain fatty acid production, and uremic markers. The second outcome refers to food consumption and the effects on biochemical markers related to chronic kidney disease, such as urea and creatinine.

2.7. Anthropometric Measures

Body weight was evaluated using an electronic platform scale (Toledo Brazil, Model 2096 PP) capable of handling up to 150 kg and providing measurements with the precision of 50 g. Height was determined using a wall-mounted stadiometer (Alturexata®, Belo Horizonte, Brazil). Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters (kg/m^2) and categorized according to the World Health Organization (WHO) guidelines, 2000 [22]. These anthropometric assessments were conducted following the conclusion of the hemodialysis session, thus allowing a 30 min period of hemodynamic stabilization.

2.8. Analysis of the Consumption of Macronutrients

The consumption of macronutrients was assessed using a 24 h dietary recall, considering one day of hemodialysis, one interdialytic day, and one weekend. The Dietpro® nutrition software system (version 5i) was used to assess the intake of nutrients.

2.9. Uremic Markers

Uremic markers, such as p-cresyl sulfate, indoxyl sulfate, and indole-3-acetic acid, were analyzed and determined in plasma samples by HPLC, according to the method proposed by LOOR et al. (2009) [23]. The method is based on the acidification and centrifugation of the plasma sample. The clear supernatant obtained was injected on a reversed phase HPLC column.

2.10. Gastrointestinal Symptoms

The gastrointestinal symptoms were identified by applying multiple-choice questions taken from the Metabolic Questionnaire adapted from DIXON [19], and the shape of the stools was classified according to the Bristol scale [20].

2.11. Fecal SCFA Concentrations

Approximately 500 mg of feces was blended while adding 1 mL of ultrapure water to extract the short-chain fatty acids from the fecal samples. Next, the samples were subjected to centrifugation at 12,000× *g* for 10 min at the temperature of 4 °C using a Himac CT 15RE centrifuge from Hitachi (Tokyo, Japan). Subsequently, the supernatants were processed as outlined by Ussar et al. (2015) [24]. The propionic and butyric acids were quantified via high-performance liquid chromatography (HPLC), using a Dionex Ultimate 3000 dual-detector HPLC system (Dionex Corporation, Sunnyvale, CA, USA) coupled with a refractive index (RI) Shodex RI-101 detector (Tokyo, Japan). The following chromatographic conditions were employed: a Bio-Rad HPX-87H column (300 mm × 4.6 mm) (Hercules, CA, USA) equipped with a Bio-Rad Cation H guard column (Hercules, CA, USA), maintained at a column temperature of 45 °C, and a 20 µL injection volume. The mobile phase consisted of concentrated sulfuric acid, EDTA, and ultrapure water, with a flow rate of 0.7 mL/min. The standard curve was calibrated using the following organic acids: acetic, succinic, formic, propionic, valeric, isovaleric, isobutyric, and butyric acid. The standard solutions of these acids were prepared with a final concentration of 10 mmol/L, except for acetic acid, which presented a concentration of 20 mmol/L. The solute levels were determined using standards and a quantitative curve.

2.12. Analysis of Intestinal Microbiota

The DNA from stool samples was extracted using the QIAmp DNA stool mini kit for human stool (Qiagen®, Venlo, The Netherlands), according to the manufacturer's protocol. The quality and quantity of the extracted DNA were verified using a µDropTM Plate (Thermo Fisher Scientific, Vantaa, Finland). Integrity and size were measured by agarose gel electrophoresis, and the samples were stored at −20 °C until the time of sequencing analyses.

The variable regions of the 16S rRNA gene of members of the bacteria domains (V3–V4) were sequenced by the company Argonne National Laboratory® (Lemont, IL, USA), using the MiSeq platform (Illumina, San Diego, CA, USA). Data processing and analysis were performed using the Mothur v.1.40.0 program [25]. The sequences were aligned using the SILVA v.132 16S rRNA gene reference database [26]. The taxonomic classification was carried out using the same database mentioned above. The operational taxonomic unit (OTU) was grouped with a cutoff point of 97% similarity.

The Chao1, Shannon, and Simpson indices were applied for α-diversity analysis. β-diversity was assessed by principal coordinate analysis (PCoA) based on the Bray–Curtis dissimilarity index and similarity test for non-parametric data (ANOSIM, permutation number = 1000), using the Past software system (HAMMER et al., 2001) [27].

The metagenome functional predictive analysis was carried out using the PICRUSt2 software system. The normalized OTU abundance was identified, and the assigned functional traits were predicted, based on reference genomes, using the Kyoto Encyclopedia of Genes and Genomes (KEGG). The most abundant metabolic processes and significant fold-change differences in functional pathways between experimental groups, adopting unpaired t-test control versus symbiotic analysis or paired t-test (for beginning- and endpoint group analysis) (α = 95%) using STAMP software version 2.1.3, were plotted.

2.13. Statistical Analysis

The dataset was tested for normality by the Kolmogorov–Smirnov test, and parametric data were submitted to ANOVA followed by Tukey's post hoc test for multiple comparisons. The non-parametric and independent data were submitted to the Kruskal–Wallis test followed by the Mann–Whitney test for multiple comparisons. T-tests were applied to compare the baseline and endpoint results of each group. The data were corrected using

the FDR (false discovery rate) criterion in the STAMP software. Statistical analyses were performed using GraphPad software version 9.0. Statistical significance was established at $p < 0.05$.

3. Results

3.1. Baseline Characteristics of Treatment Groups

Thirty-nine subjects completed the study protocol and were included in the analyses; 20 of them were from CG and 19 were from SG. The anthropometric measurements did not differ between the groups at baseline (Table 1). According to the body mass index (BMI), 15% ($n = 3$) of the participants were overweight or obese; 60% ($n = 12$) were eutrophic; and 25% ($n = 5$) were considered underweight. They were 26.81 ± 0.74 years old, with a mean waist circumference of 96.88 ± 1.04 cm.

Table 1. Baseline characteristics of the study participants.

Variables	Symbiotic Group	Control Group	*p* Value
Subjects ($n = 39$)	19	20	-
Sex	Man: 12 Woman: 7	Man: 15 Woman: 5	-
Age (years)	62.85 ± 11.74	64.22 ± 9.68	0.68
Body weight (kg)	66.06 ± 10.79	59.20 ± 9.89	0.05
BMI (kg/m^2)	25.96 ± 4.68	23.08 ± 3.09	0.05
HD time (months)	59.60 ± 72.79	50.83 ± 55.05	>0.99

BMI: body mass index; HD: hemodialysis. The data were subjected to unpaired *t*-test or Mann–Whitney test at 5% probability in GraphPad prism version 9.0.

3.2. Consumption of Macronutrients and Body Mass Index

The consumption of energy, carbohydrates, proteins, and lipids did not differ between the groups during the intervention. Every day, the CG consumed 36.24 ± 13.44 g of lipids, 73.17 ± 30.91 g of protein, and 219.94 ± 92.11 g of carbohydrates. Every day, the symbiotic group consumed 34.94 ± 13.98 g of lipids, 63.45 ± 28.54 g of protein, and 211.19 ± 73.89 g of carbohydrates. On the other hand, the symbiotic consumption reduced

the body mass index (BMI) intergroup at the endpoint, with delta equal to −0.079 ± 0.7511 for the symbiotic group and 0.59 ± 1.1204 for the control group ($p = 0.0479$).

3.3. Uremic Markers

The serum urea levels were similar inter- and intragroup and did not differ between the groups after the intervention period. Regarding the uremic markers, the symbiotic meal consumption reduced the p-cresyl sulfate and indole-3-acetic acid concentrations intragroup. Considering the delta values, intergroup differences were not observed. The creatinine and urea levels did not change after the intra- and intergroup intervention (Table 2).

Table 2. Uremic marker blood concentrations in CKD patients who received symbiotic or control meal by 7 weeks.

Variables	Symbiotic Group		p^1 Value	Control Group		p^1 Value	Delta p Value
	Baseline	Endpoint		Baseline	Endpoint		
IS (mg/dL)	140.46 ± 70.85	115.95 ± 55.65	0.1297	151.94 ± 61.13	147.74 ± 51.36	0.7075	0.1758
IAA (µg/L)	24.21 ± 13.73	18.19 ± 10.67	0.0030	18.62 ± 13.44	15.62 ± 4.91	0.9764	0.3891
p-CS (mg/L)	386.47 ± 197.99	241.13 ± 99.79	0.0001	289.21 ± 245.62	295.02 ± 127.18	0.065	0.3524
Urea	37.15 ± 16.70	37.80 ± 12.42	0.8835	43.70 ± 32.88	33.09 ± 16.35	0.1482	0.2175
Creatinine	8.31 ± 3.23	8.67 ± 2.60	0.1011	8.44 ± 3.30	9.01 ± 3.93	0.1530	0.7016

IS: indoxyl sulfate; IAA: indole-3-acetic acid; p-CS: p-cresyl sulfate. Values expressed as mean ± standard deviation (SD). Data were subjected to an unpaired *t*-test or a Mann–Whitney test at 5% probability in GraphPad prism version 9.0. p^1 means the difference comparing baseline and endpoint of each intervention group.

3.4. Gastrointestinal Symptoms

The consumption of the symbiotic drink increased the evacuation frequency and decreased the gastrointestinal symptoms assessed through the Dixon questionnaire. After the intervention period, 68.4% and 31.6% of the participants allocated to the SG reported having evacuated 5–7 and 2–4 times per week, respectively. On the other hand, 25%, 35%, and 40% of the participants allocated to the CG reported having evacuated 1 time, 5–7, and 2–4 times per week, respectively. In addition, 63.1% and 60% of the participants of the SG and CG groups, respectively, did not present constipation, nausea, heartburn, bloating, intestinal gas, diarrhea, or belching.

According to the Bristol scale, the prevalence of SG participants with a normal consistency of stool, diarrhea, and constipation was 84.2%, 10.5%, and 5.3%, respectively, while for CG, values of 90%, 5%, and 5% were found, respectively (Table S3).

Supplementary Table 3. Stool classification of treatment groups according Bristol Scale.

Stool Classification	Control Group (n=20)		Symbiotic Group (n=19)	
	Baseline	Endpoint	Baseline	Endpoint
1	2	0	4	0
2	7	1	4	1
3	4	2	3	4
4	6	16	6	10
5	0	0	0	2
6	1	1	3	2
7	0	0	0	0

The stool classification was performed by Bristol Scale.

3.5. Fecal SCFA Concentrations

The production of short-chain fatty acids (acetic, propionic, and butyric acid) increased intragroup after the consumption of symbiotic and control meals in both intervention groups when compared to the baseline, with the exception of butyric acid for the CG. The SCFA content did not differ intergroup (Table 3).

Table 3. Short-chain fatty acid fecal concentrations in patients with CKD who received symbiotic or control meal by 7 weeks.

Variables	Symbiotic Group		*p*[1] Value	Control Group		*p*[1] Value	Delta *p* Value
	Baseline	Endpoint		Baseline	Endpoint		
Acetic acid	3.71 ± 1.76	7.00 ± 2.60	<0.0001	4.91 ± 2.07	7.95 ± 3.98	0.0007	0.1758
Propionic acid	2.41 ± 2.04	6.38 ± 3.73	<0.0001	3.05 ± 2.91	5.95 ± 3.71	0.0050	0.3891
Butyric acid	2.26 ± 1.66	3.87 ± 2.38	0.040	2.57 ± 1.91	4.42 ± 4.10	0.0636	0.3524

Values expressed as mean ± standard deviation (SD). Data were subjected to an unpaired *t*-test or a Mann–Whitney test at 5% probability in GraphPad prism version 9.0. p[1] means the difference comparing baseline and endpoint of each intervention group.

3.6. Analysis of Intestinal Microbiota

The sequencing of the 16S rRNA gene from stool samples generated 2,645,395 raw sequences. After filtering and cleaning, 1,890,466 good-quality sequences were obtained. The Good's coverage obtained in the samples was > 99%, which indicates good sequencing coverage. Raw read, filtered read, and normalized read counts per group are provided in the Supplemental Materials (Table S4).

The α-diversity, an indicator of microbial richness estimated by the Chao1 index, increased in the SG compared to the baseline ($p = 0.02$) (Figure 3A). However, the Simpson and Shannon indices did not differ between groups after the intervention period (Figure 3B,C).

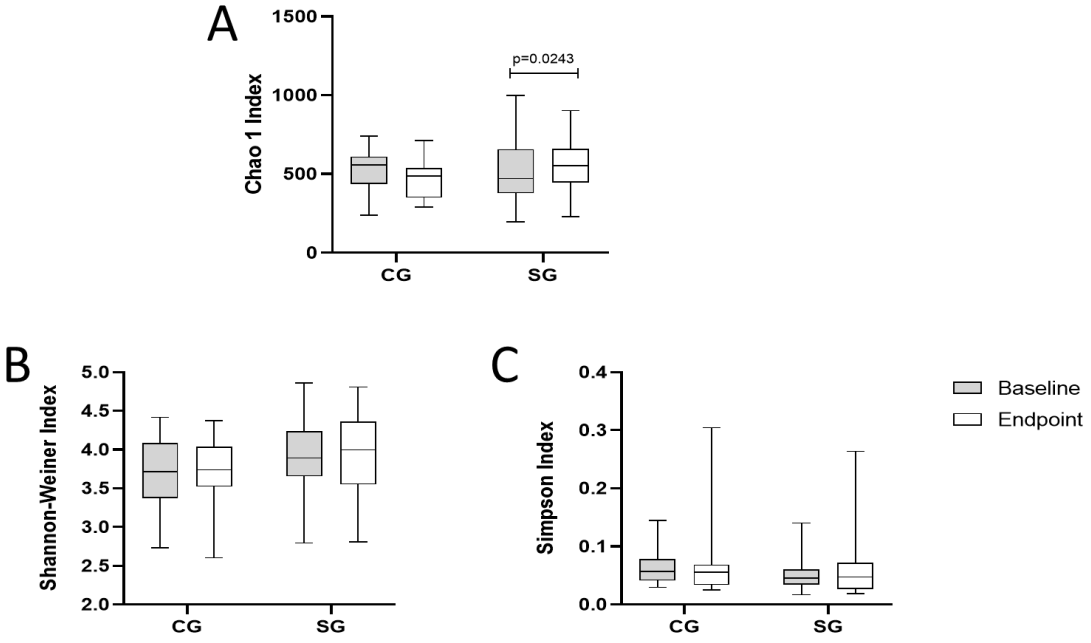


Figure 3. Effect of extruded sorghum BRS 305 plus Bifidobacterium longum on α-diversity index. (**A**) Chao1 index at baseline and endpoint, (**B**) Shannon–Weiner index at baseline and endpoint, (**C**) Simpson index at baseline and endpoint. CG: control group; SG: symbiotic group. The data were subjected to a paired t-test or unpaired *t*-test (α = 0.05) in GraphPad version 9.0.

The β-diversity was assessed at four points. The principal coordinate analysis (PCoA) represented approximately 33.31% and 30.18% of the dissimilarity in bacterial

species composition for SG and CG, respectively (Figure 4A,B). At baseline, PCoA represented approximately 30.40% of the dissimilarity in bacterial species composition (Figure 4C). At the endpoint, PCoA represented 30.6% of dissimilarity in bacterial species composition (Figure 4D). The clustering of the bacterial community did not differ between groups at the phyla, class, order, family or genera levels ($p > 0.05$).

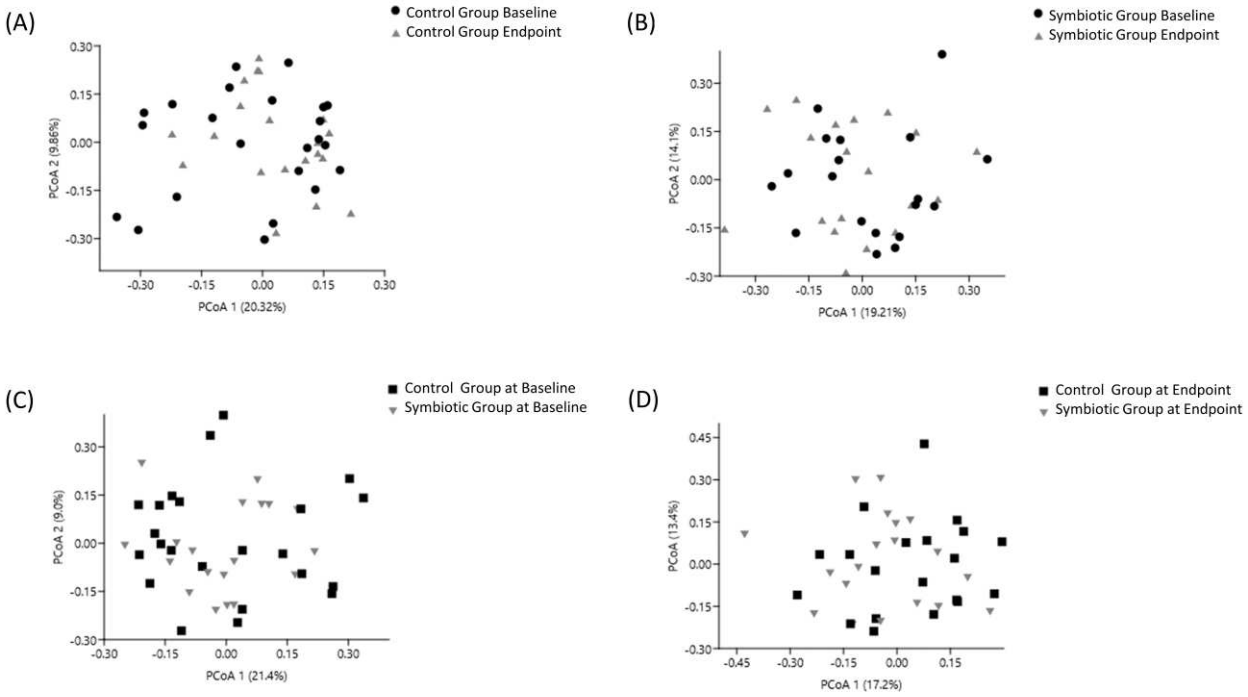
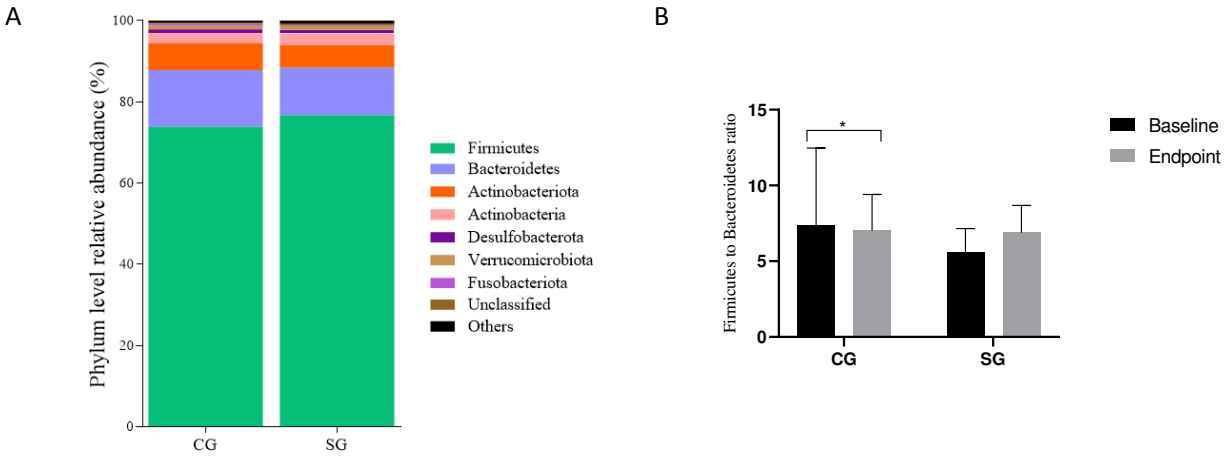


Figure 4. Effect of extruded sorghum BRS 305 plus *Bifidobacterium longum* on the β-diversity estimated by principal coordinate analysis (PcoA), based on the Jaccard similarity distance of gut microbial communities in chronic kidney disease patients. (**A**) PCoA of the symbiotic group at baseline and endpoint; (**B**) PCoA of the control group at baseline and endpoint; (**C**) PCoA of the control and symbiotic groups at baseline; (**D**) PCoA of the control and symbiotic groups at endpoint. Permutational multivariate analysis of variance (PERMANOVA) was conducted using the STAMP software system version 2.0.2 considering α = 5%.

The samples presented 18 phyla, 30 classes, 73 orders, 1141 families, and 373 genera. All groups exhibited eight predominant phyla, including Firmicutes (CG: 73.81 ±

3.89%; SG: 76.71 ± 3.39%), followed by Bacteroidetes (CG: 14.03 ± 3.25%; SG: 11.73 ± 2.33%), Actinobacteria (CG: 6.67 ± 2.20%; SG: 5.59 ± 1.91%), Desulfobacterium (CG: 0.95 ± 0.50%; SG: 0.90 ± 0.48%), and Verrucomicrobia (CG: 0.89 ± 1.05%; SG: 0.92 ± 0.64%) (Supplementary Figure S1A). In the intergroup comparison, the Firmicutes/Bacteroidetes ratio was similar ($p > 0.05$), but in the intragroup comparison, the Firmicutes/Bacteroidetes ratio of CG differed (Supplementary Figure S1B).

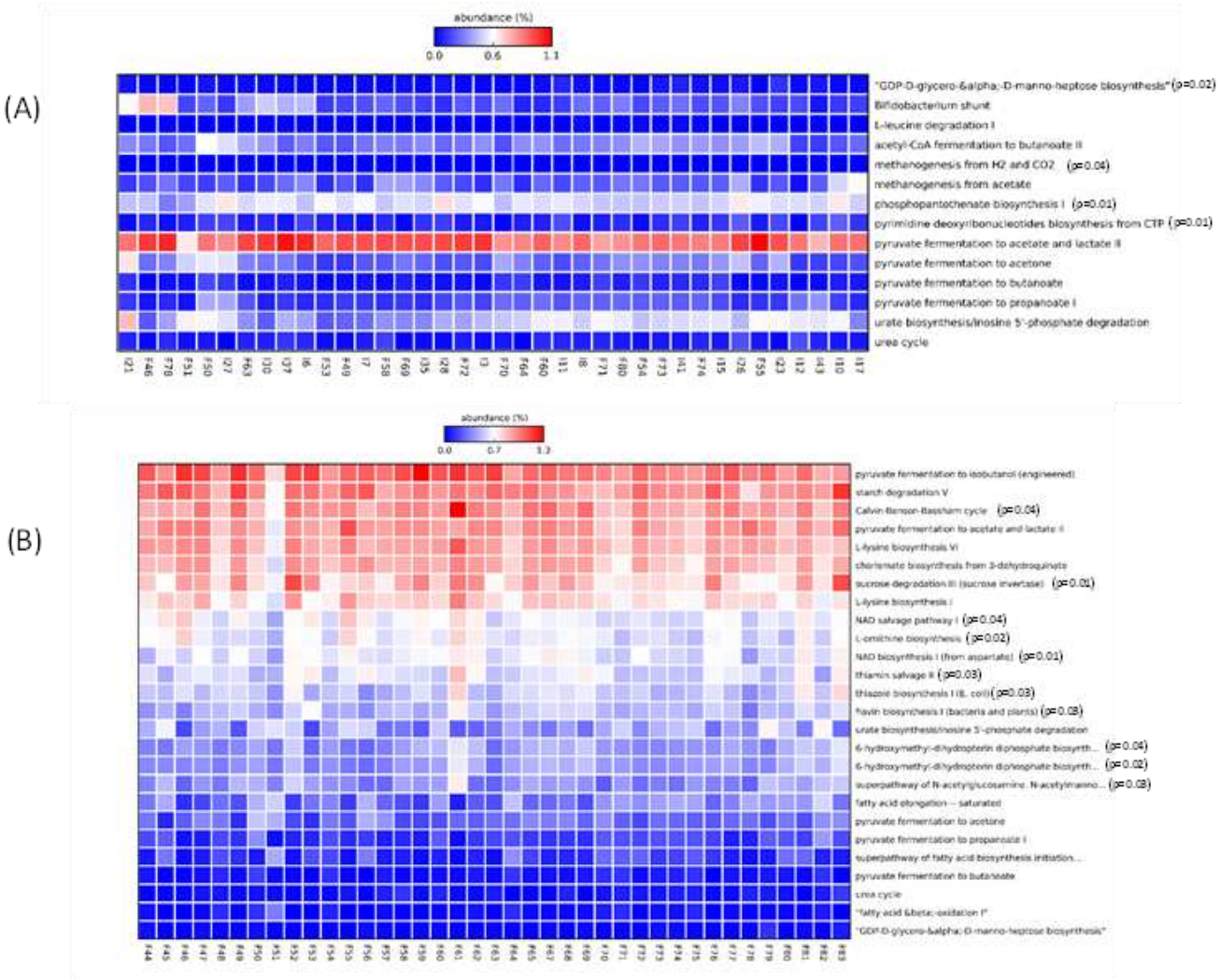


Supplementary Figure 2. Effect of extruded sorghum BRS305 plus *Bifidobacterium longum* on gut microbiota relative abundance at phylum level at the end of treatment. (A) Bacterial composition at phylum level (control group: n=20 | symbiotic group: n=19); (B) Firmicutes to Bacteroidetes ratio at baseline and endpoint. CG: control group; SG: symbiotic group; The data were submitted to paired t-test or unpaired t-test, (α=0.05) in GraphPad version 9.0 and to Dunn's test with FDR and Bonferroni corrections.

According to the KEGG metabolic pathway analysis, SG increased GPD-D-glycerol-alpha content, D-manno-heptose biosynthesis ($p = 0.02$), L-lysine biosynthesis ($p = 0.04$), methanogenesis from H_2 and CO_2 ($p = 0.04$), pantothenate content, coenzyme A biosynthesis I ($p = 0.04$), phosphopantotenate biosynthesis I ($p = 0.01$), pyrimidine deoxyribonucleotide biosynthesis from CTP ($p = 0.01$), and pyrimidine deoxyribonucleotide de novo biosynthesis IV ($p = 0.01$), while reducing the reductive TCA cycle I ($p = 0.04$) metabolic pathway intragroup (Supplementary Figure S2A).

In the comparison of intergroup differences for metabolic pathways by KEGG analysis, the SG reduced 6-hydroxymethyl-dihydropterin diphosphate biosynthesis I ($p =$

0.04), 6-hydroxymethyl-dihydropterin diphosphate biosynthesis III (Chlamydia) ($p = 0.02$), the Calvin–Benson–Bassham cycle ($p = 0.01$), chorismate biosynthesis from 3-dehydroquinate ($p = 0.04$), flavin biosynthesis I (bacteria and plants) ($p = 0.03$), L-lysine biosynthesis I ($p = 0.01$), L-ornithine biosynthesis ($p = 0.02$), N10-formyl-tetrahydrofolate biosynthesis ($p = 0.04$), NAD biosynthesis I (from aspartate) ($p = 0.01$), NAD salvage pathway I ($p = 0.04$), pyrimidine deoxyribonucleotide de novo biosynthesis IV ($p = 0.008$), sucrose degradation III (sucrose invertase) ($p = 0.01$), the superpathway of N-acetylglucosamine, N-acetylmannosamine and N-acetylneuraminate degradation ($p = 0.03$), thiamin salvage II ($p = 0.03$), and thiazole biosynthesis I (E. coli) ($p = 0.03$) metabolic pathways (Supplementary Figure S2B).



Supplementary Figure 3. Effect of extruded sorghum BRS305 plus *Bifidobacterium longum* in microbial metabolic pathways in feces of CKD patients. (A) Microbial metabolic pathways in feces of CKD patients before and after receiving a symbiotic meal, by the paired t test. (B) Microbial metabolic pathways in the stool of CKD patients that received control drink meal or symbiotic drink meal, by the unpaired t test. Variables presenting differences were pointed out next to the tittle of metabolic pathway. Statistical analyses were performed in the STAMP software system (α=0.05).

4. Discussion

The present study investigated the effects of the consumption of a symbiotic meal containing extruded BRS 305 hybrid sorghum and extruded corn on the modulation of gut

microbiota and the markers associated with uremic parameters in patients with CKD. Symbiotic meal consumption reduced indoxyl sulfate, indole-3 acetic acid (IAA), and p-cresyl sulfate serum concentration intragroup. No differences were observed intergroup. Further, the symbiotic drink ameliorated the intestinal function, enhanced evacuation frequency, reduced gastrointestinal symptoms, and enhanced the number of species at endpoint without altering the Firmicutes/Bacteroidetes ratio or varying genus composition. In this context, the symbiotic meal offered improved the Chao1 index, gastrointestinal symptoms, and SCFA production, in addition to reducing the blood level of uremic toxins in CKD patients.

Symbiotic meal consumption increased acetic, propionic, and butyric acid levels intragroup. Probiotics like *Bifidobacterium longum* helped to re-establish a healthy gut microbiota by enhancing the growth of beneficial bacteria and reducing the levels of pathogenic bacteria and uremic toxins. The sorghum, a source of dietary fiber, acts like a prebiotic, providing the necessary nutrients to support the growth and activity of these probiotics, promoting colonic fermentation, resulting in an increase in SCFA synthesis and concentration. In this context, the combined action of probiotics and prebiotics in a symbiotic meal can enhance the production of short-chain fatty acids (SCFAs), helping to maintain gut barrier integrity and reduce inflammation. This symbiotic approach not only improves gut health but also potentially mitigates CKD progression by reducing systemic inflammation and uremic toxin levels [28,29]. In the intestinal environment, SCFAs provide energy for colonocytes, thus modulating their proliferation, differentiation, and the inhibition of pathogenic bacteria growth, in addition to strengthening the intestinal barrier, reducing luminal pH and intestinal permeability, and improving the immune function of CKD patients [30,31].

Symbiotic meal consumption reduced uremic toxins, such as indole-3-acetic acid (IAA) and p-cresyl sulfate (p-CS). Uremic toxins are usually increased in CKD patients. They can alter the intestinal microbiota and promote dysbiosis by increasing intestinal permeability [32] and pH, which facilitates the growth of pathogenic microorganisms [33,34] and favors the progression of CKD [34,35]. However, the symbiotic meal increased SCFA content, which probably inactivated the bacterial families associated with the production of uremic toxins, such as p-CS and IAA. Further, probiotic intake can increase

acetic acid production, to which Bifidobacteria are mainly associated [36]. In addition, no increase in the number of lactic acid bacteria was observed in our study, since the production of butyric and propionic acids increased and may be associated with the amount of dietary fiber, resistant starch [37], and other bioactive compounds present in extruded sorghum [11], such as 3-deoxyanthocyanins and condensed tannins. It is known that butyric acid improves the intestinal barrier function and inhibits the generation of p-CS and the activation of marker inflammation [29,38]. In addition, the consumption of resistant starch was associated with reduced uremic toxin serum levels [35,38].

The bioactive compounds, such as condensed tannins and 3-deoxyanthocyanins and dietary fibers from sorghum, are related to the lower digestibility of the cereal when compared to corn [39], which can favor BMI reduction intergroup. Although no differences in intergroup food intake were observed in the present study, the administration of Bifidobacterium longum has been associated with other microorganisms and weight loss in obese individuals due to reduced microbiome lipopolysaccharides and a consequent increase in satiety [40,41].

In our study, the presence of dietary fiber and resistant starch through the consumption of the symbiotic meal increased the frequency of bowel movements. The fermentation of soluble fiber is associated with increased SCFA production, while the fermentation of insoluble fiber accelerates intestinal transit and increases the fecal bolus [32,35], which improves stool consistency and reduces intestinal constipation [42]. Beneficial effects on gastrointestinal symptoms were observed by Cruz-Mora et al. (2014) in CKD patients receiving a probiotic and inulin [43]. In addition, symbiotics containing *Bifidobacterium longum* are associated with reduced gastrointestinal symptoms, including constipation and ameliorated life quality, according to the Patient Assessment of Constipation Quality of Life (PAC-QOL) questionnaire [15]. Therefore, this symbiotic meal can promote a favorable intestinal condition.

In CKD patients, dysbiosis is frequent and characterized by reduced beneficial commensal bacteria and increased uremic toxin-producing bacteria [44,45]. In our study, the symbiotic meal improved α-diversity, thus increasing the abundance of microbial genera. Other indices related to α-diversity, such as Shannon and Simpson indices, indicated no changes in the number or dominance of species. The β-diversity supported this result,

without inter- or intragroup differences. Other factors, such as stress, aging [46], obesity/fat accumulation [47], diet quality [48], and occurrence of other diseases, can alter gut microbiota composition [49,50], which may hinder the observation of positive changes during 7 weeks of symbiotic meal intervention. In agreement with our results, the consumption of a diet rich in dietary fiber provided by whole grains did not change α-diversity or β-diversity [51,52].

The increase in the Chao1 index without changes in Shannon and Simpson indices suggests that the symbiotic meal had a specific impact on the alpha diversity of the intestinal microbiota of chronic kidney disease patients on hemodialysis, increasing the number of rare or less abundant species—that is, increasing their wealth. The results obtained for the Shannon and Simpson indices suggest that although more species may have been introduced or flourished due to treatment with the symbiotic meal, the abundance of the dominant species was not significantly changed. Therefore, the symbiotic meal may have contributed to a greater diversity of rare species in the intestinal microbiota without modifying the general structure of the microbial community [53].

The KEGG analysis demonstrated that the symbiotic meal improved pathways related to energy metabolism, amino sugar metabolism [54], essential amino acid production and metabolism [55], degradation of sucrose [56], and the biosynthesis of ribonucleotides [57]. The predictive effects observed in amino acid and amino sugar pathways are related to improved immune function, oxidative stress, and immune response [10]. Further, the predictive analysis revealed an increased L-ornithine pathway, an intermediate compound in L-arginine biosynthesis, which, in turn, is used to synthesize glutamate. Glutamate is an amino acid with beneficial effects on intestinal barrier function, which reduces the entrance of endotoxins [58,59].

The main limitations of this study included CKD patients with long hemodialysis treatment (more than 50 months, on average, for both groups), the time of the intervention, only one type of probiotic used, and the lack of control groups with sorghum and *Bifidobacterium longum*. Thus, our study revealed that the consumption of symbiotic meal with extruded BRS 305 grains associated with *Bifidobacterium longum* was effective in improving gastrointestinal symptoms, stool consistency, and SCFA production, in addition to reduc-

ing uremic toxins, such as p-CS and IAA, possibly favoring enterocyte proliferation. Despite the favorable results of this symbiotic meal consumption, further studies are necessary to evaluate the long-term effect of symbiotic meal consumption on the biochemical (creatinine, urea, and uric acid) and intestinal health (gut microbiota composition, intestinal permeability, feces pH, and stool consistency) parameters of CKD patients undergoing hemodialysis.

5. Conclusions

The symbiotic meal containing extruded sorghum BRS 305 associated with *Bifidobacterium longum* was able to improve SCFA production, reduce uremic toxin serum levels, and decrease BMI in CKD patients. Furthermore, the beverage increased bacterial richness and metabolic pathways related to energy metabolism and the biosynthesis of amino acids. Therefore, the symbiotic meal improved intestinal and systemic health status in chronic kidney disease patients.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1. Nutritional composition of the control and test meals by 100g/portion. Table S2. Nutritional composition of sorghum and corn used in test meals. Table S3. Stool classification of treatment groups according Bristol Scale. Table S4. Sequencing data at baseline and at the end of 7 weeks of treatment, according to each group. Figure S1. Analysis report on the cellular prediction of the probiotic drink containing *Bifidobacterium longum*. Figure S2. Effect of extruded sorghum BRS305 plus *Bifidobacterium longum* on gut microbiota relative abundance at phylum level at the end of treatment. Figure S3. Effect of extruded sorghum BRS305 plus *Bifidobacterium longum* in microbial metabolic pathways in feces of CKD patients.

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investigation; methodology; validation; visualization; roles/writing—original draft; writing—review and editing. H.M.: conceptualization; data curation; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; visualization; roles/writing—original draft; writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the guidelines in the Declaration of Helsinki, and all procedures involving human subjects/patients were approved by the Human Research Ethics Committee of the Federal University of Vicosa, MG, Brazil (protocol number 701.796/2014). The study was registered at www.ensaiosclinicos.gov.br under ID number RBR-2d9ny6 in August, 08, 2017. Written informed consent was obtained from all subjects/patients.

Informed Consent Statement: All subjects were informed about the objectives of the study, and informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available upon request to the corresponding author. The data are not publicly available due to the fact that they are available within an internal database of the research institution; therefore, they cannot be made publicly available.

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7. GENERAL CONCLUSIONS

The BRS305 and SC319 Sorghum are genotypes from the Empresa Brasileira de Pesquisa Agropecuária that shows a chemical composition with potential beneficial health effects. The BRS305 sorghum exerts positive effects on antioxidant response in brain and adipose tissue of *Wistar* rats fed with a high-fat-high-fructose diet as well on normalization of activity from markers related to neuroendocrine control of satiety. In addition, improve intestinal markers of chronic kidney disease patients, altering Chao1 index, reducing gastrointestinal symptoms, serum level of uremic toxins (indoxyl sulfate, indole-3-acetic acid and p-cresyl sulfate), SCFAs production and fecal pH. The SC319 genotype promoted adiposity reduction, increase the SCFAs production and altered the gut microbiota composition, reducing pathogenic bacteria, without alter inflammatory markers.

First, the paper 1 demonstrated that dry heat treatment of BRS 305 sorghum grains before milling preserved the resistant starch content, as well as condensed tannins and 3 deoxyanthocyanins. The sorghum bioactive compounds, even when subjected to dry cooking, maintained a high content of phenolic compounds and the antioxidant capacity. Furthermore, molecular docking demonstrated that 3-deoxyanthocyanins had a strong connection with leptin, the endocabinoid receptor and PPAR-alpha, demonstrating that these compounds are associated with the control of satiety mechanisms, previously altered by the consumption of a diet rich in saturated fat and fructose. Also, the consumption of 3 deoxyanthocyanins was positively correlated with improved antioxidant response in the brain and adipose tissue, with increased gene expression of nuclear factor erythroid 2-related factor 2 (NRF2), zinc-dependent superoxide dismutase (ZnSOD), peroxisome proliferator-activated receptor gamma (PPAR-gamma), heat shock protein 72 (HSP72), and SOD and catalase activity, in the brain. Furthermore, this group of anthocyanins correlated negatively with leptin in adipose tissue, endocannabinoid receptor type 1 (CB1) in the brain and adipose tissue, and neuropeptide Y in brain. Finally, although the consumption of 3DXAs did not correlate with the quantification of AGEs, it positively correlated with the gene expression of leptin and CB1, which was reduced in the sorghum group, favoring similar concentrations of AGEs between the AIN-93M and HFHF + sorghum groups.

The second study conducted (article 2) demonstrated that the consumption of extruded sorghum SC319 associated with a calorie restriction of 500kcal/day in obese male individuals, for eight weeks, is associated with the reduction of anthropometric measurements such as body weight, BMI, circumference waist size, body fat and waist/height ratio and, when compared to extruded wheat. Besides this, it promotes higher loss of body fat, without promoting changes in inflammatory markers. Furthermore, sorghum is a cereal that promotes increased synthesis of short-chain fatty acids such as acetic, propionic and butyric. As for the intestinal microbiota, extruded sorghum SC319 promoted a reduction of *Clostridium_sensu_strictu1*, *Dorea*, *Odoribacter*, and an increase of CAG-873 and *Turicibacter* genera. When compared to wheat, it tended to reduce proteobacteria. These results indicate that sorghum SC319, due its nutritional composition high content of condensed tannins and 3-deoxyanthocyanins, can assist caloric restriction in reducing body weight and reducing adiposity as a consequence of digestibility reduction of macronutrients, as well as modulating the intestinal microbiota and production of short-chain fatty acids.

Finally, the third study demonstrated that the extrusion process leads to the loss of resistant starch for sorghum grains of the BRS305 genotype, however, it is a cereal that has a high content of condensed tannins and dietary fiber. The offer of a symbiotic meal that includes the administration of extruded sorghum BRS305 and strains of Bifidobacterium longum in chronic kidney disease patients on hemodialysis, led to the improvement of gastrointestinal symptoms such as vomiting, nausea, flatulence and gastrointestinal discomfort, and improved stool consistency. Furthermore, it led to increased production of short-chain fatty acids. As for the intestinal microbiota, the consumption of this symbiotic meal led to an increase in microbial richness, however, without changes in beta diversity, intra or intergroup. Furthermore, functional prediction analysis (KEGG) revealed that symbiotic meal consumption improved pathways related to energy metabolism, amino sugars metabolism, essential amino acids production and metabolism and degradation of sucrose. These effects are associated with improved immune function, reduced oxidative stress and improved immune response.

8. CONCLUSÕES GERAIS

Os sorgos BRS305 e SC319 são genótipos da Empresa Brasileira de Pesquisa Agropecuária que apresentam composição química com potenciais efeitos benéficos à saúde. O sorgo BRS305 promoveu efeitos positivos na resposta antioxidante no cérebro e tecido adiposo bem como na ressensibilização de marcadores relacionados ao controle da saciedade de ratos *Wistar* machos alimentados com dieta rica em gordura saturada e frutose. Ainda, melhorou marcadores intestinais de pacientes renais crônicos, alterando o índice Chao1, reduzindo sintomas gastrointestinais, nível sérico de toxinas urêmicas (indoxil sulfato, ácido indole-3-acético e p-cresil sulfato), produção de AGCC e pH fecal. O genótipo SC319 promoveu redução da adiposidade, aumentou a produção de AGCC e alterou a composição da microbiota intestinal, reduzindo bactérias patogênicas, sem alterar os marcadores inflamatórios.

O primeiro estudo (paper 1) demonstrou que o processamento térmico a seco dos grãos de sorgo BRS 305 antes da moagem da farinha preservou o teor de amido resistente, bem como taninos condensados e 3 desoxiantocianinas. O sorgo BRS305, mesmo quando submetido ao cozimento a seco, manteve elevado teor de compostos fenólicos e a capacidade antioxidante, com as 3-deoxiantocianinas preservadas. Além disso, o docking molecular demonstrou que as 3-desoxiantocianinas tinham uma forte ligação com a leptina, o receptor endocabinóide e o PPAR-α, demonstrando que estes compostos estão associados ao controle dos mecanismos de saciedade, previamente alterados pelo consumo de uma dieta rica em gordura saturada e frutose. Adicionalmente, o consumo de 3 desoxiantocianinas foi positivamente correlacionado com a melhora da resposta antioxidante no cérebro e no tecido adiposo, com aumento da expressão gênica do fator nuclear 2 relacionado ao eritróide 2 (NRF2), superóxido dismutase dependente de zinco (ZnSOD), proliferador de peroxissoma- receptor gama ativado (PPAR-gama) e proteína de choque térmico 72 (HSP72), além de aumentar a atividade de SOD e Catalase no cérebro. Além disso, este grupo de antocianinas também se correlacionou negativamente com a leptina no tecido adiposo, o receptor endocanabinoide tipo 1 (CB1) no cérebro e no tecido adiposo, e com o neuropeptídeo Y (NPY) no cérebro. Por fim, embora o consumo de 3DXAs não tenha se correlacionado com a quantificação de AGEs, ele se correlacionou positivamente com a expressão gênica de Leptina e CB1, que foi

reduzida no grupo sorgo, o que pode ter favorecido concentrações semelhantes de AGEs entre os grupos AIN-93M e HFHF + sorgo.

O segundo estudo realizado (artigo 2) demonstrou que o consumo de sorgo extrusado SC319 associado à restrição calórica de 500kcal/dia em indivíduos obesos do sexo masculino, durante oito semanas, está associado à redução de medidas antropométricas como peso corporal, IMC, circunferência tamanho da cintura, gordura corporal e relação cintura/estatura e, quando comparado ao trigo extrusado, promove maior perda de gordura corporal, sem promover alterações nos marcadores inflamatórios. Além disso, é um cereal que promove o aumento da síntese de ácidos graxos de cadeia curta, como os ácidos acético, propiônico e butírico. Quanto à microbiota intestinal, o sorgo extrusado SC319 promoveu redução dos gêneros *Clostridium_sensu_strictu1*, *Dorea*, *Odoribacte*r e aumento dos gêneros CAG-873 e *Turicibacter*. Quando comparado ao trigo, tendeu a reduzir proteobactérias. Esses resultados indicam, especialmente, que o sorgo SC319, por conter em sua composição nutricional, alto teor de taninos condensados, amido resistente e 3-deoxiantocianinas, pode auxiliar a restrição calórica na redução do peso corporal e na redução da adiposidade como consequência da redução da digestibilidade dos macronutrientes, além de modular a microbiota intestinal e a produção de ácidos graxos de cadeia curta.

Por fim, o terceiro estudo demonstrou que o processo de extrusão leva à perda de amido resistente para os grãos de sorgo do genótipo BRS305, porém é um cereal que possui alto teor de taninos condensados e fibra alimentar. A oferta de uma refeição simbiótica que inclui a administração de sorgo extrusado BRS305 e cepas de *Bifidobacterium longum* em pacientes renais crônicos em hemodiálise sem intervenções dietéticas durante 7 semanas, levou à melhora de sintomas gastrointestinais como vômitos, náuseas, flatulência e desconforto gastrointestinal e melhor consistência das fezes. Além disso, levou ao aumento da produção de ácidos graxos de cadeia curta. Quanto à microbiota intestinal, o consumo desta refeição simbiótica levou a um aumento da riqueza microbiana, porém, sem alterações na diversidade beta, intra ou intergrupo. Além disso, a análise de predição funcional (KEGG) revelou que o consumo de refeições simbióticas melhorou as vias relacionadas ao metabolismo energético, metabolismo de aminoácidos, produção de aminoácidos essenciais e metabolismo e degradação da

sacarose. Esses efeitos estão associados à melhora da função imunológica, redução do estresse oxidativo e melhora da resposta imunológica.

9. FINAL CONSIDERATIONS

Actually, the sorghum is an ingredient used in the production of foods intended for feeding individuals who have a healthy diet or a diet with a restriction of wheat consumption. Finally, this study revealed that due to the nutritional composition of these Brazilian sorghum genotypes, they are foods that can be introduced and recommended in the nutritional management of metabolic disorders and diseases, such as obesity and its consequences, and also in the metabolic improvement of individuals with of chronic kidney disease. This fact can be due the bioactive compounds, such as condensed tannins found in grain that can interfere in nutrient absorption, favoring the modulation of weight and body composition. It is important to highlight that 3-deoxyanthocyanins present in food can exert antioxidant effects, increasing the antioxidant response and favoring the reduction of inflammatory status.

New studies conducted in humans using these sorghum genotypes are necessary, such as investigating the use of sorghum flour BRS305 in modulating markers related to satiety and appetite in patients with metabolic disorders. Furthermore, the use of these sorghum genotypes associated or not with *Bifidobacterium longum* in the intestinal modulation of individuals should also be investigated. These sorghum genotypes have dietary fiber, resistant starch and bioactive compounds that have potential to improve intestinal health, including gut microbiota and there are related to the immune response, inflammation, neurodegenerative diseases, and absorption of nutrients and metabolites.

10. APPENDIX

APPENDIX I. CEUA/UFV ACCEPTANCE LETTER OF STUDY 1

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.

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

APPENDIX II. CEP/UFV ACCEPTANCE LETTER OF STUDY 2

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: CARACTERIZAÇÃO QUÍMICA E COMPOSTOS BIOATIVOS DE GRÃOS, FARINHAS E PRODUTOS À BASE DE NOVOS GENÓTIPOS DE SORGO E IMPACTO DO CONSUMO NA SAÚDE HUMANA

Pesquisador: HELENA MARIA PINHEIRO SANT'ANA

Área Temática:

Versão: 4

CAAE: 13630513.0.0000.5153

Instituição Proponente: Departamento de Nutrição e Saúde

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 839.595

Data da Relatoria: 13/10/2014

Apresentação do Projeto:

Trata-se de pedido de emenda ao protocolo sob a seguinte justificativa: "A presente Emenda se faz necessária devido a inclusão de um quinto estudo (ESTUDO 5: EFEITO DO PROCESSAMENTO NO ÍNDICE GLICÊMICO DO SORGO) no qual se descreve a avaliação do índice glicêmico do sorgo em humanos. Devido a essa Emenda foram realizados pequenos ajustes no Resumo, Revisão bibliográfica (inclusão de um tópico sobre índice glicêmico do sorgo) e Justificativa do projeto. Na sessão Materiais e Métodos foram incluídas informações detalhadas sobre o novo estudo. Para a submissão da Emenda foram incluídos 5 novos arquivos: Ofício de Encaminhamento de Emenda; Projeto Detalhado (com emenda); ANEXOS 9, 10 e 11. Após a submissão da emenda foi emitido um parecer em 19/05/2014 no qual foi solicitada simplificação do TCLE. O TCLE modificado foi submetido, porém se a carta de resposta a Pendências, o que resultou na emissão de uma pendência documental"

Objetivo da Pesquisa:

Expostos anteriormente.

Endereço: Universidade Federal de Viçosa, prédio Arthur Bernardes, piso inferior
Bairro: campus Viçosa **CEP:** 36.570-000
UF: MG **Município:** VICOSA
Telefone: (31)3899-2492 **Fax:** (31)3899-2492 **E-mail:** cep@ufv.br

Avaliação dos Riscos e Benefícios:

Definidos e apresentadas as medidas protetivas necessárias para minimizar e/ou excluir os riscos.

Comentários e Considerações sobre a Pesquisa:

Alterações na revisão bibliográfica e na justificativa que não alteram os aspectos da pesquisa.

Considerações sobre os Termos de apresentação obrigatória:

Incluídos os seguintes documentos novos: Ofício de encaminhamento da emenda, projeto detalhado com a emenda, anexos 9, 10 e 11 e um TCLE que foi elaborado de forma simples e com linguagem simples para que os sujeitos pesquisados possam compreender. O Protocolo se encontra compatível com as determinações contidas na Resulão 466/2012 da CONEP.

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

Aprovadoa a emenda para as alterações informadas pela pesquisadora.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Ao término da pesquisa é necessária a apresentação do Relatório Final e após a aprovação desse, deve ser encaminhado o Comunicado de Término dos Estudos.

Emenda analisada durante a 9ª reunião de 2014, realizada nos dias 13 e 17 de outubro de 2014.

VICOSA, 21 de Outubro de 2014

Assinado por:
Patrícia Aurélia Del Nero
(Coordenador)

APPENDIX III. CEP/UFV ACCEPTANCE LETTER OF STUDY 3

UNIVERSIDADE FEDERAL DE VIÇOSA - UFV

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: PREVALÊNCIA DE DOENÇA CELÍACA ENTRE PACIENTES COM DOENÇAS RENAIS CRONICAS E IMPLEMENTAÇÃO DE ESTRATÉGIAS DIETÉTICAS

Pesquisador: Sônia Machado Rocha Ribeiro

Área Temática:

Versão: 2

CAAE: 27364314.8.0000.5153

Instituição Proponente: Departamento de Nutrição e Saúde

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 701.796

Data da Relatoria: 04/07/2014

Apresentação do Projeto:

Trata-se de um estudo prospectivo para avaliar a prevalência de doença celíaca entre pacientes com doença renal crônica e implementar estratégias nutricionais e dietéticas com intuito de corroborar para qualidade de vida e diminuir possíveis complicações clínicas associáveis entre ambas patologias.Espera-se conhecer a problemática relacionada entre a doença celíaca e a doença renal em tratamento hemodialítico para a implementação de protocolos e rotinas de atendimento nutricional, visando a melhoria da condição clínico-nutricional dos pacientes atendidos na referida unidade hospitalar.

Objetivo da Pesquisa:

Investigar a associação entre doença celíaca e a doença renal em pacientes submetidos ao tratamento hemodialítico e implementar estratégias dietéticas.

Objetivos Secundários:

Avaliar a presença das principais manifestações clínicas relacionadas à DC em pacientes portadores de DRC em tratamento hemodialítico;

Investigar parâmetros bioquímicos séricos e biópsia de mucosa intestinal sugestivos de intolerância à gliadina;

Calcular a prevalência de portadores de exames positivos para DC;

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Correlacionar os resultados encontrados para Anti-endomísio IgA anticorpos EMA com os exames bioquímicos de: PCR, IL-6, Potássio, Fósforo, Cálcio, Albumina, Glicemia de jejum, Hemoglobina glicada,Hematócrito, Hemoblobina, Ktv.
Avaliar o estresse oxidativo dos participantes.
Avaliar o estado nutricional e a ingestão alimentar dos participantes.
Realizar a intervenção dietética com o uso de probiótico.
Analisar os exames marcadores de DC e de controle metabólico da DRC após a intervenção dietética.
Realizar avaliação antropométrica dos participantes antes e após a intervenção dietética para a retirada de glúten.
Oferecer acompanhamento nutricional no programa pró-celíaco (atividade de extensão da UFV) para os participantes com exames positivos para DC.
Elaborar receitas de baixo custo adaptadas para fins especiais da doença renal crônica e doença celíaca.

Avaliação dos Riscos e Benefícios:
Descritos de forma adequada.

Comentários e Considerações sobre a Pesquisa:
Todas as alterações solicitadas foram atendidas.

Considerações sobre os Termos de apresentação obrigatória:
Apresentados de forma adequada. Foi anexado aos documentos um parecer técnico do serviço de Nefrologia e um parecer técnico do serviço de Gastroenterologia do Hospital São João Batista esclarecendo todas as pendências identificadas anteriormente.

Recomendações:
Quando da coleta de dados, o TCLE deve ser elaborado em duas vidas, rubricadas em todas as suas páginas e assinadas, ao seu término, pelo convidado a participar da pesquisa, ou por ser representante legal, assim como pelo pesquisador responsável, ou pela(s) pessoa(s) por ele delegada(s), devendo as páginas de assinaturas estar na mesma folha. Para a submissão, não é necessária a assinatura do TCLE.

Conclusões ou Pendências e Lista de Inadequações:
Não há.

Situação do Parecer:
Aprovado