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Chemical-nutritional composition of processed pearl millet (*Pennisetum glaucum* (L.) R. Br.) and its effects on intestinal health, iron metabolism, oxidative stress and inflammation

Jaqueline Maciel Vieira Theodoro
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JAQUELINE MACIEL VIEIRA THEODORO

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Thesis submitted to the Nutritional 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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Assent:

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ABSTRACT

THEODORO, Jaqueline Maciel Vieira, D.Sc., Universidade Federal de Viçosa, July, 2025. **Chemical-nutritional composition of processed pearl millet (*Pennisetum glaucum* (L.) R. Br.) and its effects on intestinal health, iron metabolism, oxidative stress and inflammation**. Adviser: Hercia Stampini Duarte Martino. Co-advisers: Carlos Wanderlei Piler de Carvalho, Bárbara Pereira da Silva and Mariana Grancieri.

Millets, in addition to being nutritious, providing carbohydrates, proteins, dietary fiber, resistant starch and minerals with iron, are low cost and have the potential to be environmentally sustainable and resistant, being a promising option to overcome situations of food and nutritional insecurity. In Brazil, the most widespread millet species is *Pennisetum glaucum*. This grain has a good chemical composition; however, the presence of phytochemicals, such as phytates, can compromise its nutritional quality. Therefore, it is recommended to consume the whole grain subjected to processing, such as germination and extrusion cooking, to preserve or improve its nutritional value. In addition, the inclusion of this cereal in the diet could minimize metabolic changes in the body, due to its good iron bioavailability, potential antioxidant and anti-inflammatory effect, and improvement of intestinal health. Objective: to evaluate the chemical-nutritional composition of millet (*Pennisetum glaucum* (L.) R. Br.) subjected to different processing methods and its effects on intestinal health, iron metabolism, oxidative stress, and inflammation. Methodology: this study was divided into three phases. In the first phase (biological assay I, article 1), the effects of the consumption of germinated pearl millet (raw millet flour) on the microbiota composition and intestinal morphology of rats fed with high saturated fat and fructose diet (HFHF) were investigated. For this, the animals were initially divided into two groups: AIN-93M and HFHF, for 8 weeks, to induce metabolic changes. Subsequently, the animals in the AIN-93M group were maintained and the HFHF group was divided into HFHF (HFHF diet) and HFHF + millet (HFHF + germinated millet flour) for 10 weeks. In the second phase (chemical-nutritional composition and in vitro iron bioavailability, article 2), the chemical-nutritional composition of pearl millet flours with different processing was performed: non-germinated open-pan cooked millet flour (NGOPCMF); germinated open-pan cooked millet flour (GOPCMF) and extrusion-cooked millet flour (ECMF), and their respective in vitro iron bioavailability using Caco-2 cells. In the third phase (biological assay II, articles 3 and 4), the effects of the consumption of pearl millet subjected to different processing (cooked millet flours) on iron metabolism, intestinal

health, oxidative stress and inflammation were analyzed in rats induced to iron deficiency, through the depletion method (animals receiving a standard iron-free diet, SD iron-free, for 28 days, to induce iron deficiency) and hemoglobin repletion (animals separated into 4 groups for treatment: 1 control group, which received ferrous sulfate as an iron source (SD+FS) and 3 test groups, which received non-germinated open-pan cooked millet flour (SD+ NGOPCMF), germinated open-pan cooked millet flour (SD+ GOPCMF) or extrusion-cooked millet flour (SD+ ECMF), for 21 days). Results: Article 1: Raw germinated pearl millet flour reduced the genus Oscillibacter and the phylum Desulfobacterota, while increasing the family Eggerthellaceae. In addition, it increased beta diversity, cecum weight, cecum/body weight ratio, crypt depth and thickness, goblet cell count, and the concentration of SCFA propionate, and reduced fecal pH and urinary mannitol excretion. Article 2: Regarding the chemical composition of cooked pearl millet flours, when compared to NGOPCMF, ECMF showed an increase in the contents of carbohydrates, tryptophan, iron, manganese, total saturated fatty acids, diosmin, and cyanidin, and a reduction in total dietary fiber, resistant starch, and total monounsaturated and polyunsaturated fatty acids. On the other hand, GOPCMF showed an increase in the content of lysine, total polyunsaturated fatty acids and vitamin C and a reduction in the contents of phytate, total saturated and monounsaturated fatty acids and riboflavin. In addition, both flours (GOPCMF and ECMF) demonstrated an increase in the concentrations of essential and non-essential amino acids and a reduction in the concentrations of total lipids and total vitamin E. In the in vitro assay, the concentration of ferritin was higher in Caco-2 cells exposed to GOPCMF and ECMF compared to NGOPCMF. Article 3: The SD+GM group showed an increase in the expression of the DcytB, ferroportin and hephaestin genes. The SD+EM group showed an increase in the concentration of transferrin compared to the SD+FS group and serum iron, compared to the other groups, and a reduction in the total antioxidant capacity (TAC) of the liver. In the SD+CM group, there was an increase in the expression of ferritin. All test groups (SD+CM, SD+GM and SD+EM) demonstrated an increase in the gene expression of DMT1 and hephaestin, as well as in the serum iron concentration and a reduction in the gene expression of transferrin, Nrf2 and TNF and in the levels of MDA, NO, SOD and CAT in relation to the SD+FS group. Article 4: Animals fed with pearl millet flours subjected to different processing (SD+NGOPCMF, SD+GOPCMF and SD+ECMF) demonstrated a higher Chao index in the microbiome and a higher number and area of goblet cells and longitudinal muscle layer width. The SD+NGOPCMF and SD+GOPCMF groups demonstrated increased cecum weight, crypt depth, crypt thickness, and circular muscle layer width; lower

fecal pH; and a higher relative abundance of Bacteroidota, while the SD+FS group showed the highest abundance of Actinobacteriota. The SD+GOPCMF group stood out for showing the lowest fecal pH, better a-diversity (Chao and Shannon index), and the highest width of the longitudinal muscle layer. Conclusion: pearl millet subjected to different processing has a good chemical-nutritional composition and beneficial effects on intestinal health, evidenced mainly by the improvement in the composition of the microbiota and intestinal morphology, on iron metabolism and in reduction of inflammation and oxidative stress in vivo. However, germinated pearl millet stands out for favoring the chemical-nutritional composition and beneficial effects on the body, both pre-cooked added to a normal diet or raw added to a high-fat diet.

Keywords: open pan cooking; extrusion; germination; pre-cooked millet flours; intestinal microbiota; iron bioavailability

RESUMO

THEODORO, Jaqueline Maciel Vieira, D.Sc., Universidade Federal de Viçosa, julho de 2025. **Composição química-nutricional de milheto pérola (*Pennisetum glaucum* (L.) R. Br.) processado e seus efeitos na saúde intestinal, metabolismo do ferro, estresse oxidativo e inflamação**. Orientadora: Hercia Stampini Duarte Martino. Coorientadores: Carlos Wanderlei Piler de Carvalho, Bárbara Pereira da Silva e Mariana Grancieri.

Os milhetos, além de serem nutritivos, fornecendo carboidratos, proteínas, fibras alimentares, amido resistente e minerais com ferro, possuem baixo custo e têm potencial para serem ambientalmente sustentáveis e resistentes, sendo uma opção promissora para superar situações de insegurança alimentar e nutricional. No Brasil a espécie mais difundida é a *Pennisetum glaucum*. Seu grão apresenta boa composição química, no entanto, a presença de fitoquímicos, como fitatos, pode comprometer sua qualidade nutricional. Portanto, recomenda-se consumir o grão inteiro submetido a processamentos, como a germinação e a extrusão termoplástica, para preservar ou melhorar seu valor nutricional. Além disso, a inclusão desse cereal na dieta poderia minimizar alterações metabólicas no organismo, devido à sua boa biodisponibilidade de ferro, potencial efeito antioxidante, anti-inflamatório e melhora da saúde intestinal. Objetivo: avaliar a composição química-nutricional do milheto (*Pennisetum glaucum* (L.) R. Br.) submetido a diferentes processamentos e seus efeitos na saúde intestinal, metabolismo do ferro, estresse oxidativo e inflamação. Metodologia: este estudo foi dividido em três fases. Na primeira fase (ensaio biológico I, artigo 1), foram investigados os efeitos do consumo de milheto pérola germinado (farinha de milheto cru) na composição da microbiota e na morfologia intestinal de ratos alimentados com dieta rica em gordura saturada e frutose (HFHF). Para isso, os animais foram inicialmente divididos em dois grupos: AIN-93M e HFHF, por 8 semanas, para indução de alterações metabólicas. Posteriormente, os animais do grupo AIN-93M foram mantidos e o grupo HFHF foi dividido em HFHF (dieta HFHF) e HFHF + milheto (HFHF + farinha de milheto germinado) por 10 semanas. Na segunda fase (composição química-nutricional e biodisponibilidade de ferro in vitro, artigo 2), foi realizada a composição química-nutricional das farinhas de milheto pérola com diferentes processamentos: farinha de milheto não germinado cozido em panela aberta (NGOPCMF); farinha de milheto germinado cozido em panela aberta (GOPCMF) e farinha de milheto cozido por extrusão (ECMF), e suas respectivas biodisponibilidades de ferro in vitro, utilizando células Caco-2. Na terceira fase (ensaio biológico II, artigos 3 e 4), foram

analisados os efeitos do consumo de milheto pérola submetido a diferentes processamentos (farinhas de milheto cozido) no metabolismo de ferro, na saúde intestinal, no estresse oxidativo e na inflamação em ratos induzidos à deficiência de ferro, por meio do método de depleção (animais recebendo dieta padrão isenta de ferro (SD iron-free) por 28 dias, para induzir a deficiência de ferro) e repleção de hemoglobina (animais separados em 4 grupos para tratamento: 1 grupo controle, que recebeu como fonte de ferro sulfato ferroso (SD+FS) e 3 grupos testes, que receberam farinha de milheto não germinado cozido em panela aberta (SD+ NGOPCMF), farinha de milheto germinado cozido em panela aberta (SD+ GOPCMF) ou farinha de milheto cozido por extrusão (SD+ ECMF), por 21 dias). Resultados: Artigo 1: A farinha de milheto pérola germinado cru reduziu o gênero Oscillibacter e o filo Desulfobacterota, enquanto aumentou a família Eggerthellaceae. Além disso, aumentou a diversidade beta, o peso do ceco, a relação ceco/peso corporal, a profundidade e espessura das criptas, a contagem de células caliciformes e a concentração do AGCC propionato e reduziu o pH fecal e a excreção urinária de manitol. Artigo 2: Em relação à composição química das farinhas de milheto pérola cozido, quando comparada à NGOPCMF, a ECMF apresentou aumento nos conteúdos de carboidratos, triptofano, ferro, manganês, ácidos graxos saturados totais, diosmina e cianidina e redução no total de fibra alimentar, amido resistente e ácidos graxos monoinsaturados e poli-insaturados totais. Por outro lado, a GOPCMF apresentou aumento no teor de lisina, ácidos graxos poli-insaturados totais e vitamina C e redução nos conteúdos de fitato, ácidos graxos saturados e monoinsaturados totais e riboflavina. Além disso, ambas as farinhas (GOPCMF e ECMF) demonstraram aumento nas concentrações de aminoácidos essenciais e não essenciais e redução nas concentrações de lipídios totais e vitamina E total. No ensaio in vitro, a concentração de ferritina foi maior nas células Caco-2 expostas GOPCMF e ECMF em comparação à NGOPCMF. Artigo 3: O grupo SD+GM apresentou aumento na expressão dos genes DcytB, ferroportina e hefaestina. O grupo SD+EM apresentou aumento na concentração de transferrina em comparação ao grupo SD+FS e de ferro sérico, em comparação aos demais grupos, e redução na capacidade antioxidante total (TAC) do fígado. No grupo SD+CM houve aumento na expressão de ferritina. Todos os grupos testes (SD+CM, SD+GM e SD+EM) demonstraram aumento na expressão gênica de DMT1 e hefaestina, bem como na concentração de ferro sérico e redução na expressão gênica de transferrina, Nrf2 e TNF e nos níveis de MDA, NO, SOD e CAT em relação ao grupo SD+FS. Artigo 4: Os animais alimentados com as farinhas de milheto pérola submetidas a diferentes processamentos (SD+NGOPCMF, SD+GOPCMF e SD+ECMF) demonstraram

maior índice de Chao no microbioma e maior número e área de células caliciformes e largura da camada muscular longitudinal. Os grupos SD+NGOPCMF e SD+GOPCMF demonstraram maior peso do ceco, profundidade da cripta, espessura da cripta e largura da camada muscular circular; menor pH fecal; e maior abundância relativa de Bacteroidota, enquanto o grupo SD+FS apresentou a maior abundância de Actinobacteriota. O grupo SD+GOPCMF destacou-se por apresentar o menor pH fecal, melhor diversidade a (índice de Chao e Shannon) e maior largura da camada muscular longitudinal. Conclusão: o milheto pérola submetido a diferentes processamentos apresenta boa composição química-nutricional e efeitos benéficos na saúde intestinal, evidenciado principalmente pela melhora da composição da microbiota e da morfologia intestinal, no metabolismo do ferro e na redução da inflamação e do estresse oxidativo in vivo. Porém, o milheto pérola germinado se destaca por favorecer a composição químico-nutricional e os efeitos benéficos ao organismo, tanto pré-cozido adicionado a uma dieta normal quanto cru adicionado a uma dieta rica em gordura.

Palavras-chave: cozimento em panela aberta; extrusão; germinação; farinhas de milheto pré-cozido; microbiota intestinal; biodisponibilidade de ferro

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LIST OF ACRONYMS AND ABBREVIATIONS

AMPK: AMP-activated protein kinase

CAT: catalase

CM: conventional millet

DcytB: duodenal cytochrome b

DMT-1: divalent metal transporter 1

ECMF: extrusion-cooked millet flour

EM: extruded millet

Fe: iron

FER: feed efficiency ratio

GM: Germinated millet

GOPCMF: germinated open-pan cooked millet flour

Hb: hemoglobin

HFHF: high-fat high-fructose diet

HRE: hemoglobin regeneration efficiency

IFN-γ: interferon-γ

LPS: lipopolysaccharide

MDA: malondialdehyde

NF-κB: nuclear factor kappa B

NGOPCMF: non-germinated open-pan cooked millet flour

NO: nitric oxide

Nrf2: nuclear factor erythroid 2

RBV HRE: biological value of hemoglobin regeneration efficiency

ROS: reactive oxygen species

SD iron-free: standard iron-free diet

SD+CM: standard diet + conventional millet flour

SD+EM: standard diet + extruded millet flour

SD+ECMF: standard diet + extrusion cooked millet flour

SD+FS: standard diet + ferrous sulfate

SD+GM: standard diet + germinated millet flour

SD+GOPCMF: standard diet + germinated millet flour cooked in an open-pan

SD+NGOPCMF: standard diet + non-germinated millet flour cooked in an open-pan

SCFA: short-chain fatty acids

SGLT1: sodium-glucose transport protein 1

SOD: superoxide dismutase

TAC: total antioxidant capacity

TLR4: Toll-like Receptor 4

TJ: tight junctions

TNF: tumor necrosis factor

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1. INTRODUCTION

Millets are the sixth most cultivated cereal in the world, with India being the world's largest producer. In Brazil, the most widespread species is *Pennisetum glaucum*, also known as pearl millet (DIAS-MARTINS et al., 2018; FAOSTAT, 2024). This grain stands out because, in addition to being nutritious, it has the potential to be environmentally sustainable and resistant to drought and heat, facilitating its production even in adverse climatic conditions (ANITHA et al., 2021; VETRIVENTHAN et al., 2021). In view of these benefits, the use of this grain fits into the Sustainable Development Goals (SDGs) of the United Nations (UN) 2030 Agenda, including zero hunger and sustainable agriculture (SDG number 2), health and well-being (SDG number 3), and responsible consumption and production (SDG number 12) (GTSCA2030, 2020; ONU, 2015). In addition the United Nations General Assembly promoted in 2023 the "Year of Millets" (FAO, 2023), encouraging more research and dissemination of knowledge about this cereal.

Pearl millet contains significant amounts of carbohydrates (>60%), proteins (~10%), dietary fiber (~8%), resistant starch (3%) and minerals such as calcium (300mg/kg), zinc (43 mg/kg) and iron (18 mg/kg), in addition to monounsaturated and polyunsaturated fatty acids and bioactive compounds, such as phenolic, present mainly in the grain pericarp (DIAS-MARTINS et al., 2018; NANJE GOWDA et al., 2022; PATIL; CHIMMAD; ITAGI, 2015; THEODORO et al., 2021b). However, the presence of phytochemicals, such as phytates, in this food matrix can compromise the nutritional quality of this cereal and may interfere with the bioavailability of some minerals, including iron. Therefore, it is recommended to consume whole millet followed by some type of processing that preserves or improves its nutritional value (NANJE GOWDA et al., 2022; SHARMA; GUJRAL, 2019; SIHAG et al., 2016).

Among the available processing methods, germination is a good alternative because it is simple, inexpensive, and has been shown to be beneficial, by increasing the content of dietary fiber and minerals (OWHERUO; IFESAN; KOLAWOLE, 2019; SARITA; EKTA SINGH, 2016), antioxidant and anti-inflammatory activity (THEODORO et al., 2021a), and reducing the concentration of phytic acid (SARITA; EKTA SINGH, 2016). Another option is the extrusion cooking, which is a versatile, short-term process with high potential for developing millet-based products and offering ready-

to-eat products. This process improves grain digestibility and promotes higher sensory acceptance, in addition to inactivating lipases, leading to an increase of shelf life of the products (DIAS-MARTINS et al., 2018; PESSANHA et al., 2023). Pearl millet can also be cooked by boiling in an open pan, under pressure or in a microwave, with open pan cooking being the most commonly used method for human consumption. In addition, the grains can be ground to obtain flour to be used as an ingredient in culinary preparations. Cooking and grinding the grains are the most common processes used for pearl millet, as they are simple and inexpensive, and can be applied as pretreatment, alone or in conjunction with other processing (DIAS-MARTINS et al., 2018; NANJE GOWDA et al., 2022).

The inclusion of pearl millet in the diet, by providing nutrients and bioactive compounds such as proteins, dietary fiber, resistant starch and minerals such as iron, can be a strategy to minimize metabolic changes in the body, such as those resulting from compromised intestinal health or an unbalanced intake, whether due to excess, such as high-fat diets, or deficiency, such as insufficient diets and or diets with low bioavailability of nutrients (DIAS MARTINS, 2019; SIHAG et al., 2016; THEODORO et al., 2021a).

Diets rich in sugar and fat can affect the composition of the microbiota and the function of the intestinal barrier, enabling the rupture and increased permeability in the intestine, allowing the translocation of lipopolysaccharides (LPS) that can activate inflammatory mediators and lead to a cycle of changes in the body (MALESZA et al., 2021; SCHEITHAUER et al., 2016), compromising the absorption of nutrients and its homeostasis. In addition, inadequate absorption of nutrients can also occur due to metabolic changes resulting from the imbalance of the intestinal microbiota. Iron bioavailability, for example, is related to intestinal health, since the composition of the microbiota and the production of short-chain fatty acids (SCFA), promoted by the fermentation of prebiotics, reduces intestinal pH, making iron more soluble and improving the proliferation of intestinal cells, increasing iron absorption. However, when the microbiota is unbalanced, this mechanism is altered, which can compromise the absorption of this mineral and other compounds (GANNON et al., 2021; RUSU et al., 2020). Insufficient iron supply in the body can lead to iron deficiency anemia, which is a public health problem that affects low, middle and high-income populations worldwide, contributing to increased morbidity and mortality (SIHAG et al., 2016; TURAWA et al., 2021).

Therefore, the consumption of prebiotics, such as dietary fiber and resistant starch, in addition to other bioactive compounds and iron, present in pearl millet, can be an alternative for modulating intestinal health and improving the supply and absorption of nutrients (RODRÍGUEZ-CABEZAS et al., 2010; RUSU et al., 2020). In addition, prebiotics improve the diversity and function of the microbiota, intestinal morphology and help to reduce intestinal permeability, reducing inflammation, oxidative stress and intestinal dysbiosis (ABDI; JOYE, 2021; DIAS-MARTINS et al., 2018; RODRÍGUEZ-CABEZAS et al., 2010), contributing to better homeostasis of the organism. In addition to favoring the chemical-nutritional composition, the use of processing in pearl millet contributes to the development of a product with technological innovation, improving the sustainable potential of a resilient crop, being a promising option to overcome situations of food and nutritional insecurity (ONU, 2015; VETRIVENTHAN et al., 2021).

Our research group has observed several benefits of pearl millet consumption in vivo. In our previous study, we demonstrated that raw germinated pearl millet flour reduced adiposity, inflammation, oxidative stress, and insulin resistance in a model of metabolic alteration induced by consumption of high-fat high-fructose (HFHF) diet (THEODORO et al., 2021a, 2021b). Furthermore, other authors, who worked with other millet species and a high-fat diet, found an increase in SCFA and greater diversity of microbial species (Li et al., 2019), reduced serum LPS levels and abundance of potentially protective bacteria (SARMA et al., 2017), in addition to increased hemoglobin synthesis compared to the synthetic diet in animals fed millet-based diets fortified with iron and vitamin A (SIHAG et al., 2016). Therefore, in the present study, we concluded the investigations of raw germinated pearl millet flour on the microbiota composition and intestinal morphology of rats fed with HFHF diet. In addition, we evaluated the chemical-nutritional composition of pearl millet subjected to different processing types (cooked grains) and its effects on intestinal health, iron metabolism, oxidative stress, and inflammation. We proposed to continue the work using cooked pearl millet to better reflect the form of use in human consumption.

2. HYPOTHESIS

Raw germinated pearl millet flour contributes with intestinal health by beneficially modulating the metabolic changes that occurred in rats fed a HFHF diet, through improvements in the composition and function of the intestinal microbiota and in the morphology of the intestine. In addition, extrusion cooking and germination

followed by cooking in an open-pan contribute to a good chemical-nutritional composition of pearl millet, providing higher in vitro iron bioavailability, in addition to beneficial effects on iron metabolism, intestinal health, oxidative stress and inflammatory mechanisms in vivo.

3. OBJECTIVES

3.1. General objective

To evaluate the chemical-nutritional composition of pearl millet (*Pennisetum glaucum* (L.) R. Br.) subjected to different processing and its effects on intestinal health, iron metabolism, oxidative stress and inflammation.

3.2. Specific objectives

- To investigate the effect of consumption of raw germinated pearl millet on the composition and function of the intestinal microbiota and intestinal morphology in rats with metabolic alterations induced by HFHF diet;
- To determine the chemical-nutritional composition and in vitro iron bioavailability of pearl millet subjected to different processing methods (non-germinated open-pan cooked millet flour, germinated open-pan cooked millet flour and extrusion-cooked millet flour);
- To evaluate the effect of consumption of cooked processed pearl millet (non-germinated, germinated and extruded) on iron metabolism and antioxidant capacity in rats;
- To investigate the effect of cooked processed pearl millet consumption (non-germinated, germinated and extruded) on the intestinal health of rats induced to iron deficiency.

4. LITERATURE REVIEW

4.1. Millet: Nutritional quality and grain processing

4.1.1. Nutritional quality of millet

Cereals, especially whole grains, have aroused interest among researchers because they are recognized as beneficial in reducing metabolic disorders and improving health (LI et al., 2023; MURTAZA et al., 2014; SANDERS et al., 2023). Millet, belonging to the Poaceae family of grasses, is the sixth most cultivated cereal in the world, with India

being the world's largest producer. In Brazil, the most widespread species is *Pennisetum glaucum*, also known as pearl millet, occupying a total planting area of approximately 5.5 million hectares and representing one of the main cover crops in the Cerrado and in the South region (DIAS-MARTINS et al., 2018; FAOSTAT, 2020, 2024; LU et al., 2009).

Pearl millet has an extensive root system that allows the extraction of water and nutrients from the deepest layers of the soil, being resistant to drought, low soil fertility, high salinity and high temperature tolerance (DIAS-MARTINS et al., 2018; VETRIVENTHAN et al., 2021). Due to climate change, which directly affects agricultural production, this cereal stands out as an option to overcome situations of food insecurity, since in addition to being nutritious, it is environmentally sustainable and resistant, facilitating its production even in adverse weather conditions (ANITHA et al., 2021; VETRIVENTHAN et al., 2021). Furthermore, millet has a low incidence of mycotoxin contamination compared to other crops such as wheat and corn (DIAS-MARTINS et al., 2018).

Pearl millet grains have, in their chemical composition, approximately 62 to 76% carbohydrates, which are mainly in the form of starch and dietary fiber (average content of 7.8%), 8 to 11% protein and 2 to 9% lipids. Among the minerals present, the following stand out: calcium (300 mg/kg), zinc (43 mg/kg), iron (18 mg/kg), potassium (3.9 mg/kg), phosphorus (3.3 mg/kg) and magnesium (1.3 mg/kg) (ANNOR et al., 2015; DIAS-MARTINS et al., 2018; NANJE GOWDA et al., 2022; TAYLOR, 2015). In addition, the grain is also composed of fatty acids, mainly linoleic acid (39-45%), oleic acid (21-27%) and palmitic acid (20-21%) and, to a lesser extent, stearic acid (5%) and linolenic acid (3%) (LI et al., 2020b; NANI et al., 2015).

This grain also contains resistant starch, condensed tannins, and other phytochemicals such as phenols, lignans, β-glucan, inulin, phytates, sterols, tocopherol, and carotenoids (SALAR; PUREWAL; SANDHU, 2017; SARITA; EKTA SINGH, 2016; SIROHA; SANDHU; KAUR, 2016). The main polyphenols are phenolic acids and tannins, while flavonoids are present in small amounts (CHANDRASEKARA; SHAHIDI, 2010; DIAS-MARTINS et al., 2018). Polyphenols are the most important phytochemicals in pearl millet due to their nutraceutical potential, with antioxidant, anti-inflammatory and antimicrobial activity, being useful in the management of physiological disorders and maintenance of the health of the gastrointestinal tract (DIAS-MARTINS et al., 2018; SINGH et al., 2015).

Although pearl millet is recognized as a source of protein (~10%) and iron (18 mg/kg), the presence of phytates and polyphenols can compromise the nutritional quality of this cereal, requiring some treatment to improve the bioavailability of nutrients (NANJE GOWDA et al., 2022; SHARMA; GUJRAL, 2019; SIHAG et al., 2016). Furthermore, variations in the chemical composition of this grain are possible due to the genotype, climatic conditions, nutrient content in the soil and the type of processing performed, which may or may not improve its composition and, consequently, its health benefits (BONCOMPAGNI et al., 2018; ELNOUR et al., 1997; SINGH et al., 2015; THEODORO et al., 2021a).

4.1.2. Processing of millet grains

After harvesting, the grains can be subjected to different processes such as germination, extrusion, fermentation, milling, decortication, roasting, blanching, or cooking by boiling in an open-pan, under pressure or in a microwave, with cooking in an open-pan being the simplest and most widely used for human consumption (DIAS MARTINS, 2019). Some of these processes can modify the composition of the millet, reducing or improving its nutritional value (DIAS MARTINS, 2019; NANJE GOWDA et al., 2022; PESSANHA et al., 2021).

Some nutrients present in pearl millet are found mainly in the pericarp of the grain, for example, minerals and bioactive phytochemicals, which is the reason of recommending the consumption of the whole gain followed by some type of processing that would preserve or improve its nutritional quality (NANJE GOWDA et al., 2022; SALEH et al., 2013; SHARMA; GUJRAL, 2019).

Germination, which begins by soaking the grains in water and then place them under controlled temperature and time (for example, 30 ± 2 ºC and 90% humidity for 24 h), is a good alternative to improve the composition of the grain, as it is a simple and inexpensive process that allows the production of healthy foods (BENINCASA et al., 2019; HAN; YANG, 2015; JIMÉNEZ et al., 2024). During this processing, hydrolytic enzymes promote biochemical changes, structural modification and synthesis of new compounds that can increase the nutritional value and chemical stability of grains (DIAS-MARTINS et al., 2018; NANJE GOWDA et al., 2022). This bioprocess degrades macronutrients such as proteins, carbohydrates and lipids into simpler structures,

improving the bioavailability of nutrients and the functionality of grains (ELKHALIFA; BERNHARDT, 2010; JIMÉNEZ et al., 2024).

Millets germination has been shown to be beneficial in vitro and in vivo, since it increases protein digestibility (NKAMA; GBENYI; HAMAKER, 2015), dietary fiber content (SARITA; EKTA SINGH, 2016), some minerals such as calcium, iron, zinc and magnesium (OWHERUO; IFESAN; KOLAWOLE, 2019), essential amino acids such as lysine (ADEBIYI et al., 2017; ARORA; JOOD; KHETARPAUL, 2011), vitamin C (GAN et al., 2017), B vitamins (ARORA; JOOD; KHETARPAUL, 2011), in addition to polyphenols and antioxidant and anti-inflammatory activities (ALVAREZ-JUBETE et al., 2010; THEODORO et al., 2021a; VAN HUNG et al., 2012). Furthermore, this processing reduces phytochemicals, such as phytic acid, which form complexes, decreasing the bioavailability of nutrients (KRISHNAN; DHARMARAJ; MALLESHI, 2012; SARITA; EKTA SINGH, 2016). The magnitude of changes in the chemical composition of the grain may vary between studies due to differences in soaking practices, germination duration and temperature, in addition to the variety of millet species available (DIAS-MARTINS et al., 2018; JIMÉNEZ et al., 2024; SINGH et al., 2015).

Another option is extrusion cooking, which consists of a combination of thermal and mechanical treatment under conditions of low moisture, high pressure, temperature and shear (high screw speed inside the extruder and narrow die outlet), for a short period of time (PATEL, 2015; SALAZAR-LÓPEZ et al., 2018). This is an alternative process because it is a versatile that has been shown to increase the total content of phenolic compounds and the antioxidant capacity of some cereals (PATEL, 2015; SALAZAR-LÓPEZ et al., 2018). In addition, it has great potential for use in the development of millet-based products, with improved digestibility, greater sensory acceptance and provision of ready-to-eat food (BALASUBRAMANIAN; KAUR; SINGH, 2014; DIAS MARTINS, 2019). During this process, there may be an increase in carbohydrates digestability, due to the breakdown of the starch structure into small molecules by the action of heat and humidity, a reduction in lipids, due to the formation of complexes with other compounds present in the food matrix, such as starch (OBILANA; ODHAV; JIDEANI, 2018; PESSANHA et al., 2023), in addition to inactivation of lipases and reduction of lipid oxidation, thus improving the shelf life of extruded millet-based products (OBILANA; ODHAV; JIDEANI, 2018; SUMATHI; USHAKUMARI; MALLESHI, 2007).

Therefore, understanding the influence of processing on nutritional properties is important to increase the availability of pearl millet for human consumption, aiming to assist in choosing an appropriate processing technique for this cereal, in order to maximize the use of nutrients and beneficial effects on health, improve palatability and increase the shelf life of its products (NANJE GOWDA et al., 2022).

4.2. Nutritional interventions and intestinal health

Intestinal health can be negatively or positively altered by eating habits and nutritional interventions (KONIKOFF; GOPHNA, 2016). The unbalanced supply of nutrients can interfere with the composition of the microbiota and the functioning of the intestine. Both excesses, such as the consumption of high-fat diets or the use of iron salts with high bioavailability (ferrous sulfate), and deficiencies, such as the intake of a diet low in dietary fiber or with low bioavailability of nutrients, can lead to intestinal dysbiosis and changes in the morphology of the intestine (BOTTA et al., 2021; MALESZA et al., 2021; PARMANAND et al., 2019).

The high-fat diet, considered pro-inflammatory and oxidative, generally presents high amounts of sugar and fat, mainly saturated, and a low amount of dietary fiber (TER HORST; SERLIE, 2017). Consuming this type of diet favors the progression of metabolic changes in the body, enabling the rupture of the barrier and increased permeability in the intestine, allowing the translocation of lipopolysaccharides (LPS) that can activate inflammatory and oxidative mediators, compromising the composition of the microbiota, intestinal function and the homeostasis of the organism (MALESZA et al., 2021; SCHEITHAUER et al., 2016; TER HORST; SERLIE, 2017).

The intestinal epithelium is responsible for regulating the passage of substances from the intestinal cavity to the bloodstream. Under normal conditions, this passage occurs via the transcellular route, but some molecules can cross the intercellular space between enterocytes and reach the bloodstream via the paracellular route (FRANCE; TURNER, 2017). In this way, the epithelium acts as a wall, and is then referred to as the intestinal barrier (LUISSINT; PARKOS; NUSRAT, 2016). The integrity of this barrier is guaranteed by the existence of tight junctions (TJ), which involve proteins, including occludins, claudins and adhesion molecules, which prevent the passage of unwanted substances (TALADRID et al., 2021).

The urinary excretion of unmetabolized sugars, such as lactulose and mannitol, administered orally, is the most widely used technique to assess the integrity of this barrier

through the analysis of intestinal permeability. The absorption of the monosaccharide mannitol is used to reflect transcellular permeability, demonstrating the absorption of small molecules, while the absorption of the disaccharide lactulose, which is capable of passing between the paracellular junctions, reflects the permeability of larger molecules. Therefore, higher excretion of lactulose and mannitol is related to damage to mucosal cells and tight junctions, reflecting higher intestinal permeability (TALADRID et al., 2021; WIEST; RATH, 2003).

In addition to controlling the flow of nutrients and other substances, the intestinal barrier also offers protection against pathogens and toxins (TALADRID et al., 2021). Compromised integrity of this barrier is known as "leaky gut", a condition in which several toxic food compounds and LPS (a component of the cell membrane of gram-negative bacteria) reach the bloodstream. This condition leads to a subclinical inflammatory state, called metabolic endotoxemia, and the onset of gastrointestinal and metabolic diseases, such as inflammatory intestinal disease, diabetes and obesity (DAMMS-MACHADO et al., 2017). LPS induces inflammation through stimulation of Toll-like receptor 4 (TLR4), which in turn generates intracellular responses that culminate in the release of nuclear factor Kappa B (NF-κB), which increases the expression of pro-inflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor (TNF) (TALADRID et al., 2021).

Intestinal integrity is also guaranteed by nutritional interventions that favor a healthy intestinal microbiota and by the presence of short-chain fatty acids (SCFA), produced by bacterial fermentation, including butyrate, propionate and acetate, which can decrease LPS translocation by improving the intestinal barrier, helping to minimize the effect of pathogenic bacteria (WANG et al., 2012). These SCFAs, such as butyrate, exert beneficial functions on the intestinal barrier by increasing the gene expression of TJ proteins, through the activation of AMP-activated protein kinase (AMPK) and inhibition of inflammatory mediators such as NF-κB and TNF (MALESZA et al., 2022; MCNABNEY; HENAGAN, 2017; PENG et al., 2009).

The intestinal microbiota is a complex ecosystem, composed mainly of bacteria. These microorganisms synthesize essential micronutrients, such as vitamins, metabolize toxins, ensure the maturation of the immune system, aid in the growth and differentiation of enterocytes, and protect against enteric pathogens (GÉRARD; BERNALIER-DONADILLE, 2007). Dietary components have a fundamental impact on the intestinal

microbiota, influencing its composition in terms of richness and diversity (RINNINELLA et al., 2019).

A high intake of saturated fat, sugar, iron and animal protein can stimulate the growth of potentially pathogenic bacteria, such as some Firmicutes, in detriment of potentially beneficial bacteria, such as some Bacteroidetes, leading to changes in the balance of the intestinal microbiota (SINGH et al., 2017; WU et al., 2011). Dysbiosis influences the functioning of the intestine, which can increase permeability and result in a cycle of changes in the body (SCHEITHAUER et al., 2016). Furthermore, inadequate nutrient absorption, caused by impaired intestinal health, is also a significant metabolic alteration that can interfere with the bioavailability of micronutrients, such as iron, for example, and can affect intestinal and systemic functionality, since iron is essential for bacterial growth, cellular respiration, immune response, and homeostasis of the organism (GANNON; GLAHN; MEHTA, 2021; PARMANAND et al., 2019).

On the other hand, the consumption of complex polysaccharides, phenolic compounds, and vegetable protein is associated with a reduction in the growth of potentially pathogenic bacteria and an increase in potentially beneficial bacteria, stimulating the production of SCFA (DINGEO et al., 2020; SINGH et al., 2017). Furthermore, studies show that the consumption of dietary fiber, resistant starch and bioactive compounds, such as polyphenols, for example, is directly linked to an increase in the number and diameter of goblet cells and greater mucus production, providing greater protection for the membrane, in addition to an increase in the surface area, height and width of the villi, greater depth of the crypts and improvement in the integrity of the barrier, improving intestinal functionality and nutrient absorption (DA SILVA et al., 2019b; DINGEO et al., 2020; GAO et al., 2020; HAN et al., 2023). These functional and morphological effects appear to occur due to increased motility of the digestive tract, leading to cell hyperplasia and/or hypertrophy (DA SILVA et al., 2016b; GAO et al., 2020). Furthermore, omega-3 also appears to have the potential to confer health benefits through modulation of the intestinal microbiota, favoring the growth of potentially beneficial bacteria (RINNINELLA et al., 2019). The beneficial effects of polyphenols on intestinal health may be due to the synergy between their prebiotic and antimicrobial properties and are closely related to the food matrix (DINGEO et al., 2020).

In view of this, the consumption of prebiotics, which are non-digestible food constituents used by intestinal microorganisms that beneficially affect the physiology of the host (GIBSON et al., 2004; HOLSCHER, 2017), is a strategy to minimize or avoid

metabolic changes in the body through modulation of the intestinal microbiota (NICOLUCCI et al., 2017). Cereals, as one of the main components of the human diet, contain substantial levels of dietary fiber, resistant starch, phenolic compounds, and proteins that are used as fermentable substrates for probiotic microorganisms, providing prebiotic effects, such as production of SCFA, growth of potentially beneficial bacteria, production of functional proteins, and hypertrophy of the intestinal mucosa, improving intestinal function (ABDI; JOYE, 2021; DINGEO et al., 2020; GOMES et al., 2021; KASPRZAK-DROZD et al., 2021; NOORI et al., 2017). Therefore, the consumption of whole grains, such as millet, may be an option to provide essential components for maintaining or improving intestinal health and promoting nutrient absorption.

4.3. Iron metabolism

Iron is one of the most abundant elements in nature (SILVA; MURA, 2011). However, its ability to form highly insoluble oxides when in contact with oxygen reduces its bioavailability (GONZÁLEZ-DOMÍNGUEZ et al., 2020). Therefore, iron deficiency anemia, resulting from insufficient intake and/or low bioavailability of iron from the diet, is a public health problem that affects more than 2 billion people in low, middle and high-income populations worldwide, contributing to increased morbidity and mortality (DRAKESMITH et al., 2021; SIHAG et al., 2016).

Iron deficiency anemia negatively affects growth and cognitive development in children, cognitive, physical and psychological health in women of childbearing age, pregnant women and postpartum women, physical performance and productivity at work in adults, as well as health and quality of life in the elderly, and is associated with several negative outcomes, such as longer hospital stays and increased risk of disability (DEV; BABITT, 2017; TURAWA et al., 2021). Many factors are associated with iron deficiency anemia, including malabsorption due to compromised intestinal health, impaired iron bioavailability, inadequate folic acid, vitamin C, vitamin A and vitamin B12 in the diet, as well as iron losses due to inflammation and disease states (ANITHA et al., 2021; GANNON; GLAHN; MEHTA, 2021; TURAWA et al., 2021).

Nutrient bioavailability is a critical component that links nutrient intake, absorption, and physiological function, resulting in health maintenance (GANNON; GLAHN; MEHTA, 2021). Inadequate iron absorption, caused by impaired intestinal health, for example, is a significant metabolic alteration that interferes with the bioavailability and function of this mineral. In addition, the availability of iron in the food

matrix is also important and may interfere with its use by the body. Therefore, inadequate absorption and/or impairment of iron bioavailability can result in health problems, as previously mentioned (CAI et al., 2017; GANNON; GLAHN; MEHTA, 2021).

To assess iron bioavailability in vivo, using an animal model, a widely used method is the hemoglobin depletion/repletion technique proposed by AOAC (1998). In this technique, anemia is first induced in the animals and then test products are offered in the diet to assess hemoglobin gain. This result is calculated using the Hemoglobin Regeneration Efficiency (HRE), using a mathematical formula that takes into account the amount of iron consumed in the diet, the body weight and the hemoglobin level in the animals' blood. This equation considers that the blood volume corresponds to 6.7% of the animal's body weight and the iron content of hemoglobin is 0.335% (HARO-VICENTE et al., 2008).

In developing countries, the majority (>80%) of the diet is composed of staple foods with low iron content. Due to limited access to animal products (meat, fish and eggs) that provide a high concentration of heme iron, the main dietary sources of iron are cereals and legumes, making it difficult to achieve sufficient iron requirement through the rest of the diet (20%). Therefore, it is important to diversify staple foods, including food crops that are naturally rich in iron (ANITHA et al., 2021; NANJE GOWDA et al., 2022; VETRIVENTHAN et al., 2021).

Animal sources of heme iron are known for their high bioavailability, however, they are not always accessible to the poorest segments of the population (ANITHA et al., 2021). On the other hand, iron from plant sources is more accessible, but has lower bioavailability. This is in the form of non-heme iron and, in order to be absorbed, it needs to be solubilized and present in the reduced ferrous state, allowing it to be transported through the intestinal enterocyte (GANNON; GLAHN; MEHTA, 2021).

When transported into the enterocyte, iron passes through three barriers: apical membrane, translocation through the cytosol and release into the basolateral membrane, following it into the circulation (GROTTO, 2008). Dietary iron is absorbed into the apical membrane of enterocytes in the form of ferritin, heme iron or as reduced Fe^{2+}. Internalized ferritin undergoes degradation in lysosomes and Fe^{2+} is released. Endocytosis of heme iron is mediated by heme transport protein 1 (HCP1). Heme is degraded in endosomes resulting in the release of Fe^{2+}. Ferric iron must first be reduced from Fe^{3+} to Fe^{2+} by duodenal cytochrome b ferroreductase (DcytB) and is taken up by enterocytes via divalent metal transporter 1 (DMT-1). Fe^{2+} is exported from the enterocytes across the basolateral

membrane by ferroportin. The released Fe^{2+} is oxidized by a transmembrane ferroxidase, hephaestin, into Fe^{3+}, which is transported by transferrin in the circulation (BIELIK; KOLISEK, 2021).

Transferrin is a protein that transports serum iron. This protein captures free ferric iron (Fe^{3+}) in the blood and transports it to cells and tissues (GAO et al., 2019). Free iron, when not used in metabolic processes, can be incorporated into ferritin, which is a cytosolic iron storage protein, acting as the main cellular iron store in the body (GAO et al., 2019; PENG; UPRICHARD, 2017).

The body regulates iron concentrations, controlling both the threats of inadequate supply that would limit critical functions, and the excess of uncontrolled iron, which could be toxic to the body. Systemic iron homeostasis is maintained by regulating intestinal iron absorption, iron concentration in blood plasma and extracellular fluid, iron distribution between organs and tissues, and the amount of iron stored (NEMETH; GANZ, 2021).

In situations of inflammation, ferritin levels may be elevated in the body (MAHROUM et al., 2022). This occurs because ferritin acts as a positive acute phase protein that sequesters and stores iron intracellularly, in order to prevent excess iron from being available in the body, which can react with oxygen radicals, generating reactive species and oxidative stress that compromise cells and damage tissues, or can stimulate the growth of potentially pathogenic bacteria, stimulating intestinal dysbiosis (KERNAN; CARCILLO, 2017; MAHROUM et al., 2022). Furthermore, hyperferritinemia during inflammation may also be secondary to immunological stimulation of ferritin synthesis by inflammatory cytokines (KERNAN; CARCILLO, 2017; MAHROUM et al., 2022).

Another important point is the action of hepcidin, a hormone that regulates iron homeostasis and controls the plasma concentration and tissue distribution of this mineral, inhibiting its intestinal absorption, recycling by macrophages and mobilization of hepatic stores when necessary (SIHAG et al., 2016). When iron levels in the body are high, intestinal cells increase the capture of iron from the plasma. The increased concentration of iron in enterocytes stimulates the expression of hepcidin, which leads to a reduction in the synthesis of proteins involved in iron absorption, such as DMT-1 and DcytB, reducing the entry of iron into the enterocyte. Furthermore, hepcidin also interacts with ferroportin to cause the internalization of this protein and subsequent degradation, which decreases the transfer of iron into the bloodstream. Therefore, when activated, hepcidin reduces iron bioavailability (COZZOLINO, 1997; NEMETH; GANZ, 2021). However, in the state of iron deficiency and hypoxia, hepcidin expression decreases and duodenal expression of

DMT-1, DcytB and ferroportin is stimulated, increasing iron absorption. In contrast, the inflammatory state can lead to an increase in hepcidin expression, leading to a reduction in iron absorption, this occurs to prevent more iron from entering the body and causing oxidative reactions (COZZOLINO, 1997; NEMETH; GANZ, 2021; RIVERA et al., 2005).

Some factors can favor intestinal iron absorption, such as acidity and the presence of solubilizing agents such as sugars, which can favor the bioavailability of the mineral and the body's needs. In situations where there is a lack of iron or an increased need, such as pregnancy, puberty or hemolysis, there is greater iron absorption (COZZOLINO, 1997; GROTTO, 2008; NEMETH; GANZ, 2021). To meet this greater demand, there is a greater expression of proteins involved in iron absorption, such as ferroportin and DMT-1 (GROTTO, 2008). On the other hand, mutations that inactivate ferroportin or hephaestin can lead to impaired absorption and accumulation of iron in enterocytes and macrophages, which can result in impairment of the body's homeostasis (COZZOLINO, 1997; GROTTO, 2008).

The bioavailability of this mineral depends not only on the content and type of iron, but also on the presence and absence of enhancers and inhibitors. Absorption promoters are organic acids, such as ascorbic and citric acid. Inhibitors include oxalic acid and polyphenols, found in tea, coffee, cocoa and some legumes, and phytate, found in whole grains and legumes (SIHAG et al., 2016). Regarding polyphenols, there is variation, since some polyphenols have been shown to inhibit, while others appear to increase iron absorption. However, in the presence of both, inhibitors generally have more potent effects compared to promoters, resulting in a net inhibitory effect (GANNON; GLAHN; MEHTA, 2021).

In addition, there are interactions between dietary iron, the food matrix, the composition of the intestinal microbiota and the bioavailability of iron (KORTMAN et al., 2014). The physiological form of iron and the food matrix can affect the absorption of this mineral in the upper intestine. The cell wall of grain cotyledons, for example, is a significant barrier to iron bioavailability, since it is often not degraded by cooking and by human gastric and intestinal enzymes, reducing the absorption of this mineral (GLAHN et al., 2016). Therefore, the use of processing that improves iron availability in the food matrix may be interesting (ALBARRACÍN et al., 2019; NANJE GOWDA et al., 2022).

Therefore, it is worth considering that bioavailability is not necessarily constant as food intake increases, since, as previously described, it is related to the homeostatic

regulation of absorption, enhancers and inhibitors, food matrix, competition with other nutrients (e.g. zinc) and intestinal health (GANNON; GLAHN; MEHTA, 2021).

4.4. Iron metabolism, gut health, oxidative stress and inflammation

Iron, which is an essential nutrient for the host and the microbiome, can be ingested both in excess and in insufficient quantities to meet the organism's demands (PARMANAND et al., 2019). The host needs iron for several metabolic functions, such as cellular respiration, oxygen transport, immune response, etc. Likewise, for most bacteria, iron is essential for the proper functioning of some proteins and enzymes, being used for the growth and proliferation of bacteria (BOTTA et al., 2021). Therefore, iron deficiency can inhibit these metabolic processes, which can impair bacterial growth and the organism's homeostasis (PARMANAND et al., 2019).

On the other hand, excessive levels of iron can favor oxidative stress and increase the prevalence of potentially pathogenic bacteria (PHIPPS et al., 2020). The literature highlights evidence of side effects of oral iron supplementation in relation to exacerbation of inflammation, changes in the intestinal microbiota and adverse gastrointestinal events, since this mineral has the ability to generate free radicals, which can form reactive oxygen species (ROS), in addition to serving as an essential growth factor for pathogenic microorganisms, as well as for neoplastic cells (MALESZA et al., 2022; TOLKIEN et al., 2015).

In anemic or Fe-deficient patients, the use of iron salts (ferrous sulfate) optimizes and increases the absorption of this mineral. However, in some cases, this strategy is accompanied by unpleasant side effects, including constipation, gastric irritation, nausea and metallic taste in the mouth (AUERBACH; SCHRIER, 2017). Furthermore, the consumption of iron salts can increase Fe in the large intestine and, consequently, unabsorbed iron can cause changes in the proportion of potentially protective bacteria and potentially pathogenic ones, reducing the number of lactic bacteria, such as *bifidobacteria* and *lactobacilli*, and increasing bacteria that are associated with intestinal inflammation, such as *Escherichia coli* (PAGANINI; ZIMMERMANN, 2017).

The overproduction of free radicals, caused by excess iron, can lead to cellular changes and increase the permeability of the intestinal barrier (QI et al., 2019). ROS initiate lipid peroxidation and generate stress in the mitochondria and endoplasmic reticulum, leading to the destruction of cell membranes, violating the intestinal barrier

function and contributing to the increased permeability of bacterial toxins in the bloodstream, favoring inflammation (MALESZA et al., 2022; QI et al., 2019).

On the other hand, regarding iron bioavailability, the pH of the cecal content and SCFAs are factors that determine the absorption of this mineral. The fermentation of microorganisms can decrease the pH in the intestine, releasing iron from protein complexes, thus increasing its absorption (GANNON; GLAHN; MEHTA, 2021b). In addition, the production of SCFA by the intestinal microbiota has been positively related to the increase in iron bioavailability, since SCFAs can also increase the absorption area in the intestine by stimulating the proliferation of epithelial cells (RUSU et al., 2020). Another mechanism is explained by González et al., (2017), who demonstrated that *Lactobacillus fermentum*, one of the main probiotics found in the microbiota, exhibits a remarkable iron-reducing activity. P-hydroxyphenyllactic acid, a metabolite produced by this strain, reduces Fe^{3+} to Fe^{2+} and increases iron absorption by DMT 1 transporters in enterocytes. Thus, we can conclude that the relationship between iron and microbiota is complex and bilateral, where iron deficiency and excess modulate the intestinal microbiota, just as the composition of the intestinal microbiota also influences iron bioavailability and the effect of this mineral on health.

4.5. Potential functional effects of pearl millet on gut health, iron metabolism, oxidative stress and inflammation

Although millet has high concentrations of phytate and polyphenols, which are potent inhibitors of iron absorption, iron-fortified millet-based diets resulted in higher hemoglobin (Hb) synthesis compared to the synthetic diet, and this was attributed to the processing employed in millet, which is known to improve iron bioavailability and reduce potential inhibitors (SIHAG et al., 2016). In addition, millet has a higher amount of dietary fiber compared to other cereals such as rice and wheat (ANITHA et al., 2021), and this acts positively on the composition of the intestinal microbiota, improving abundance and altering the composition of the microbiota in a beneficial way (PAGANINI; ZIMMERMANN, 2017), enabling better nutrient absorption (PAGANINI; UYOGA; ZIMMERMANN, 2016).

Li et al., (2019) evaluated the intestinal microbiota in rats fed a high-fat diet supplemented with millet (*Setaria itálica L*) for 4 weeks and found an increase in SCFA (acetate, propionate and butyrate), greater diversity of microbial species, in addition to a reduction in Firmicutes and an increase in Actinobacteria. Similarly, Sarma et al., (2017)

investigated the supplementation of kodo millet (*Paspalum scrobiculatum*) in mice fed a high-fat diet for 16 weeks and observed a reduction in serum LPS levels, abundance of potentially protective bacteria (*Lactobacillus sp., Bifidobacteria, Akkermansia and Roseburia spp.*) and higher acetate production, evidencing that millet supplementation can reduce the changes induced by the high-fat diet in these animals. In vivo and in vitro studies have reported adverse effects of oral Fe supplementation on microbiota composition and intestinal health, as previously mentioned, in patients with chronic diseases (KORTMAN; REIJNDERS; SWINKELS, 2017; LEE et al., 2017). On the other hand, Cai et al., (2017) demonstrated, in an in vitro study, that Fe bioaccessibility in the colon is higher than in the small intestine, suggesting that the intestinal microbiota can increase Fe extraction from vegetables. In this scenario, increasing Fe absorption from natural foods, such as cereals, can serve as an alternative to dietary supplements to overcome Fe deficiency situations, in addition to having a beneficial effect on health (PAGANINI; ZIMMERMANN, 2017). Millet has considerable amounts of iron in its composition (18 mg/kg), in addition to also presenting prebiotics and bioactive compounds that can contribute to improved intestinal function, favoring nutrient absorption. Furthermore, the use of processing can improve iron availability by reducing the formation of complexes, allowing iron to be more available in the food matrix and can be adequately absorbed (DIAS-MARTINS et al., 2018; NANJE GOWDA et al., 2022).

Our research group has already evaluated the effect of pearl millet in vivo. In our previous study, we demonstrated that germinated pearl millet flour reduced inflammation, oxidative stress, and insulin resistance in rats fed a diet high in saturated fat and fructose (HFHF) (THEODORO et al., 2021a, 2021b). In addition, Li et al. (2020) explored the antioxidant activity and neural protective function of millet in oxidative stress induced by a high-fat diet (for six weeks) in rat brains and suggested that millet intake could alleviate oxidative stress in the hippocampus and negatively regulate the expression of genes related to neurodegenerative diseases. The authors demonstrated that millet polyphenols play important roles during this process, decreasing oxidative stress levels in cells and the expression of pro-inflammatory factors.

In view of this, pearl millet (*Pennisetum glaucum*), which is the most common in Brazil, as it is a cereal that is naturally a source of prebiotics, bioactive compounds and iron, in addition to being easily produced and accessible to the poorest segments of the population (DIAS MARTINS, 2019), may be an option for inclusion in the diet, since in

addition to providing dietary iron, it can beneficially modulate the microbiota and provide health benefits. Furthermore, germination and extrusion can improve the chemical composition and bioactive compounds of pearl millet, adding more functional effect to the grain, favoring intestinal health, iron bioavailability, and the body's homeostasis.

5. GENERAL METHODOLOGY

This study was carried out at the Department of Nutrition and Health of the Federal University of Viçosa, in collaboration with national institutions (State University of Campinas (SP), EMBRAPA agroindustry (RJ) and Atto Agrícola LTDA (MT)) and international universities (Cornell University and University of Wisconsin-Madison).

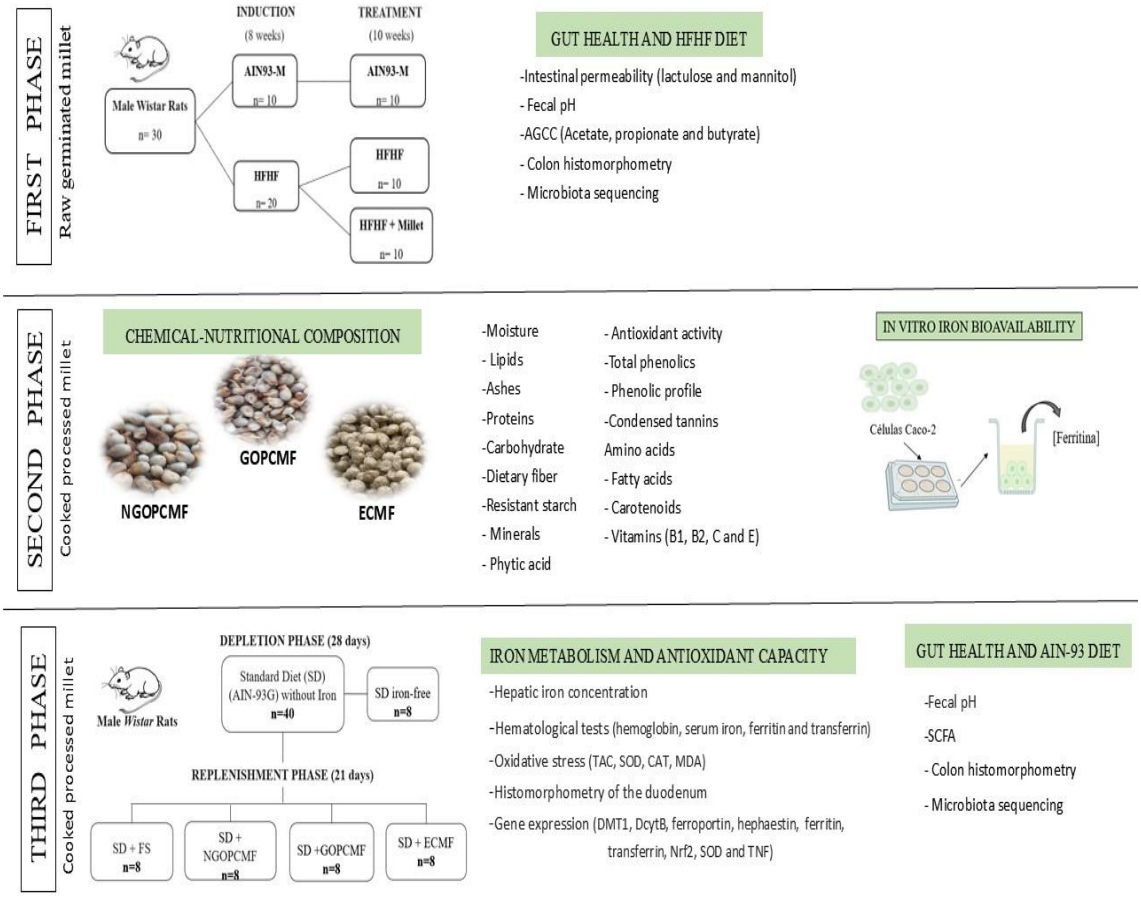
This study was divided into three stages **(Figure 1)**, as follows:

1st phase: Experiment 1**:** Investigation of the effects of the consumption of raw germinated pearl millet on the composition and function of the intestinal microbiota and on the intestinal morphology of rats subjected to metabolic changes through the consumption of a HFHF diet;

2nd phase: Determination of the chemical-nutritional composition and in vitro iron bioavailability of whole pearl millet subjected to different processing methods (non-germinated open-pan cooked millet flour, germinated open-pan cooked millet flour and extrusion-cooked millet flour);

3rd phase: Experiment 2: Evaluation of the effects of pearl millet subjected to different processing methods (pre-cooked pearl millet flour) on iron metabolism, intestinal health, oxidative stress and inflammation in rats induced to iron deficiency.

Figure 1. Experiments design



The study was divided into three phases and evaluated the chemical-nutritional composition of processed pearl millet and its effects on intestinal health, iron metabolism, oxidative stress and inflammation. In the first phase, the effects of the consumption of raw germinated pearl millet on the composition of the intestinal microbiota and intestinal function were investigated in a model of induction of metabolic alterations (high-fat high-fructose (HFHF) diet). In the second phase, the chemical-nutritional composition and the in vitro iron bioavailability of pearl millet subjected to different processing methods (non-germinated open-pan cooked millet flour (NGOPCMF); germinated open-pan cooked millet flour (GOPCMF); extrusion-cooked millet flour (ECMF)) were evaluated. In the third phase, the effects of pearl millet subjected to different processing methods (pré-cooked pearl millet) on intestinal health, iron metabolism, oxidative stress and inflammation in a model of iron deficiency (hemoglobin depletion/repletion and use of ferrous sulfate) were investigated. SCFA: short-chain fatty acids; AIN-93G: standard diet for growing rodents; DMT1: divalent metal transport protein; DcytB: duodenal cytochrome B; Nrf2: nuclear factor erythroid 2; TNF: tumor necrosis factor; TAC: total antioxidant capacity; SOD: superoxide dismutase; CAT: catalase; MDA: malondealdehyde.

5.1. Study locations

The analyses were performed in some laboratories of Federal University of Viçosa- Department of Nutrition and Health/UFV: Experimental Nutrition Laboratory

(biological tests in rats and their respective analyses of intestinal permeability, fecal pH, and biochemicals; and some analyses on millet flours, such as proximate composition, resistant starch, phytic acid, antioxidant activity, total phenolics and condensed tannins) and Vitamin Analysis Laboratory (carotenoids and vitamins); Department of Soils: Soil Analysis Laboratory (minerals); Department of Biochemistry and Molecular Biology: Laboratory of Parasitology and Molecular Epidemiology and Laboratory of Metabolism and Fermentation (Gene expression and protein quantification); Department of Microbiology: Laboratory of Microorganism Physiology (Short-chain fatty acid analyses); Department of General Biology: Laboratory of Structural Cell Biology and Department of Veterinary Medicine: Histopathology Laboratory (histological analyses).

Further, Liquid Chromatography Laboratory, EMBRAPA/RJ (fatty acids and amino acids profile) and Multidisciplinary Laboratory of Food and Health of the State University of Campinas (Unicamp, São Paulo) (phenolic profile) were used.

The in vitro bioavailability of iron was performed at Cornell University: Laboratory of Plants, Soil and Nutrition. Microbiota sequencing was performed at the Environmental Sample Preparation and Sequencing Facility/Lemont, Illinois, USA (Argonne National Laboratory) or at NovoGene Co., Ltd (Beijing, China) in conjunction with the Department of Nutritional Sciences/University of Wisconsin-Madison, Madison, USA (College of Agriculture and Life Science).

5.2. Raw material

The study was conducted with pearl millet (*Pennisetum glaucum* (L.) R. Br.). The first stage of the Thesis is a following of my master's project entitled "Effect of germinated millet flour (*Pennisetum glaucum* (L.) R. Br.) on metabolic changes of rats fed with high-fat high-fructose diet ". In this study we only worked with the germination of grains and did not use a cooking process, so we only have one treatment group, using raw germinated millet flour. However, as we continued our studies with millet, we proposed using another processing method in addition to germination, so we also included extrusion, which provides a product ready for consumption. We also wanted to work with millet in the form in which it would be used for human consumption, so in steps 2 and 3 we used cooked millet grains.

5.3. First phase - Effects of the consumption of raw germinated pearl millet on the composition and function of the intestinal microbiota and on the intestinal morphology of rats subjected to metabolic changes through the consumption of a HFHF diet

In first phase, the cultivar BRS1502 was used. The grains were planted and harvested in 2016, in the experimental field of Embrapa Milho e Sorgo, in Sete Lagoas, MG. The germinated millet flour was prepared at Embrapa Agroindústria de Alimentos (RJ), according to its own protocol.

Whole millet grains (99% of germination index) were soaked in distilled water for 30 min and placed in a controllable heat (30° ± 2 °C) and moisture chamber (> 90% relative humidity) (National Mfg. Co., Lincoln, USA) in order to germinate for 24 h, which allowed the emission of roots (up to 20 mm long). Next, the germinated millets were dried (50 °C, 4 h) in an oven (MS-G-EG; Macanuda Hauber, Joinville, Brazil). These grains were then ground in a LM3100 hammer mill (Perten Instruments AB, Huddinge, Sweden) equipped with a sieve with 1.0 mm openings. The flour was vacuum-packed in polyethylene packaging and sent to the Experimental Nutrition Laboratory of the Department of Nutrition and Health of the Federal University of Viçosa (DNS/UFV), where it was stored in a freezer (-18 °C ± 1 °C) until the diet was processed (THEODORO et al., 2021b).

Experiment 1 consisted of two phases. In the first phase, thirty male *Wistar* rats, 45 to 50 days old (*Rattus norvegicus*, from the Central Bioterium of the Federal University of Viçosa-Brazil), were distributed into 2 groups: AIN-93M (normal control group; n = 10), which received the AIN-93M diet (REEVES; NIELSEN; FAHEY, 1993) and HFHF (n = 20), which received a diet rich in saturated fat (31% lard) and fructose (20%), for 8 weeks to induce metabolic changes (adapted from Marineli et al., 2015). In the second phase, the AIN-93M group (n = 10) was maintained, and the HFHF group was split into a HFHF group (n = 10, receiving the HFHF diet) and a HFHF + millet group (which received the HFHF diet added with 286.3 g (28.63%) of germinated millet flour) for another 10 weeks (adapted from Marineli et al., 2015).

The amount of millet added to the diet replaced the dietary fiber (43.6%), starch (100%), protein (36%) and oil (39%) of the HFHF diet. Throughout the experiment, the animals received water and diet ad libitum. The diets had the same amount of fiber (55.8%), however, in the AIN93-M and HFHF diets, cellulose was added and, in the HFHF + millet diet, 43.6% of the fiber came from germinated millet flour **(Table 1)**.

Table 1. Composition of experimental diets in biological trial 1.

Ingredients (g/kg)	Experimental Diets		
	AIN93-M	HFHF	HFHF + Millet**
Albumin*	136.6	136.6	87.3
Cornstarch	463.5	135.0	-
Dextrinized starch	155.0	45.0	-
Sucrose	100.0	28.6	8.6
Pork lard	-	310.0	310.0
Soybean oil	40.0	40.0	24.5
Germinated millet	-	-	286.3
Fructose	-	200.0	200.0
Cellulose	55.8	55.8	34.0
Mineral mix	35.0	35.0	35.0
Vitamin mix	10.0	10.0	10.0
L-cystine	1.8	1.8	1.8
Choline bitartrate	2.5	2.5	2.5
Diet composition			
Carbohydrate (%)	73.22	42.5	40.3
Protein (%)	12.0	12.0	12.0
Lipid (%)	4.0	35.0	35.0
Caloric density (kcal/g)	3.8	5.3	5.3

*Albumin based on 88% protein content. AIN-93M: standard rodent diet; HFHF: high-fat high-fructose; HFHF + Millet: germinated millet flour test diet. **Chemical composition of millet in g/100g: carbohydrates- 64.47g; total dietary fiber- 7.60g; lipids- 5.41g; protein- 15.08g; moisture- 5.55g.

Body weight and food intake were monitored once a week. One week before euthanasia, urine was collected from the animals to analyze intestinal permeability. After the 18th week, the animals were anesthetized (Isoforine®, Cristália, Brazil) and euthanized by cardiac puncture. The proximal colon was collected, washed with PBS (phosphate buffer solution) and kept in formaldehyde (10%) at room temperature for histomorphometric analysis. The cecum was weighed and its contents collected and immediately stored at -80°C, for analysis of SCFA, fecal pH and intestinal microbiota.

5.4. Second phase - Chemical-nutritional composition and in vitro iron bioavailability of whole pearl millet subjected to different processing methods

For the second phase of the study the ADRg 9070 hybrid, provided by Atto Sementes (Rondonópolis, Brazil), was used. The grains were harvested in the municipality of Itiquira (MT, Brazil) during the 2nd Harvest of 2021. The processing, germination and extrusion were carried out by Embrapa Food Technology (Rio de Janeiro-RJ).

Germination was carried out at 30° ± 2 °C and 90% humidity for 24 h, with subsequent drying of the grains at 50 °C for 4 h, as previously described **(**first phase). These grains were packaged and transported to the Federal University of Viçosa, where they were stored at 4 °C until flour was prepared.

Extrusion cooking of whole millet flour, with moisture content adjusted to 13%, was carried out in an Evolum HT25 co-rotating twin-screw extruder (Clextral Inc., Firminy: France) equipped with an 8 mm diameter round die, length/diameter ratio of 40:1, and ten temperature zones defined from the feed to the die: (25, 40, 50, 90, 100, 100, 110, 110, 120, 120 °C). The expanded extrudates were dried in a ventilated oven (Hauber Macanuda, Joinville, Brazil) at 60 °C for 2 h, packaged, and transported to the Federal University of Viçosa, where they were stored at 4 °C until flour preparation (PESSANHA et al., 2023).

For thermal treatment and preparation of flours, the non-germinated and germinated millet grains were cooked in an open-pan at 98 °C for 40 minutes in the proportions (grain/water) of 1:4 for the non-germinated millet grains and 1:5 for the germinated millet grains. After cooking, the grains were dried in a ventilated oven 400/6ND (Nova Ética®, São Paulo, Brazil) at 50 °C for 24 h (DIAS-MARTINS et al., 2018). Subsequently, the non-germinated cooked, germinated cooked, and cooked by extrusion whole millet grains were ground in a stainless-steel knife mill at 500 kg/h equipped with a 1 mm sieve (TREU, Rio de Janeiro, Brazil) to obtain the pre-cooked flours called: non-germinated open-pan cooked millet flour (NGOPCMF), germinated open-pan cooked millet flour (GOPCMF) and extruded cooked millet flour (ECMF). These flours were stored in a freezer (-20ºC ± 1ºC) until analysis and preparation of the diets for the experiment 2.

Moisture, lipids, ashes, proteins (AOAC, 2016); carbohydrate (by difference: [100 - (% moisture + % lipids + % proteins + % total dietary fiber + % ash)]; dietary fiber (kit from Megazyme®, Wicklow, Ireland: K-TDFR-200 A); resistant starch (kit from

Megazyme®, Wicklow, Ireland: AACC Method 32–40); minerals (GOMES; SILVA; SILVA, 1996); phytic acid kit (kit from Megazyme®, Wicklow, Ireland: K-PHYT Phytic Acid (Phytate)/Total Phosphorus); antioxidant activity (BLOOR, 2001; RUFINO et al., 2007); total phenolics compounds (SINGLETON; ORTHOFER; LAMUELA-RAVENTO, 1999); phenolic compounds profile (PIZANI et al., 2024; RUTZ et al., 2021); condensed tannins (PRICE; VAN SCOYOC; BUTLER, 1978; RUFINO et al., 2007); amino acids (AOAC, 2016; LIU et al., 1995); fatty acids (ANTONIASSI et al., 2018); carotenoids (CARDOSO et al., 2014); and vitamins (B1, B2, C and E) (CAMPOS et al., 2009; NDAW et al., 2000; PINHEIRO et al., 2021) analyses were performed on millet flour.

In addition, the in vitro bioavailability of iron assay was also performed using Caco-2 cells. Caco-2 cells synthesize ferritin in response to increases in intracellular Fe concentration. Therefore, we used the ferritin/total protein ratio (expressed as ng ferritin/mg protein) as an indicator of cellular Fe uptake. The protocols used in the analyses of ferritin and total proteins of Caco-2 cells were similar to those described previously by Dias et al., (2018); Glahn, (2022) and Tako; GlahnL, (2010).

5.5. Third phase - Evaluation of the effects of pearl millet subjected to different processing methods (pre-cooked pearl millet flour) on iron metabolism, intestinal health, oxidative stress and inflammation in rats induced to iron deficiency

In experiment 2, the ADRg 9070 hybrid was used, according to specifications previously provided in the second phase.

Forty (40) male *Wistar* rats (*Rattus norvegicus*), 21 days old, were distributed in individual stainless-steel cages, with controlled temperature (22 ± 2 °C) and a 12 h photoperiod. The hemoglobin depletion/repletion method was used to evaluate iron bioavailability. In the depletion phase, the animals received a modified AIN-93G standard diet (SD) (REEVES; NIELSEN; FAHEY, 1993), using an iron-free mineral mixture and deionized water, *ad libitum*, for 28 days to induce iron deficiency. The weight gain and food consumption of the animals were evaluated weekly. At the end of this period, a drop of blood was collected by caudal puncture, and hemoglobin was measured in all animals using commercial colorimetric kits (Labtest®, MG, Brazil). A group of eight animals was euthanized to determine the effects of iron deficiency in these animals (SD iron-free, n =

8). The remaining animals (32 rats) were redistributed into four groups so that the mean hemoglobin levels and weight were homogeneous among the groups.

In the repletion phase, ferrous sulfate and millet flour were used as iron sources. The four experimental groups used were: standard diet with ferrous sulfate (SD+FS, n = 8), standard diet with non-germinated open-pan cooked millet flour (SD+NGOPCMF, n = 8), standard diet with germinated open-pan cooked millet flour (SD+GOPCMF, n = 8) and standard diet with extruded cooked millet flour (SD+ECMF, n = 8). Millet flours were supplied in different amounts because the objective of our study was to control the iron concentration in the diet (average of 13.29 ppm in each treatment) (DA SILVA et al., 2016a) to demonstrate the bioavailability of iron in each food matrix. The chemical composition of the flours was used to calculate the ingredients added to the experimental diet, balancing the iron concentration, macronutrients (carbohydrates, lipids, proteins) and dietary fiber (**Table 2**).

Tabela 2. Composition of experimental diets for biological trial 2.

	Depletion Phase	**Repletion phase**			
Ingredients (g/kg-1)	**SD iron-free**	**SD + FS**	**SD + NGOPCMF**	**SD + GOPCMF**	**SD + ECMF**
Ferrous sulfate	-	0.04	-	-	-
NGOPCMF	-	-	267.8	-	-
GOPCMF	-	-	-	287.80	-
ECMF	-	-	-	-	212.80
Albumin*	254.36	254.36	216.34	212.41	224.67
Corn starch	346.14	346.11	163.11	149.02	185.97
Dextrinized starch	132.00	132.00	132.00	132.00	132.00
Sucrose	100.00	100.00	100.00	100.00	100.00
Soybean oil	70.00	70.00	55.06	55.55	64.00
Cellulose	50.00	50.00	18.19	15.72	33.06
Iron-free mineral mix	35.00	35.00	35.00	35.00	35.00
Vitamin mix	10.00	10.00	10.00	10.00	10.00
Choline bitartrate	2.50	2.50	2.50	2.50	2.50
Fit. Ac./iron molar ration			14.51	12.76	11.54
Diet composition					
Carbohydrate (%)	61.79	61.79	61.78	61.70	61.76
Protein (%)	21.38	21.38	21.38	21.43	21.40
Lipid (%)	7.00	7.00	7.00	7.00	7.00
Caloric density (kcal/g)	3.74	3.74	3.74	3.74	3.74

Composition of experimental diets based on the standard diet for rodents (AIN-93G) (REEVES; NIELSEN; FAHEY, 1993). SD iron-free: standard diet (AIN-93G) without iron; SD + FS: standard diet + ferrous sulfate; SDP + NGOPCMF: standard diet + non-germinated open-pan cooked millet flour; SD + GOPCMF: standard diet + germinated open-pan cooked millet flour; SD + ECMF: standard diet + extruded cooked millet flour. In diets using millet as an iron source, substitutions were made according to the centesimal composition of millet flours. *Albumin based on 78.63% protein content.

The animals received deionized water ad libitum and a controlled diet weighed daily for a period of 21 days. During this phase, weight gain and feed intake were also monitored to calculate the feed efficiency ratio (FER [weight gain (g)/feed intake (g)] x 100) and the weekly hemoglobin level. On the 50th day, after a 12-h fast, the animals were anesthetized with isoflurane (Isoforine, Crist´alia®) and euthanized by cardiac puncture. Blood was collected for hematological analyses (hemoglobin, serum iron, ferritin, and transferrin). In addition, the liver and intestine were also collected to assess iron bioavailability (DMT1, DcytB, ferroportin, hephaestin, ferritin, transferrin), intestinal health (fecal pH, SCFA, and histomorphometry of the duodenum and colon and microbiota sequencing), inflammation (TNF gene expression) and oxidative stress (total antioxidant capacity (TAC), superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), nitric oxide (NO), and gene expression of nuclear factor erythroid 2 (Nrf2) and SOD).

5.6. Ethical aspects

The studies with animals were approved by the Animal Research Ethics Committee of the Federal University of Viçosa (CEUA/UFV protocol no. 39/2019 and 05/2022) (Appendix A and B). All experimental procedures with animals were carried out in accordance with the ethical principles of animal experimentation.

5.7. Statistical analyses

For the analysis of the compounds present in the millet flours and biological assays, the results were subjected to the Shapiro-Wilk and Kolmogorov-Smirnov normality tests. One-way ANOVA analysis of variance was applied, followed by the Newman-Keuls post-hoc test for the analyses of biological assays I and II and Tukey for the analyses of chemical composition in the flours. In addition, we also used the Student's t-test to compare the chemical composition of the NGOPCMF flour (control) with the GOPCMF or ECMF flour.

Regarding the microbiota, the Chao1, Shannon and Simpson indices were used to estimate alpha diversity and the differences between beta diversity were analyzed by the Pairwise PERMANOVA test. Statistically significant p-values associated with microbial clades and functions were corrected for multiple comparisons using the Benjamini Hochberg false discovery rate (FDR) correction.

A Pearson correlation analysis was used to assess the relationship between changes in intestinal microbiota abundance and modulations in markers of metabolic alterations, inflammation, and oxidative stress.

Analyses were performed using SPSS version 20.0 (for microbiota in study 1), MicrobiomeAnalyst (for microbiota in study 2) and GraphPad Prism (GraphPad Software, San Diego, CA), version 9.0 (for the other analyses). The significance level set for all tests was 5%.

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7. THESYS PUBLICATION

Table 3. Description of papers published in the thesis.

Paper	Title	Journal (Impactor factor)	Status / Year of Publication
Original paper 1	Germinated Millet (Pennisetum glaucum (L.) R. Br.) Flour Improved the Gut Function and its Microbiota Composition in Rats Fed with High-fat High-fructose Diet	International Journal of Environmental Research and Public Health (IF: 4.614)	Published in 2022
Original paper 2	Chemical composition and in vitro iron bioavailability of extruded and open-pan cooked germinated and ungerminated pearl whole millet "Pennisetum glaucum (L.) R. Br."	Food Chemistry (IF: 8.5)	Published in 2024
Original paper 3	Conventional and germinated pearl millet flour (Pennisetum glaucum (L.) R. Br.) improves iron metabolism and antioxidant capacity in Wistar rats	Journal of Cereal Science (FI: 4.075)	Published in 2024
Original paper 4	Processed pearl Millet improves the morphology and gut microbiota in Wistar rats	Foods (FI: 5.1)	Published in 2025

8. RESULTS

8.1. Paper 1: Germinated Millet (*Pennisetum glaucum* (L.) R. Br.) Flour Improved the Gut Function and Its Microbiota Composition in Rats Fed with High-Fat High-Fructose Diet

International Journal of *Environmental Research and Public Health*

MDPI

Article

Germinated Millet (*Pennisetum glaucum* (L.) R. Br.) Flour Improved the Gut Function and Its Microbiota Composition in Rats Fed with High-Fat High-Fructose Diet

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Abstract: Germinated millet (*Pennisetum glaucum* (L.) R. Br.) is a source of phenolic compounds that has potential prebiotic action. This study aims at evaluating the action of germinated pearl millet on gut function and its microbiota composition in *Wistar* rats fed with a high-fat high-fructose (HFHF) diet. In the first stage, lasting eight weeks, the experiment consisted of two groups: AIN-93M (n = 10) and HFHF group (n = 20). In the second stage, which lasted ten weeks, the animals of the AIN-93M group (n = 10) were kept, while the HFHF group was dismembered into HFHF (HFHF diet, n = 10) and HFHF + millet (HFHF added 28.6% of germinated millet flour, n = 10) groups. After the 18th week, the urine of the animals was collected for the analysis of lactulose and mannitol intestinal permeability by urinary excretion. The histomorphometry was analyzed on the proximal colon and the fecal pH, concentration of short-chain fatty acids (SCFA), and sequencing of microbiota were performed in cecum content. The Mothur v.1.44.3 software was used for data analysis of sequencing. Alpha diversity was estimated by Chao1, Shannon, and Simpson indexes. Beta diversity was assessed by PCoA (Principal Coordinate Analysis). The functional predictive analysis was performed with PICRUSt2 software (version 2.1.2−b). Functional traits attributed to normalized OTU abundance were determined by the Kyoto Encyclopedia of Genes and Genomes (KEGG). In the results, germinated millet flour reduced *Oscillibacter genus* and Desulfobacterota phylum, while increasing the Eggerthellaceae family. Furthermore, germinated millet flour: increased beta diversity, cecum weight, and cecum/body weight ratio; improved gut histological parameters by increasing the depth and thickness of the crypt and the goblet cell count ($p < 0.05$); reduced ($p < 0.05$) the fecal pH and mannitol urinary excretion; increased ($p < 0.05$) the propionate short-chain fatty acid concentration. Thus, germinated millet has the potential to improve the composition of gut microbiota and the intestinal function of rats fed with an HFHF diet.

Keywords: beta diversity; goblet cells; intestinal health; prebiotic; intestinal permeability; whole grain

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1.Introduction

Diets rich in sugar and fat are characteristics of modern society, which favors the progression of metabolic changes, such as insulin resistance, dyslipidemia, adiposity (BALAKUMAR et al., 2016; LOZANO et al., 2016), and intestinal dysbiosis (YANG; HUR; LEE, 2017). Furthermore, changes in the composition of the intestinal microbiota can affect the body's homeostasis and enable barrier rupture, thus increasing permeability, and allowing lipopolysaccharide (LPS) translocation (AMAR et al., 2011). This fact

leads to metabolic disorders that culminate in the promotion of non-communicable chronic diseases (SCHEITHAUER et al., 2016).

The gut microbiota is mainly modulated by eating habits and nutritional interventions (KONIKOFF; GOPHNA, 2016). The beneficial modulation of microbiota has been considered to interfere with the regression of metabolic changes in the body. Short-chain fatty acids, mainly butyrate, acetate, and propionate, which are metabolites of the intestinal microbiota, can change the diversity of microorganisms and improve the microbiota (LI et al., 2020a). In addition, dietary fiber and protein are associated with increased intestinal cell proliferation, which benefits intestinal health (ADAM et al., 2015; CHEN et al., 2018). Therefore, some foods that can improve the action of microorganisms that colonize the gastrointestinal tract can help to reduce metabolic changes in the body and prevent associated diseases (ADAM et al., 2015; LI et al., 2020a).

Pearl millet (*Pennisetum glaucum*) is a whole grain abundant in proteins, dietary fiber, minerals, and bioactive compounds (DIAS-MARTINS et al., 2018; SHARMA; GUJRAL, 2019; TAYLOR, 2015). It is known that the germination process of the grain can increase the content of these compounds (OWHERUO; IFESAN; KOLAWOLE, 2019; SARITA; EKTA SINGH, 2016) and reduce the phytochemicals, such as phytic acid (SARITA; EKTA SINGH, 2016). In our previous studies, we demonstrated that germinated millet flour had functional activities, acting as a hypoglycemic, antioxidant, and anti-inflammatory food (THEODORO et al., 2021a, 2021b). In addition, germinated millet can reduce hyperlipidemia and hepatic steatosis in animals fed a high-fat high-fructose diet (THEODORO et al., 2021a). Therefore, the present study aimed to evaluate the effect of the consumption of germinated millet flour on intestinal function and intestinal microbiota composition in rats fed with a high-fat high-fructose diet.

2. Materials and Methods

2.1. Grain Germination

For germination, pearl millet (*Pennisetum glaucum* (L.) R. Br.), cultivar BRS1502, was kept at 30 ± 2ºC and 90% of relative humidity for 24 h, then dried at 50 °C for 4 h, and ground to obtain flour, as described by (THEODORO et al., 2021a). The resulting flour is composed of carbohydrate (64.5%), protein (15.1%), dietary fiber (7.6%), lipids (5.4%), and resistant starch (3.5%) (THEODORO et al., 2021b), which were analyzed according to AOAC (AOAC, 2016).

2.2. Animals and Diets

The experiment consisted of two stages. In the first stage (induction of metabolic changes), thirty male, 45-50-day old *Wistar* rats (*Rattus norvegicus*) were randomized by body weight, into: AIN-93M (normal control group; wt 155.98 ± 17.05g, n = 10), which received AIN-93M diet (REEVES; NIELSEN; FAHEY, 1993) and high-fat high-fructose (HFHF) (wt 158.03 ± 17.81g, n = 20), which received a diet rich in saturated fat (31%) and fructose (20%) groups, during 8 weeks (adapted from (MARINELI et al., 2015)). In the second stage (intervention), the AIN-93M group (wt 349.94 ± 30.71g, n = 10) was maintained, while the HFHF group was dismembered into: HFHF (wt 366.89 ± 36.90g, n = 10), which maintained the HFHF diet, and HFHF + millet group (wt 370.12 ± 36.49g, n = 10), which received the HFHF diet added with 286.3g germinated millet flour, that replacing dietary fiber (43.6%), starch (100%), protein (36%), and oil (39%) from HFHF diet, for another 10 weeks. Throughout the experiment, the animals received water and diet *ad libitum* (THEODORO et al., 2021a).

Body weight and food intake were monitored weekly. One week before euthanasia, the urine of the animals was collected for the analysis of intestinal permeability. After the 18th week, the animals were anesthetized with isoflurane (Isoforine®, Cristália, Brazil) and euthanized by cardiac puncture. The proximal colon was collected, washed in saline solution (PBS), fixed in 10% formaldehyde, and kept at room temperature for later histological analysis. The cecum was weighed and cecum content was collected in a sterile microtube and immediately stored at -80°C until the time of analysis. The chemical analyses, the markers of inflammation and oxidative stress, and other markers of metabolic alterations were previously performed and published (THEODORO et al., 2021a, 2021b).

The Ethics Committee on the Use of Animals of the Federal University of Viçosa approved the study (CEUA/UFV; process n◦ 39/2019).

2.3. Intestinal Permeability

The animals were fasted for 12 h (in the last week of the experiment) and, subsequently, received by gavage 0.5 mL of a solution containing 19 mg of lactulose and 9 mg of mannitol. After administration, the animals were placed in individual metabolic cages where they fasted for 5 h. Then, the 24-hour urine was collected, measured, recorded, and stored at -80ºC (SONG et al., 2011). Urinary lactulose and mannitol were performed by high-performance liquid chromatography (HPLC) analysis, according to

De Sá et al., (2011). The concentration of lactulose and mannitol in urine was determined by a standard curve using the standard lactulose (24-0.14 g/L) and mannitol (9-0.14g/L) (GARCIA VILELA et al., 2008; SONG et al., 2011).

2.4. Fecal pH

To quantify fecal pH, about 0.4 g of cecum content was homogenized, with vortex, in 4 mL of distilled water. Subsequently, the glass electrode of the pHmeter (Bel Engineering®) was inserted to measure the pH (GRANCIERI et al., 2017).

2.5. Short Chain Fatty Acids (SCFA) content

The concentration of SCFA was analyzed in cecum as the methodology proposed by (SMIRICKY-TJARDES et al., 2003), and the quantification of SCFA was determined by HPLC, as described by Gomes et al. (GOMES et al., 2022). Acetic, propionic, and butyric acids were used as standards in the calibration curve.

2.6. Colon Histomorphometry Analysis

Semi-serialized 3 μm-thick histological colon fragments were obtained on an automated rotating microtome (Reichert-Jung®, Genossen, Germany) and stained by hematoxylin and eosin technique. The slides were examined under an Olympus BX43 light microscope, using a 20x objective. Twenty random fields per animal were selected to measure crypt depth, crypt thickness, goblet cell number, and thickness of the circular and longitudinal muscle layers. The images were processed using the ImagePro-Plus® software version 4.5 (Media Cybernetics, Rockville USA).

2.7. DNA Extraction and Sequencing

The extraction of genomic DNA from the cecal contents was performed in accordance with phenol/chloroform extraction protocol, after a mechanical rupture (STEVENSON; WEIMER, 2007). PCR amplicons from the V4 region of the 16S rRNA gene were generated using primers 515F (5'GTGYCAGCMGCCGCGGTAA3') and 806R (GGACTACNVGGGTWTCTAAT3') in addition to primers adapted for the Illumina MiSeq platform (Illumina, San Diego, California, USA (CAPORASO et al., 2011, 2012). Paired-end sequencing reactions were loaded into an Illumina flow cell, using the Illumina MiSeq platform, at the Environmental Sample Preparation and Sequencing Facility (ESPSF), Argonne National Laboratory (Lemont, Illinois, USA).

The sequences obtained in this study were deposited to Sequence Read Archive (SRA) on the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sra) under the accession number PRJNA808059.

The Mothur v.1.44.3 software was used for data analysis (SCHLOSS et al., 2009), according to Gomes et al. (GOMES et al., 2022). The Operational Taxonomic Units (OTUs) were grouped with a 97% sequence similarity cutoff. The normalized data table was used to calculate alpha- and beta-diversity and the relative abundance of OTUs. Alpha-diversity was estimated by using Chao1, Shannon, and Simpson indexes. Beta-diversity among groups was assessed by Principal Coordinate Analysis (PCoA) based on the Bray-Curtis dissimilarity index (LOZUPONE; KNIGHT, 2005). The PICRUSt2 software was used for the functional predictive analysis of the metagenome (DOUGLAS et al., 2020). Functional traits attributed to normalized OTU abundance were predicted based on reference genomes, using the Kyoto Encyclopedia of Genes and Genomes (KEGG).

2.8. Statistical Analysis

The data of the characterization analysis of animals were submitted to a Kolmogorov-Smirnov normality test, followed by a *one-way* analysis of variance (ANOVA) and the *post-hoc* of Newman-Keuls. Differences between beta-diversity were analyzed by the Pairwise PERMANOVA test. Statistically significant *p*-values associated with microbial clades and functions were corrected for multiple comparisons, using the Benjamini Hochberg false discovery rate (FDR) correction. Pearson's correlation analysis was used to assess the relationship between changes in gut microbiota abundance and modulations in markers of metabolic change.

The data were evaluated for homogeneity of variance by the Kolmogorov-Smirnov test, and nonparametric and independent samples were submitted to Kruskal-Wallis with Dunn's multiple comparison test. Statistical analysis was performed using SPSS software version 20.0 and GraphPad Prism version 8.0. The level of significance was established at $p < 0.05$.

3. Results

3.1. Effect of germinated millet flour on food consumption, body weight, and cecum weight

After 18 weeks of the experiment (end of phase 2), the AIN-93M group had higher food consumption, compared to the other groups **(Figure 1A)**. There was no difference ($p>0.05$) in body weight **(Figure 1B)**, but the consumption of germinated millet flour increased ($p <0.05$) the cecal weight and the cecum/body weight ratio of the animals in the HFHF + millet group, compared to the HFHF group **(Figure 1 C and D)**.

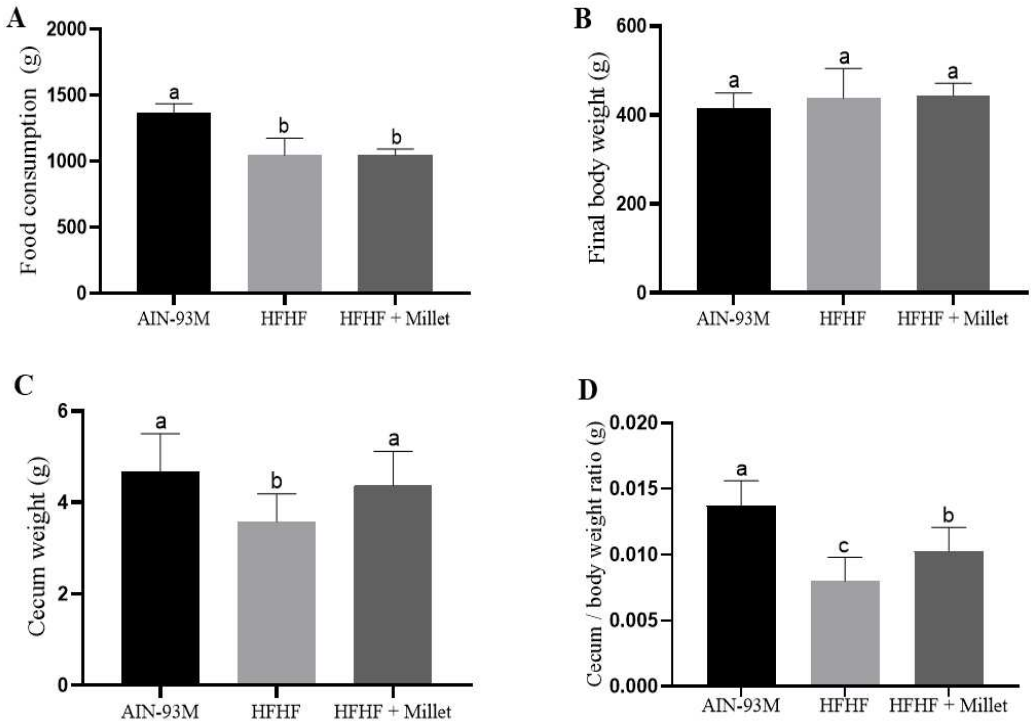

Figure 1. Effect of germinated millet flour on food consumption (A), body weight (B), cecum weight (C), and cecum/ body weight ratio (D), after 10 weeks of intervention. Data were analyzed by one-way ANOVA and *post-hoc* of Newman-Keuls at 5% probability. Values are expressed as means and standard deviation (n = 10/group). Different letters indicate significant differences ($p<0.05$). AIN-93M: animals fed with a standard diet; HFHF: high-fat high-fructose diet; HFHF + Millet: high-fat high-fructose diet with added germinated millet flour.

3.2. Germinated millet flour improves intestinal health

As for permeability, the consumption of germinated millet flour reduced ($p<0.05$) the urinary excretion of mannitol, compared to the HFHF group. Urinary lactulose excretion did not differ among the groups **(Table 1)**.

Regarding other variables related to intestinal health, the group that consumed germinated millet flour (HFHF + millet flour) presented reduced fecal pH ($p<0.05$), compared to the groups that did not consume it (AIN-93M and HFHF groups) **(Table 1)**. As for short-chain fatty acids (SCFA), the consumption of germinated millet flour increased ($p<0.05$) propionate production, while acetate concentration was higher ($p<0.05$) in the AIN-93M group, and butyrate concentration did not differ among the

groups **(Table 1)**. The number of goblet cells and depth of the crypts were higher ($p<0.05$) in the HFHF + millet group, compared to the others groups. The HFHF + millet and AIN-93M groups presented crypts thickness similar; however, this parameter was higher ($p<0.05$), comparing HFHF + millet with the HFHF group **(Table 1 and Figure 2)**. The longitudinal and circular mucus layer widths did not differ ($p>0.05$) among the experimental groups.

Table 1. Effect of germinated millet flour intake on intestinal health in *Wistar* rats (n = 8), for 10 weeks.

Variables	AIN-93M	HFHF	HFHF + Millet
Fecal pH	9.18 ± 0.26^{a}	9.28 ± 0.16^{a}	8.63 ± 0.38^{b}
Short Chain Fatty Acids (mM)			
Propionic acid	4.58 ± 1.13^{b}	4.36 ± 1.55^{b}	9.96 ± 3.64^{a}
Acetic acid	21.36 ± 5.11^{a}	13.71 ± 2.90^{b}	13.81 ± 3.05^{b}
Butyric acid	2.51 ± 0.77^{a}	3.60 ± 1.01^{a}	3.19 ± 0.82^{a}
Number of goblet cells	24.64 ± 3.93^{b}	23.88 ± 3.91^{b}	30.57 ± 2.10^{a}
Crypts depth (µM)	127.2 ± 12.06^{b}	134.3 ± 12.21^{b}	168.7 ± 22.34^{a}
Crypts thickness (µM)	18.27 ± 1.54^{ab}	17.16 ± 1.79^{b}	21.12 ± 2.22^{a}
Longitudinal Mucus Layer Width (µM)	86.51 ± 22.88^{a}	74.05 ± 27.48^{a}	81.93 ± 5.81^{a}
Circular Mucus Layer Width (µM)	28.50 ± 7.72^{a}	25.99 ± 8.36^{a}	32.41 ± 8.69^{a}
Mannitol urinary excretion (%)	2.82 ± 0.85^{b}	9.37 ± 6.70^{a}	4.67 ± 2.09^{b}
Lactulose urinary excretion (%)	5.44 ± 1.37^{a}	9.07 ± 2.99^{a}	10.03 ± 4.91^{a}

Means followed by the same letter in the lines do not differ by the Newman-Keuls test, at the 5% level of significance. Short-chain fatty acids, mannitol, and lactulose were analyzed by HPLC. Histology was performed using a 20x objective and staining with hematoxylin and eosin. AIN-93M: animals fed with a standard diet; HFHF: high-fat high-fructose diet; HFHF + Millet: high-fat high-fructose diet with added germinated millet flour.

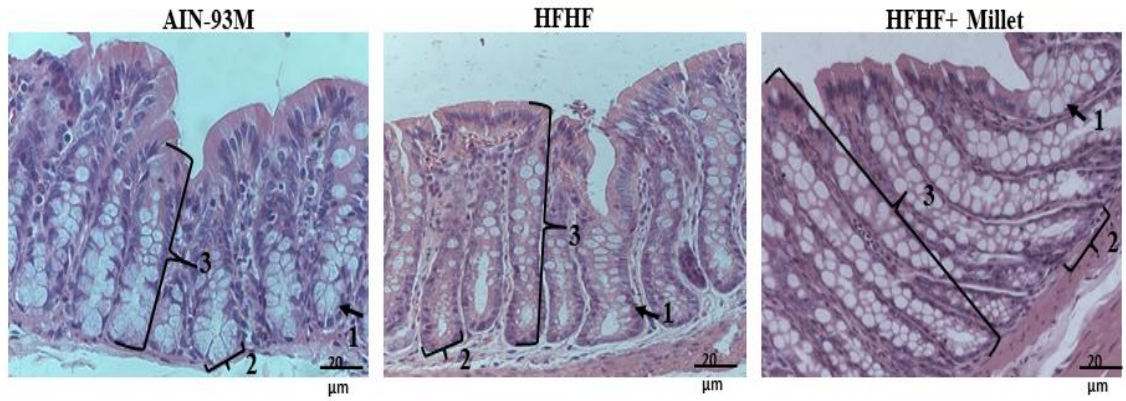

Figure 2. Effect of germinated millet flour on the histomorphometry of the colon. Number of goblet cells (1) and crypt thickness (2) and depth (3), after 10 weeks of intervention. AIN-93M: animals fed with a standard diet; HFHF: high-fat high-fructose diet; HFHF + Millet: high-fat high-fructose diet with added germinated millet flour. Staining was carried out with hematoxylin and eosin. Bar: 20 μm. Objective: 20×.

3.3. Effects of germinated millet flour consumption on the diversity of the microbial community

To evaluate the effect of germinated millet flour consumption on intestinal microbiota, 16S rRNA gene sequencing was performed on the cecal content. This sequencing generated 765.057 raw sequences. After filtering and cleaning the sequences, 583.130 sequences of good quality were obtained. The Good's coverage obtained in the samples was > 99%, which indicates good sequencing coverage.

The alpha diversity analysis presented by Chao, Shannon, and Simpson indexes revealed no difference among the groups ($p>0.05$) **(Figure 3A-C)**. According to PERMANOVA, the clustering differed among all experimental groups ($p<0.001$) **(Figure 3D)**.

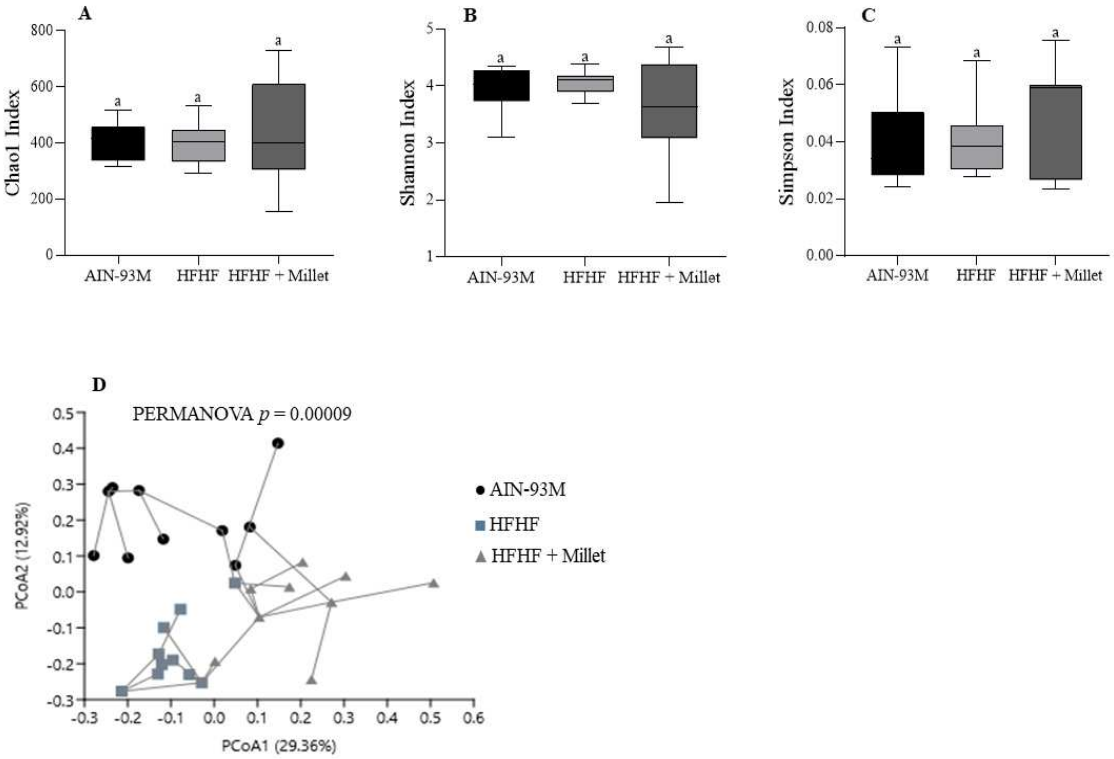

Figure 3. Microbial diversity of the cecal microbiome of different experimental groups, after 10 weeks of the experiment. (A) Measure of α-diversity using the Chao Index; (B) Measure of α-diversity using the Shannon Index; (C) Measure of α-diversity using the Simpson

Index; (D) Measure of β-diversity using Bray-Curtis dissimilarity distances separated by two principal components (PCoA). Columns with the same letter do not differ by the Newman-Keuls test, at the 5% level of significance. PCoA was analyzed by the PERMANOVA test. Each dot refers to one animal. n = 10/group (AIN-93M, HFHF groups) and n = 9/group (HFHF + Millet group). AIN-93M: animals fed with a standard diet; HFHF: high-fat high-fructose diet; HFHF + Millet: high-fat high-fructose diet with added germinated millet flour.

The taxonomic classification of the samples presented 22 phyla, 33 classes, 88 orders, 137 families, and 268 genera. As for the phyla, Firmicutes and Bacteroidetes were the most predominant in the three groups, with no difference among them ($p>0.05$). Desulfobacterota phylum significantly decreased ($p<0.05$) in the HFHF + millet group, compared to the AIN-93M and HFHF control groups, while Actinobacteria phylum was higher ($p<0.05$) in the HFHF + millet group, compared to HFHF, but similar ($p>0.05$) to the AIN-93M control group. Further, the relative abundance of Spirochaetota and Patescibacteria phyla decreased ($p<0.05$) in the HFHF + millet treatment group, compared to the AIN-93M control, but did not differ ($p>0.05$) concerning the HFHF group **(Figure 4A)**. Although no difference ($p>0.05$) was observed in the relative abundance of Firmicutes and Bacteroidetes phyla between the groups, the Firmicutes to Bacteroidetes ratio was higher ($p<0.05$) in the animals that received HFHF + millet diet, compared to the control groups **(Figure 4B)**.

The most abundant or key microorganisms related to SCFA production and gut health are presented in **Figure 4C**. The Oscillospirales order was predominant in the AIN-93M group, and its relative abundance was lower ($p<0.05$) in the HFHF + millet group. At the family level, Eggerthellaceae relative abundance increased ($p<0.05$) in the HFHF + millet group, compared to the AIN-93M control, while Desulfovibrionaceae relative abundance decreased ($p<0.05$), compared to the AIN-93M and HFHF controls. The relative abundance of the Lachnospiraceae family did not differ between treatments ($p>0.05$). According to our data, the HFHF + millet group decreased ($p<0.05$) levels of the genera *Muribaculaceae_ge*, compared to the HFHF and AIN-93M controls, while displayed increased ($p<0.05$) levels of *Faecalibaculum* in their gut microbiota, compared to the HFHF and AIN-93M controls. In addition, the treatment group with HFHF + millet reduced ($p<0.05$) levels of *Oscillibacter*, compared to the AIN-93M control. However, no difference ($p>0.05$) was found in the *Lactobacillus* genus among the treatment groups **(Figure 4C)**.

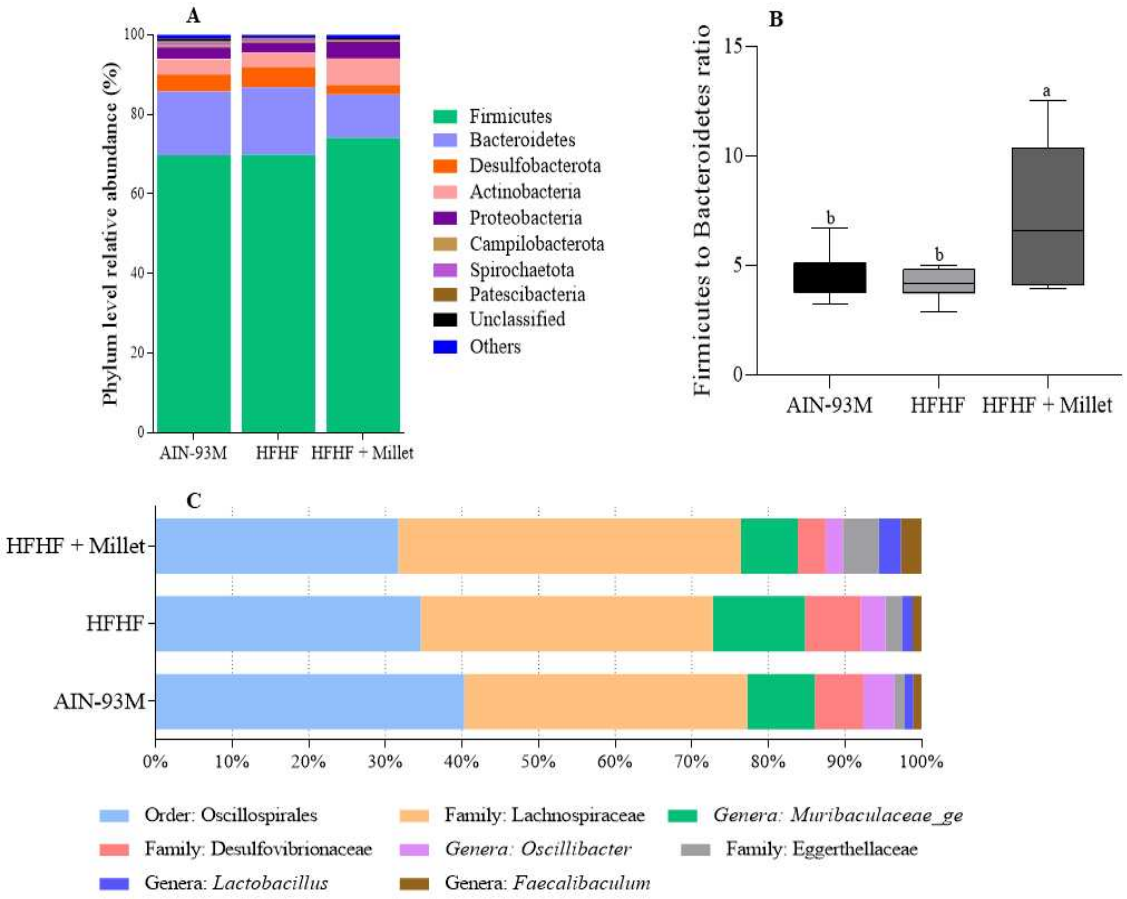

Figure 4. Compositional changes of gut microbiota in response to a germinated millet flour diet, after 10 weeks of treatment. A: Relative abundance of each identified phylum; B: Firmicutes/Bacteroidetes ratio; C: Taxonomy level changes in each experimental group. Columns with different letters indicate differences by the Newman-Keuls test, at the 5% level of significance. n = 10/group (AIN-93M, HFHF groups) and n = 9/group (HFHF + Millet group). AIN-93M: animals fed with a standard diet; HFHF: high-fat high-fructose diet; HFHF + Millet: high-fat high-fructose diet with added germinated millet flour. Only phyla with abundance > 0.3% in at least one group were displayed.

The HFHF + millet group presented increased ($p<0.05$) KEGG metabolic pathways related to the urea cycle, L-lysine, L-threonine, and L-methionine biosynthesis and sucrose degradation, compared with the controls. Furthermore, it was observed increased relative abundance of Glycerol degradation, L-phenylalanine biosynthesis, and L-tyrosine biosynthesis pathways in HFHF + millet group compared to the HFHF group ($p<0.05$) **(Figure 5)**.

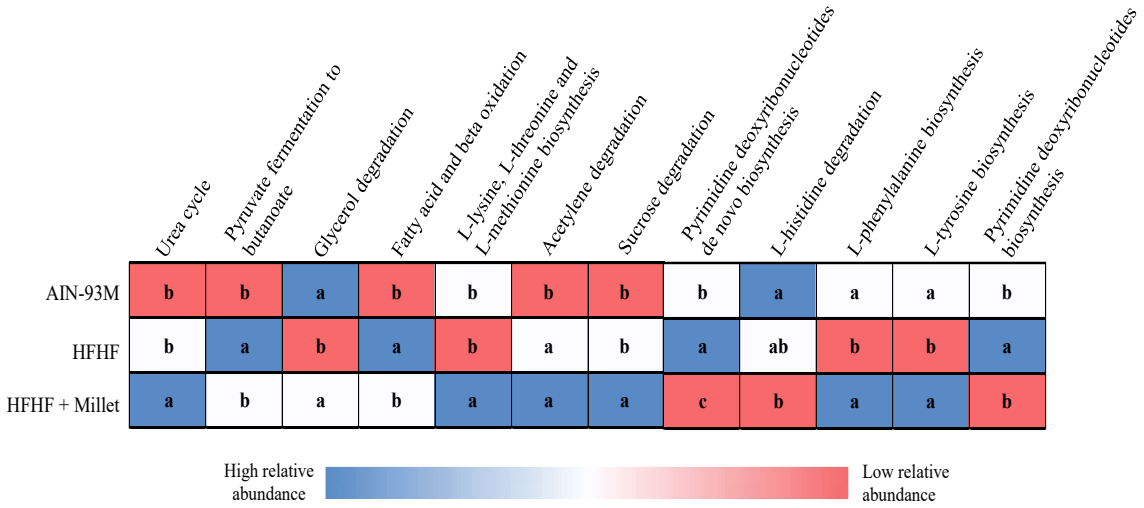

Figure 5. Relative significance of differentially enriched microbial metabolic pathways in cecal microbiota, after the consumption of high-fat high-fructose diet associated with millet. Different letters among the columns indicate a difference by the Duncan test, at a 5% significance level. n = 10/group (AIN-93M, HFHF groups) and n = 9/group (HFHF + Millet group). AIN-93M: animals fed with a standard diet; HFHF: high-fat high-fructose diet; HFHF + Millet: high-fat high-fructose diet with added germinated millet flour.

3.4. Microbial correlation with markers of metabolic alterations

When evaluating correlations with inflammatory markers, the anti-inflammatory interleukin-10 (IL-10) was positively correlated with the Egggerthellaceae family (rs= 0.520; p= <0.05) and negatively correlated with the Desulfobacterota phylum (rs= -0.544; p= <0.05) and the *Oscillibacter* genus (rs= -0.490; p= <0.05). The nuclear factor Kappa B (p65- NF-κB) was positively correlated with the Desulfobacterota (rs= 0.797; p= <0.05) and negatively correlated with the Eggertellaceae (rs= -0.711; p= <0.05).

In addition, the glucose homeostasis marker phospho-AKT1 [pS473] (pAKT) was negatively correlated with the Desulfobacterota (rs= -0.607; p= <0.05) and positively correlated with the Egggerthellaceae (rs= 0.836; p= <0.05). When evaluating correlations with short chain fatty acids, the acetic acid was positively correlated with the *Oscillibacter* (rs= 0.578; p= <0.05), the propionic acid was positively correlated with the Eggertellaceae (rs= 0.695; p= <0.05), the IL-10 (rs= 0.781; p= <0.05), and the pAKT (rs= 0.743; p= <0.05). On other hand, acetic acid was negatively correlated with the Desulfobacterota (rs= -0.480; p= <0.05), the glucose (rs= -0.650; p= <0.05), and the triglycerides (rs= -0.611; p= <0.05). The butyric acid was positively correlated with the insulin (rs= 0.706; p= <0.05) and the acetic acid (rs= 0.466; p= <0.05) **(Figure 6)**.

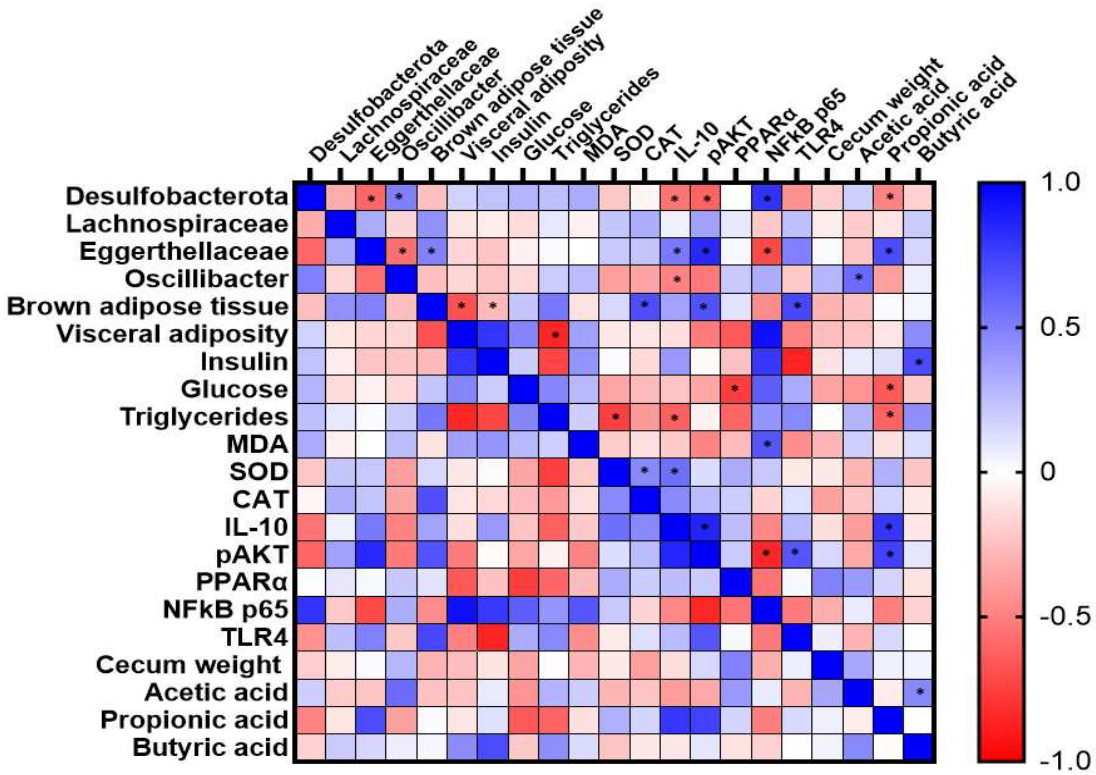

Figure 6. Heat map of Pearson correlations between changes in gut microbiota and markers of metabolic change, after 10 weeks of treatment with germinated millet flour. Malondialdehyde (MDA); Superoxide dismutase (SOD); Catalase (CAT); Interleukin 10 (IL-10); phospho-protein kinase B (pAKT); Peroxisome proliferator-activated receptor alpha (PPARα); Nuclear factor-kappa B (NFκB p65); Toll-like receptor 4 (TLR4). n = 10/group. *p < 0.05. Data from chemical analyses, markers of inflammation and oxidative stress, and other markers of metabolic changes were previously published (THEODORO et al., 2021a, 2021b).

4. Discussion

In the present study, we evaluated the capacity of germinated millet flour to improve the intestinal dysbiosis caused by HFHF diet consumption, since the germinated *Pennisetum glaucum* is rich in dietary fiber, resistant starch, and proteins. The consumption of germinated millet flour improved the characteristics of the gastrointestinal tract since it was observed, for example, greater beta diversity, cecum weight, and cecum/ body weight ratio, increased depth, and thickness of the crypts, and the number of goblet cells. Furthermore, the fecal pH and mannitol urinary excretion were reduced, while propionate SCFA production increased **(Figure 7)**.

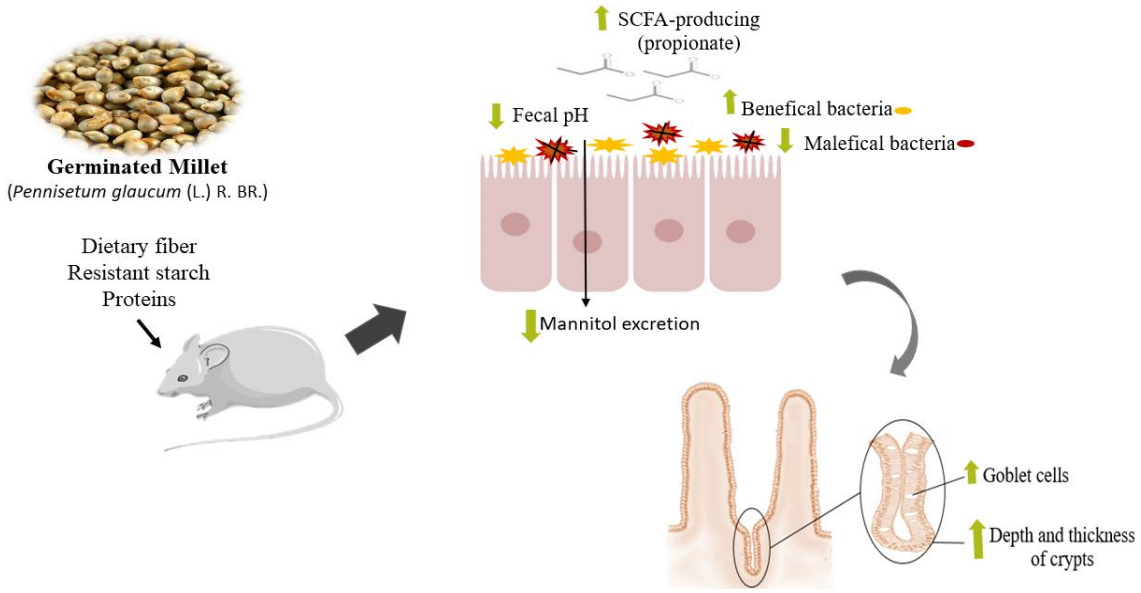

Figure 7. Schematic figure of the effects of germinated millet flour on intestinal health in rats fed with high-fat high-fructose diet. The presence of dietary fiber, proteins, and resistant starch in the germinated millet reduced the fecal pH which improved the beta diversity providing substrate for the growth of SCFA-producing bacteria (propionate). In addition, the germinated millet flour reduced intestinal permeability and increased the depth and thickness of the crypts and the number of goblet cells, improving the intestinal dysbiosis caused by HFHF diet consumption. SCFA: Short Chain Fatty Acids.

The pH was reduced by the germinated millet flour, thus improving microbial diversity and providing substrate for the growth of SCFA-producing bacteria, such as Firmicutes, Bacteroidetes, and Actinobacteria. The dietary fiber and undigested proteins present in germinated millet flour were probably fermented in the intestine and produced SCFAs, such as propionate, as observed in this research. The largest production of propionate may have stimulated the growth of beneficial bacteria, such as Actinobacteria, which increases mucus secretion and the ability to promote hypertrophy of intestinal mucosa cells, consequently increasing the area of the surface of the intestine.

These results can be related to the reduction of intestinal permeability by less urinary mannitol excretion in the group treated with millet, which improved the dysfunction caused by the HFHF diet. It is demonstrated that a prolonged intake of a diet rich in saturated fat induces inflammation and impairs the intestinal barrier function, thus leading to intestinal dysfunction, which is a feature of diet-induced metabolic dysregulation (LAM et al., 2012).

Furthermore, the improvement in the intestinal permeability caused by germinated millet consumption can be related to the reduction of the *Oscillibacter* genus, which are microorganisms that mediate intestinal dysfunction induced by a high-fat diet (LAM et

al., 2012). *Oscillibacter* may directly regulate the components involved in the maintenance of the integrity of the intestinal barrier, or its relationship to intestinal permeability may be a secondary effect of the changes in the general composition of the microbial community (LAM et al., 2012). Besides, Lachnospiraceae increased in the millet group, and this family has been associated with improved intestinal barrier function (LI et al., 2019a).

The Desulfobacterota phylum decreased and the Eggerthellaceae family increased with the consumption of germinated millet flour. These results may be related to the high production of propionic acid, which was correlated with higher pAKT and IL-10, and reduction of NFκB p65. This result is in agreement with our previous study, using the same experimental design, which demonstrated the ability of germinated millet flour to improve glucose homeostasis and reduce inflammation (THEODORO et al., 2021a, 2021b). The *Lactobacillus* genus increased in the animals fed with millet diet (HFHF + millet), but the increase was not significant. Members of this genus have been investigated for probiotic properties and demonstrated their ability to improve inflammation and intestinal barrier function in experimental models of bowel dysfunction (LAM et al., 2012).

Besides this, the millet consumption increased the cecum weight and cecum/ body weight ratio and increased the depth and thickness of the crypts and the number of goblet cells, due to the higher content of dietary fiber and resistant starch present in the germinated millet. Dietary fiber acts as prebiotic and affects the solubility and viscosity of the fecal content, besides stimulating or inhibiting its distension and improving the weight and motility in the intestine (GOMES et al., 2021).

In general, large amounts of soluble and insoluble dietary fiber improve intestinal motility and increase fecal volume, which can increase the thickness of muscle layers. However, we did not observe significant changes in the thickness of the circular and longitudinal muscle layers of the colon, which agrees with Gomes et al. (GOMES et al., 2021) and Moraes et al. (MORAES et al., 2012). This is possibly due to the predominance of insoluble fiber in our germinated millet flour sample (THEODORO et al., 2021b) since the soluble fiber is mainly responsible for muscular layer increase (HWANG et al., 2014). The largest number of goblet cells observed in the millet group suggests an increased production of the mucus that lines the intestinal lumen. This may increase the digestive and absorptive capacity and, indirectly, improve the bioavailability of dietary components (DA SILVA et al., 2019a), possibly improving the intestinal health and systemic nutrition.

The lower food consumption observed in the groups that received a high-fat diet (HFHF and HFHF+ millet) was probably due to the high energy density provided by this type of diet, which increases satiety and reduces consumption. Other studies that offered a high-fat diet for the animals also observed reduced consumption compared to the control diet (AIN-93M)(DA SILVA et al., 2019a; MEDINA MARTINEZ et al., 2021; THEODORO et al., 2021b).

The functional analysis of the KEGG microbial metabolic pathway observed that the HFHF + millet group positively regulated the pathways related to the urea cycle, amino acid biosynthesis (L-lysine, L-threonine, L-methionine, L-phenylalanine, and L-tyrosine) and degradation of sucrose and glycerol, which demonstrates that the germinated millet flour was able to modulate essential metabolic pathways. After amino acid metabolism, the activation of the urea cycle, which is a vital process, converts ammonia, a toxic byproduct, into urea, thus allowing unwanted hydrogen to be eliminated from the body (HONG et al., 2021; STEWART; SMITH, 2005). The degradation of sucrose and glycerol is also essential to generate energy and maintain homeostasis, in addition to amino acid biosynthesis, which still plays a crucial role in the regulation of the immune response and oxidative stress. In our previous studies, we demonstrated that polyphenols and prebiotics (dietary fiber and resistant starch) present in germinated millet flour were able to improve glucose metabolism (THEODORO et al., 2021b) and reduce inflammation and oxidative stress (THEODORO et al., 2021a). These dietary components altered the microbiota allowing such modulations.

All this remodeling of the microbiota through the consumption of germinated millet flour evidenced the prebiotic action of this cereal and demonstrated its potential to improve the composition and function of the microbiota.

5. Conclusions

Our results demonstrate that the germinated millet flour improves the morphology, function, and gut microbiota composition in rats fed with high-fat high-fructose diet. Therefore, the present study highlights the potential of this flour to improve intestinal health, however, further in vivo studies are needed to verify whether these effects are maintained in a microbiota similar to that of humans.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijerph192215217/s1, Figure S1: Experimental

design. The experiment was divided into two stages: Stage 1: induction of metabolic changes: the control group received a standard rodent diet (AIN-93M); and high fat high fructose group (HFHF) received the AIN-93M diet rich in saturated fat (31%) and fructose (20%), for eight weeks. Stage 2: Intervention: control group AIN-93M was maintained and group HFHF was divided into: HFHF group and HFHF + millet group (HFHF with germinated millet flour replacing 43.6% dietary fiber, 100% starch, 36% protein and 39% oil in experimental diet) for 10 weeks; Table S1: Effect of germinated Millet flour intake on biochemical variables, markers of inflammation, oxidative stress, and other metabolic alterations, in Wistar rats (n = 8), for 10 weeks.

Author Contributions: J.M.V.T.: Conceptualization, data curation, formal analysis, roles/writingoriginal draft, and software; M.G.: formal analysis, visualization and the writing-review and editing; B.P.d.S.: formal analysis, visualization, and the writing-review and editing; M.J.C.G.: data curation and formal analysis; R.C.L.T.: data curation, formal analysis, methodology, and supervision; V.P.B.d.S.J.: data curation and formal analysis; H.C.M.: data curation and formal analysis; C.W.P.C.: conceptualization, funding acquisition, investigation, project administration, resources, and the writing-review and editing; H.S.D.M.: conceptualization, data curation, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, visualization, roles/writing-original draft, and the 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 protocol was approved by the Ethics Committee on the Use of Animals of the Federal University of Viçosa (CEUA/UFV;

process n_ 39/2019). All the experimental procedures with animals were performed following the Directive 86/609/EEC of 24 November 1986.

Data Availability Statement: Not applicable.

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

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8.2. Paper 2: Chemical composition and in vitro iron bioavailability of extruded and open-pan cooked germinated and ungerminated pearl whole millet "*Pennisetum glaucum* (L.) R. Br."

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Chemical composition and in vitro iron bioavailability of extruded and open-pan cooked germinated and ungerminated pearl whole millet "*Pennisetum glaucum* (L.) R. Br."

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ABSTRACT

This study aimed to evaluate the effect of extrusion and of open-pan cooking on whole germinated and non-germinated grains of pearl millet (*Pennisetum glaucum* L. R. Br.), on its chemical-nutritional composition and in vitro iron bioavailability. The experimental design consisted of three flours: non-germination open-pan cooked millet flour (NGOPCMF), germination open-pan cooked millet flour (GOPCMF), and extrusion cooked millet flour (ECMF). The ECMF increased the carbohydrates, iron, manganese, diosmin, and cyanidin and decreased the total dietary fiber, resistant starch, lipids, and total vitamin E, in relation to NGOPCMF. The GOPCMF increased the lysine and vitamin C and decreased the phytate, lipids, total phenolic, total vitamin E, and riboflavin concentration, in relation to NGOPCMF. Furthermore, germinated cooked millet flour and extruded millet flour improved iron availability in vitro compared to non-germinated cooked millet flour. GOPCMF and ECMF generally preserved the chemical-nutritional composition of pearl millet and improved in vitro iron bioavailability; therefore, they are nutritionally equivalent and can be used to develop pearl millet-based products.

1. INTRODUCTION

Millets belong to the Poaceae family and are the sixth most planted cereals worldwide (Faostat, 2020). Their grains stand out as a promising option to overcome food insecurity conditions, since, in addition to being nutritious, they have the potential to be environmentally sustainable and resistant to adverse climate and soil conditions, which favors their production even under unfavorable weather conditions (Dias-Martins et al., 2018; Nanje Gowda et al., 2022).

Millets are primarily composed of carbohydrate (mainly starch and dietary fiber), followed by protein and lipid, in addition to resistant starch and minerals (Theodoro et al., 2021). These cereals also contain fatty acids and bioactive compounds, mainly in the pericarp of the grain. On the other hand, the occurrence of phytates and polyphenols compromises their nutritional quality and may affect the bioavailability of some nutrients,

including minerals (NANJE GOWDA et al., 2022; SARITA; EKTA SINGH, 2016). Thus, it is recommended to consume millets in whole form to preserve or improve their nutritional value (Dias-Martins et al., 2018; Theodoro et al., 2021).

Among the processes available for pearl millet, germination stands out for being a simple and cheap method, proven beneficial for increasing dietary fiber and mineral content (OWHERUO; IFESAN; KOLAWOLE, 2019; SARITA; EKTA SINGH, 2016), complex B vitamins (ARORA; JOOD; KHETARPAUL, 2011), and the antioxidant and anti-inflammatory activity of the grain (THEODORO et al., 2021a). Besides, this process reduces the concentration of phytochemicals, such as phytic acid, which decreases the bioavailability of nutrients (SARITA; EKTA SINGH, 2016). Furthermore, extrusion cooking is another versatile, short-time process with high potential to develop millet-based products, thus improving the nutrition properties of extruded millet products (DIAS-MARTINS et al., 2018; PESSANHA et al., 2023).

Germinated, extruded or cooked millet has been used in product development, due to the technological quality attributed to this grain (Dias Martins, 2019; Pessanha et al., 2023). However, it is necessary to characterize the processing and treatment of the grain to prepare flour, since the nutritional quality of the pearl millet flours may vary due to the genotype and process performed on the grain (Dias-Martins et al., 2018; Sarita; Ekta, 2016). The hypothesis is that the germination and extrusion processes improve the chemical and nutritional composition of millets. Therefore, the present study aimed to evaluate the effect of extrusion and open-pan cooking on whole germinated and non-germinated grains of pearl millet (*Pennisetum glaucum* L. R. Br.), on its chemical-nutritional composition and in vitro iron bioavailability.

2. MATERIAL AND METHODS

2.1. Raw Material

The pearl millet grains (*Pennisetum glaucum* (L.) R. Br.), hybrid ADRg 9070, kindly donated by Atto Sementes (Rondonopolis, Brazil), were harvested in the municipality of Itiquira (MT, Brazil), in 2021.

2.1.1. Pearl Millet Germination

Whole millet grains (99% of germination index) were soaked in distilled water for 30 min and placed in a controllable heat (30° ± 2 °C) and moisture chamber (> 90% relative humidity) (National Mfg. Co., Lincoln, USA) in order to germinate for 24 h,

which allowed the emission of roots (up to 20 mm long). Next, the germinated millets were dryed (50 °C, 4h) in an oven (MS-G-EG; Macanuda Hauber, Joinville, Brazil) (THEODORO et al., 2021a).

2.1.2. Pearl Millet Extrusion Cooking

The extrusion cooking of whole millet grain with moisture content adjusted to 13% was performed in an extruder (Evolum HT25 twin screw co-rotating, Clextral Inc., Firminy: France) equipped with round die (8mm) and length/diameter ratio of 40:1. The following temperatures were used: 25, 40, 50, 90, 100, 110, 120 °C. The extrudates were dried in a ventilated oven (Hauber Macanuda, Joinville, Brazil), at 60 °C, for 2 h, packaged and stored at 4ºC (PESSANHA et al., 2023).

2.1.3. Wet Heat Treatment of Grains and Flour Preparation

Whole grains of non-germinated and germinated millet were cooked in an open pan (98° ± 2 °C, 40 min) using the proportion grain/water of 1:4 or 1:5 to non-germinated and germinated millet, respectively. This cooking was carried out to imitate the way humans consume this grain. Then, the grains were dried (50°C, 24 h) in a ventilated oven (400/6ND; Nova Ética®, São Paulo, Brazil) (DIAS-MARTINS et al., 2018).

After preparation stage, the whole cooked millet grains were ground in a stainless-steel knife mill at 500 kg/h with sieve (1 mm) (TREU, Rio de Janeiro, Brazil). The resulting flours, denominated non-germination open-pan cooked millet flour (NGOPCMF), open-pan cooked germinated millet flour (GOPCMF), and extrusion-cooked millet flour (ECMF), were stored in a freezer at -20ºC ± 1ºC, until analysis.

2.2. Characterization: Chemical Analysis of Pearl Millet Flours

2.2.1. Moisture, Lipids, Ash, Protein, Dietary Fiber, Carbohydrate and Resistant Starch

The centesimal composition was analyzed as Association of Official Analytical Chemistry (AOAC, 2016), and the results were expressed in g/100g. Moisture content was determined using an air circulation oven (12 h, 105 °C; AOAC 934.01) (Nova Ética®, model 400/6ND, São Paulo, Brazil). The lipid content was measured by Soxhlet extraction (AOAC 945.16) (Tecnal, model TE-0364, Piracicaba/SP), for 8 h, under reflux. The total ash was quantified after incineration (550 °C, 6 h) in a muffle (Quimis, model Q320 M, Brazil) (AOAC 923.03). The protein content was measured by semimicro

Kjeldhal method (protein conversion factor of 6.25; AOAC 2001.11). The total, insoluble and soluble dietary fiber analysis was performed by enzymatic gravimetric method (K-TDFR-200A, Megazyme®). The carbohydrate content was determined as the following equation: [100 - (% moisture + % lipids + % proteins + % total dietary fiber + % ash)]. The calories were estimated considering: 4 kcal/g (protein or carbohydrate) and 9 kcal/g (lipid).

The resistant starch content of each flour was determined by an enzymatic kit from Megazyme®, according to the merchant's instructions (AACC Method 32-40).

2.2.3. Mineral Composition

For this analysis, all materials used were previously demineralized using Nitric acid solution (10%), during 24 h with subsequent rinsing in deionized water. The minerals (iron, phosphorus, zinc, calcium, potassium, copper, magnesium and manganese) were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) (Perkin Elmer, Optima 8300), according Gomes et al. (1996). The concentrations of each mineral in flour were expressed in mg/100 g.

2.2.4. Amino Acid Profile

Amino acid profile analyses were executed by HPLC (SCL 10AT VP; Shimadzu, Tokyo, Japan), according to AOAC 994.12/2000 (AOAC, 2016; Liu et al., 1995). The following amino acids were quantified: cysteine, histidine, arginine, isoleucine, leucine, alanine, lysine, methionine, phenylalanine, threonine, glutamic acid, tyrosine, valine, tryptophan, aspartic acid, glycine, serine, and proline. These were expressed in g/100 g.

2.2.5. Fatty Acids Profile

Lipids extraction from flour was carried out with hexane at room temperature. Methylation of the oil to obtain methyl esters was carried out according to Antoniassi et al. (2018) and analyzed by gas chromatography (Agilent 7890) using the following conditions: flame ionization detector operated at 280 ºC; fused silica capillary column made of cyanopropylsiloxane film (60 m x 0.32 mm x 0.25 µm); temperature of 100 ºC for 3 min (initial), 100-150 ºC (ramp of 50 ºC/min), 150-180 ºC (ramp of 1 ºC/min), 180-200 ºC (ramp of 25 ºC/min), and 200 ºC for 10 min (final temperature). For analysis, 1 µL of the sample was injected into a heated injector (250 ºC) using a 1:50 flow split mode.

Identification as well as the quantification of each fatty acid were performed by comparing retention times with standards from NU-CHEK PREP, Inc., Elysian, MN and Supelco, St. Louis, MO (C14:0, C15:0, C16:0, C16:1, C17:0, C18:0, C18:1, C18:2, C18:3, C20:0, C20:1, C22:0, C24:0). The values were expressed as % of each fatty acid in the total fatty acids.

2.2.6. Phytic Acid

The phytic acid content was determined using a commercial kit (K-PHYT Phytic Acid (Phytate)/Total Phosphorus; Megazyme®, Wicklow, Ireland). The spreadsheet available on the kit supplier's online platform (www.megazyme.com) was used to calculate the samples' phytic acid content (mg/g of flour).

2.2.7. Total Phenolics Compounds

Millet flour (1.0 g) was added to an extractor solution (20 mL) (50% ethanol) and stirred (60 min, 50°C) (PIZANI et al., 2024). Then, the solution was centrifuged (10 min, 3000 *g*) and the supernatant was stored under refrigeration until the time of analysis.
The total phenolic compounds were analyzed using the Folin-Ciocalteau reagent (SINGLETON; ORTHOFER; LAMUELA-RAVENTO, 1999). The absorbance reading was performed on a Multiskan™ GO spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), at 600 nm. The gallic acid was used as standard and results were expressed as mg of gallic acid equivalent/g (mg GAE/g) of flour.

2.2.8. Phenolic compounds profile

The extraction of phenolic in millet flours was carried out in an ultrasonic bath (P60H, Elmasonic, Singen, Germany, 2.75 L, 37 kHz, 135 W) for 60 minutes, starting at room temperature and reaching until 50 °C (PIZANI et al., 2024). The extraction medium was composed of EtOH: H_2O (1:1, v/v), with a solvent-feed ratio of 20 mL/g. Subsequently, the extracts were centrifugated, diluted 5-fold in water, filtered in a nylon filter of 0.22 µm, and further injected in an UPLC-PDA-MS (Acquity, Waters Co., Milford, MA, USA). The separation system was in Acquity UPLC BEH C18 50 × 2.1 mm, 1.7 µm analytical column and the mobile phases were consisted of water (A) and acetonitrile (B), both acidified with 0.1% acetic acid (v/v). The gradient elution was from 5-100% B in 0-10 min. The chromatography conditions were: 0.5 mL/min of flow rate, column oven at 40 °C, and 5 µL of injection volume. The UV spectra were 210 to 400

nm at λmax of 254. The MS analysis was performed in positive and negative ionization mode (100–1000 Da) with cone voltage of 15 V and 30 V, respectively, capillary voltage of 0.8 kV, and probe at 600 ºC. The software Empower 3 was applied for data processing (Waters Alliance, Milford, MA, USA). Additionally, *m/z* and UV/Vis spectra of compounds previously identified in *Pennisetum glaucum*, as recorded in the LOTUS database (https://lotus.naturalproducts.net/) and literature studies, were compared with the data obtained from UPLC-PDA-MS to tentatively identify potential candidates (RUTZ et al., 2021). Calibration curves for each compound was based on hesperidin equilvalent ($y = 14788x + 73215$; $R^2 = 0.9932$) and were performed using seven concentrations of the standards vs. the area of the respective chromatographic peak.

2.2.9. Antioxidant Activity

To prepare the extract, pearl millet flour (1.0 g) was added to an extractor solution (10 mL) (4:4:2 v/v, respectively of 50% methanol, 70% acetone, and distilled water) (RUFINO et al., 2007) and stirred for 16 h. Then, the solution was centrifuged (10 min, 3000 *g*) and the supernatant was stored under refrigeration until the time of analysis.

The antioxidant activity of the flours was determined using the DPPH (2,2-diphenyl-1-picryl-hydrazyl) method (Bloor, 2001). The samples and control were then read at 517 nm in spectrophotometer (Multiskan™ GO, Thermo Fisher Scientific, Waltham, MA, USA). An analytical curve was obtained using Trolox and results were expressed as µM Trolox equivalent/g (µM TE/g) of flour.

2.2.10. Condensed Tannins

The extract for the analysis of condensed tannins was prepared in a similar way to DPPH, but using another extracting solution (1% HCl in methanol) (RUFINO et al., 2007), and the analysis was carried out using the vanillin/HCl reaction method (Price et al. 1978). The absorbance was measured at 500 nm in a spectrophotometer (Multiskan Go, ThermoScientific, USA). The standard curve were carried out using catechin and the results were expressed in mg catechin equivalent/g (mg CE/g) of flour.

2.2.11. Carotenoids

Carotenoids were extracted according to Cardoso et al., (2014). For extraction, cooled acetone was used, and then the carotenoids were partitioned from acetone into petroleum ether. For analysis, the extract was evaporated under a stream of nitrogen gas,

and the dried residue was taken up in acetone. Carotenoids were analyzed in HPLC (Shimadzu, model VP SCL 10AT, Japan) with a high-pressure pump (Shimadzu, LC-10AT model VP, Japan), an automatic sampler (500 µL loop) (Shimadzu, model SIL-10AF, Japan) and a diode array detector (Shimadzu, model SPD-M10A, Japan). Futhermore, were used a RP-18 column (Phenomenex Gemini), pre-column (C18) (Phenomenex ODS, 4 mm x 3 mm); mobile phase was hexane: isopropanol (95:5 v/v), flow rate of 2.0 mL/min, and 50 µL of injection volume. Chromatograms were obtained at 450 nm and standarts were used to identify the peaks.

2.2.12. Vitamin E

The analysis of vitamin E components (α-, β-, γ- and δ-tocopherols, and tocotrienols) in millet flours were investigated. The extraction and analysis were performed as Pinheiro et al. (2021) methodology. For extraction was used heated ultrapure water (80 ± 1 °C), added with hexane + 0.05% BHT, anhydrous sodium sulfate, isopropyl alcohol and a mix of hexane: ethyl acetate (85:15 v/v). The vitamin E components were identified by UPLC using standards.

2.2.13. Ascorbic acid (vitamin C)

The extraction and analysis of ascorbic acid were performed according to Campos et al. (2009). The extraction solution was composed by 3% metaphosphoric acid, 8% acetic acid, sulphuric acid 0.3 N, and 1 mM EDTA. The extract was centrifuged at 3000rpm, 15 min (Hermle®, model Z216MK, Germany) and filtered. The analysis was performed on the HPLC system (Shimadzu, model SCL 10AT VP, Japan) at 245 nm.

2.2.14. Thiamine and riboflavin

Total thiamine and total riboflavin were extracted simultaneously according to Ndaw et al. (2000). Before analysis, both extracts obtained were filtered through cellulose acetate (0.22 µm) (Milipore, USA). Riboflavin and thiamine analyzes were conducted individually, using HPLC system with methodology proposed by Pinheiro et al. (2021). The running time for thiamine and riboflavin was 13 and 14 minutes, respectively. The vitamin standards were thiamine hydrochloride and riboflavin (Sigma-Aldrich®, St. Louis, MO, USA) that were used for identifying and constructing analytical curves.

2.3. Assessment of In Vitro Iron Bioavailability

The bioavailability of Fe in pre-cooked millet flour samples was caried-out using Caco-2 cell culture as methodology propoused by Glahn (2022). All glassware used during the procedure was previously washed with acid. Then, the freeze-dried cooked flours (0.5 g) were subjected to simulated gastric and intestinal digestion. The protocol is based on the total ferritin retained in cells after sample treatment and total proteins of Caco-2 cells (GLAHN, 2022). The results are expressed as ferritin/mg total protein ratio as an indicator of cellular Fe uptake.

2.4. Statistical Analysis

The samples were analyzed in triplicate. The results were expressed as mean ± SD (standard deviation). The data were subjected to Kolmogorov Smirnov and Shapiro-Wilk normality tests and analyzed by One-way analysis of variance (ANOVA), followed by the Tukey's test (to compare the three flours) or Student's t-test (to compare two samples). The significance level was 5% and the analyses were performed using the GraphPad Prism software system (version 9.0, GraphPad Software, San Diego, CA).

3. RESULTS

3.1. Chemical Characterization of Flours

The ECMF decreased total lipids, total dietary fiber, and resistant starch concentration and increased the carbohydrate content, compared to the other flours (NGOPCMF and GOPCMF) **(Table 1)**. The germination open-pan cooked millet flour (GOPCMF) decreased total lipids and ash content and did not change the total dietary fiber and carbohydrate concentrations, when compared to the non-germination open-pan cooked millet flour (NGOPCMF) **(Table 1)**. The concentration of soluble and insoluble dietary fiber, moisture, protein and the total energy value did not differ among the flours **(Table 1)**.

Table 1. Chemical composition of pre-cooked millet flours submitted to different processes (g/100 g^{-1}).

Variables**	NGOPCMF	GOPCMF	ECMF
Carbohydrate	68.24 ± 1.47^{b}	67.68 ± 0.14^{b}	74.87 ± 0.44^{a}
Total fibers	11.88 ± 1.17^{a}	11.91 ± 0.30^{a}	7.96 ± 0.12^{b}
Insoluble fiber	10.45 ± 2.17^{a}	11.43 ± 0.23^{a}	6.82 ± 0.04^{a}
Soluble fiber	1.43 ± 0.99^{a}	0.49 ± 0.06^{a}	1.14 ± 0.08^{a}

Protein	11.16 ± 0.56[a]	11.46 ± 0.85[a]	10.97 ± 0.46[a]
Lipids	5.58 ± 0.17[a]	5.03 ± 0.11[b]	2.82 ± 0.29[c]
Moisture	2.03 ± 0.06[a]	2.08 ± 0.52[a]	1.72 ± 0.26[a]
Ash	1.52 ± 0.02[a]	1.40 ± 0.05[b]	1.58 ± 0.02[a]
Resistant starch	3.38 ± 0.63[a]	3.53 ± 0.04[a]	0.32 ± 0.09[b]
Calories (kcal.100 g^{-1})	367.90 ± 2.17[a]	361.80 ± 3.74[a]	369.10 ± 3.07[a]

**Values expressed in dry matter. Values expressed as mean±SD. Different letters on the line indicate significant differences by the Tukey's test, at 5% probability. NGOPCMF: non-germination open-pan cooked millet flour; GOPCMF: germination open-pan cooked millet flour; ECMF: extrusion-cooked millet flour.

Regarding the mineral composition, ECMF presented a higher concentration of iron and manganese, compared to the others (NGOPCMF and GOPCMF), besides a higher concentration of magnesium and zinc, compared to GOPCMF **(Table 2)**. The GOPCMF decreased phosphorus and potassium content, compared to the others (NGOPCMF and ECMF), and presented lower Zn and Mn concentrations, compared to NGOPCMF **(Table 2)**. Calcium and copper did not differ among the flours **(Table 2)**.

Table 2. Mineral composition of pre-cooked millet flours submitted to different processes (mg/100g).

Minerals	NGOPCMF	GOPCMF	ECMF
Potassium	380.00 ± 1.73[a]	331.30 ± 4.04[b]	382.00 ± 5.29[a]
Phosphorus	236.00 ± 0.00[a]	217.70 ± 5.51[b]	235.30 ± 2.89 [a]
Magnesium	119.30 ± 0.58[ab]	115.00 ± 4.00[b]	123.30 ± 3.79[a]
Calcium	10.30 ± 0.06[a]	10.00 ± 0.00[a]	10.05 ± 0.07[a]
Iron	4.49 ± 0.16[b]	4.18 ± 0.11[b]	5.64 ± 0.19[a]
Zinc	2.84 ± 0.10[ab]	2.61 ± 0.08[b*]	2.93 ± 0.09[a]
Manganese	1.36 ± 0.01[b]	1.19 ± 0.02[b*]	1.62 ± 0.16[a]
Copper	0.46 ± 0.01[a]	0.48 ± 0.27[a]	0.47 ± 0.00[a]

Values expressed as mean±SD. Different letters on the line indicate significant differences by Tukey's test, while * indicates difference by the t-test between NGOPCMF and the processing (GOPCMF or ECMF), at 5% probability. NGOPCMF: non-germination open-pan cooked millet flour; GOPCMF: germination open-pan cooked millet flour; ECMF: extrusion-cooked millet flour.

The amino acid profile analysis demonstrated that both germination open-pan cooked millet flour (GOPCMF) and extrusion-cooked millet flour (ECMF) increased most essential and non-essential amino acids **(Table 3)**. Further, the GOPCMF increased lysine concentration, compared to the NGOPCMF, but did not alter the histidine levels **(Table 3)**. The extrusion-cooked millet flour (ECMF) increased tryptophan concentration, but did not differ from NGOPCMF in relation to the histidine and lysine

content. Besides, this ECMF presented a higher concentration of histidine, compared to the GOPCMF. The methionine and cysteine concentrations did not differ among the flours **(Table 3)**.

Table 3. Amino acid profile of pre-cooked millet flours submitted to different processes (g/100g).

Amino acids**	NGOPCMF	GOPCMF	ECMF
Essentials			
Leucine	0.45 ± 0.04^{b}	0.59 ± 0.03^{a}	0.66 ± 0.01^{a}
Phenylalanine	0.24 ± 0.02^{b}	0.32 ± 0.01^{a}	0.35 ± 0.01^{a}
Valine	0.23 ± 0.02^{b}	0.31 ± 0.01^{a}	0.34 ± 0.01^{a}
Isoleucine	0.18 ± 0.01^{b}	0.24 ± 0.01^{a}	0.26 ± 0.01^{a}
Threonine	0.14 ± 0.01^{b}	0.19 ± 0.01^{a}	0.21 ± 0.00^{a}
Lysine	0.06 ± 0.01^{b}	0.10 ± 0.00^{a}	0.08 ± 0.01^{ab}
Histidine	0.06 ± 0.02^{ab}	0.05 ± 0.01^{b}	0.11 ± 0.01^{a}
Tryptophan	0.15 ± 0.00^{b}	0.15 ± 0.00^{b}	0.16 ± 0.01^{a}
Methionine	0.26 ± 0.02^{a}	0.27 ± 0.00^{a}	0.26 ± 0.01^{a}
Non-essential			
Glutamine	0.82 ± 0.06^{b}	1.12 ± 0.05^{a}	1.23 ± 0.00^{a}
Aspartate	0.38 ± 0.02^{b}	0.53 ± 0.01^{a}	0.59 ± 0.00^{a}
Alanine	0.32 ± 0.01^{b}	0.43 ± 0.02^{a}	0.47 ± 0.00^{a}
Proline	0.30 ± 0.01^{b}	0.42 ± 0.02^{a}	0.47 ± 0.01^{a}
Arginine	0.19 ± 0.01^{b}	0.26 ± 0.01^{a}	0.28 ± 0.01^{a}
Serine	0.15 ± 0.01^{b}	0.22 ± 0.01^{a}	0.24 ± 0.00^{a}
Tyrosine	0.15 ± 0.01^{b}	0.20 ± 0.01^{a}	0.22 ± 0.00^{a}
Glycine	0.10 ± 0.01^{b}	0.15 ± 0.01^{a}	0.16 ± 0.01^{a}
Cysteine	0.26 ± 0.01^{a}	0.24 ± 0.01^{a}	0.26 ± 0.02^{a}

Values expressed as mean±SD. Different letters on the line indicate significant differences by the Tukey's test, at 5% probability. NGOPCMF: non-germination open-pan cooked millet flour; GOPCMF: germination open-pan cooked millet flour; ECMF: extrusion-cooked millet flour.

The analysis of the profile of fatty acids presented the highest concentration of linoleic acid and γ-linolenic acid and the lowest concentration of palmitic acid in the germination open-pan cooked millet flour (GOPCMF), compared to the other flours (NGOPCMF and ECMF). Furthermore, the GOPCMF increased the concentration of stearic acid relative to NGOPCMF; of pentadecylic acid in relation to ECMF; and did not differ from NGOPCMF in relation to the arachidonic, behenic, arachidonic, palmitoleic, margaric and pentadecyl acids **(Table 4)**. ECMF presented the highest concentration of palmitic acid and stearic acid and the lowest concentration of arachidic, behenic, arachidonic, palmitoleic, and linoleic acids, compared to the others (NGOPCMF and GOPCMF), in addition to a higher concentration of margaric acid, compared to

NGOPCMF, but did not differ from GOPCMF **(Table 4)**. The GOPCMF did not differ from NGOPCMF regarding the pentacylic acid **(Table 4)**. The NGOPCMF presented the highest concentration of oleic acid compared to the others (ECMF and GOPCMF) **(Table 4)**. The myristic and gadolinium acids did not differ among the flours **(Table 4)**. In general, the extrusion-cooked millet flour and germination open-pan cooked millet flour presented reduced monounsaturated fatty acids, mainly the GOPCMF. Furthermore, the extrusion-cooked millet flour increased the saturated fatty acids and decreased the polyunsaturated fatty acids. In contrast, the germination open-pan cooked millet flour increased the polyunsaturated fatty acids and decreased the saturated fatty acids, compared to the non-germination open-pan cooked millet flour (NGOPCMF) **(Table 4)**.

Table 4. Profile of fatty acids of pre-cooked millet flours submitted to different processes (g/100g).

Fatty acids**	NGOPCMF	GOPCMF	ECMF
Myristic acid (C14:0)	0.04 ± 0.00^{a}	0.04 ± 0.00^{a}	0.04 ± 0.00^{a}
Pentadecylic acid (C15:0)	0.01 ± 0.01^{ab}	0.02 ± 0.00^{a}	0.01 ± 0.00^{b}
Palmitic acid (C16:0)	16.83 ± 0.02^{b}	16.36 ± 0.01^{c}	16.94 ± 0.01^{a}
Margaric acid (C17:0)	0.05 ± 0.00^{b}	0.05 ± 0.01^{ab}	0.06 ± 0.00^{a}
Stearic acid (C18:0)	4.67 ± 0.00^{c}	4.75 ± 0.03^{b}	4.91 ± 0.04^{a}
Arachidic acid (C20:0)	0.89 ± 0.01^{a}	0.90 ± 0.00^{a}	0.87 ± 0.01^{b}
Behenic acid (C22:0)	0.23 ± 0.00^{a}	0.23 ± 0.00^{a}	0.22 ± 0.00^{b}
Arachidonic acid (C24:0)	0.24 ± 0.00^{a}	0.24 ± 0.00^{a}	0.20 ± 0.00^{b}
Palmitoleic acid (C16:1)	0.21 ± 0.00^{a}	0.21 ± 0.00^{a}	0.20 ± 0.00^{b}
Oleic acid (C18:1)	26.54 ± 0.04^{a}	26.06 ± 0.03^{c}	26.21 ± 0.06^{b}
Gadolinic acid (C20:1)	0.26 ± 0.02^{a}	0.27 ± 0.00^{a}	0.26 ± 0.01^{a}
Linoleic acid (C18:2)	45.58 ± 0.12^{b}	46.08 ± 0.09^{a}	44.70 ± 0.12^{c}
γ-linolenic acid (C18:3)	3.07 ± 0.01^{b}	3.19 ± 0.01^{a}	3.09 ± 0.01^{b}
Total			
Saturated	22.98 ± 0.03^{b}	22.60 ± 0.04^{c}	23.24 ± 0.02^{a}
Monounsaturated	27.01 ± 0.05^{a}	26.54 ± 0.02^{c}	26.68 ± 0.06^{b}
Polyunsaturated	48.66 ± 0.13^{b}	49.27 ± 0.09^{a}	47.79 ± 0.13^{c}

Values expressed in dry matter. Values expressed as mean and standard deviation. Different letters on the line indicate significant differences by the Tukey's test, at 5% probability. NGOPCMF: non-germination open-pan cooked millet flour; GOPCMF: germination open-pan cooked millet flour; ECMF: extrusion-cooked millet flour.

The germination open-pan cooked millet flour (GOPCMF) decreased the concentration of phytic acid and total phenolic compounds, while the antioxidant activity (DPPH) did not differ among the flours **(Table 5)**. Condensed tannins and carotenoids were not detected in any millet flours **(Table 5)**.

Regarding the vitamins, both germination open-pan cooked millet flour and extrusion-cooked millet flour decreased the concentration of total vitamin E. The main fractions of vitamin E identified in the flours were β and γ-tocopherol. Regarding the components of this vitamin, both GOPCMF and ECMF increased δ-tocopherol, and the highest levels were found in GOPCMF. The GOPCMF and ECMF decreased γ-tocopherol, γ-tocotrienol, and δ-tocotrienol; and the lowest concentration of δ-tocotrienol was detected in the GOPCMF. The highest rate of α-Tocopherol was found in ECMF, while β-tocopherol did not differ among the flours. The highest rate of vitamin C was detected in GOPCMF and did not differ in ECMF, compared to NGOPCMF **(Table 5 and Supplementary Figure 1)**. Riboflavin lowest level was found in GOPCMF and did not differ in ECMF, compared to NGOPCMF. Thiamine did not differ among the flours. α and β-tocotrienol was not detected in any of the millet flours **(Table 5).**

Table 5. Phytochemical, antioxidant and vitamin compounds in pre-cooked millet flours submitted to different processes.

Compounds	NGOPCMF	GOPCMF	ECMF
Phytic acid (mg/g)	7.75 ± 0.13^{a}	6.32 ± 0.08^{b}	7.69 ± 0.12^{a}
DPPH (μM TE/g)	9.09 ± 0.29^{a}	9.52 ± 0.28^{a}	9.08 ± 0.30^{a}
Total phenolics compounds (mg GAE/g)	3.45 ± 0.63^{a}	2.38 ± 0.21^{b}	3.27 ± 0.01^{a}
Condensed tannins (mg CE/g)	ND	ND	ND
Carotenoids	ND	ND	ND
Total vitamin E (mcg/100g)	1458.00 ± 18.94^{a}	1073.00 ± 85.19^{b}	831.40 ± 124.70^{b}
α-tocopherol	26.90 ± 0.14^{b}	35.30 ± 6.08^{b}	79.10 ± 6.22^{a}
α-tocotrienol	ND	ND	ND
β-tocopherol	545.00 ± 0.21^{a}	670.60 ± 61.09^{a}	483.50 ± 64.28^{a}
β-tocotrienol	ND	ND	ND
γ-tocopherol	795.80 ± 17.11^{a}	304.50 ± 16.97^{b}	198.50 ± 53.88^{b}
γ-tocotrienol	35.45 ± 0.92^{a}	20.45 ± 1.77^{b}	21.40 ± 1.56^{b}
δ-tocopherol	13.95 ± 0.35^{c}	31.05 ± 0.35^{a}	20.65 ± 1.63^{b}
δ-tocotrienol	40.95 ± 2.47^{a}	10.90 ± 0.42^{c}	28.30 ± 2.83^{b}
Vitamin C (mg/100g)			
Ascorbic acid	0.49 ± 0.08^{b}	9.32 ± 0.92^{a}	0.26 ± 0.11^{b}
Complex B vitamins (mg/100g)			
Thiamine	0.79 ± 0.22^{a}	0.51 ± 0.02^{a}	0.81 ± 0.10^{a}
Riboflavin	0.50 ± 0.01^{a}	0.23 ± 0.04^{b}	0.60 ± 0.07^{a}

Values expressed as mean±SD. Different letters on the line indicate significant differences by the Tukey's test, at 5% probability. NGOPCMF: non-germination open-pan cooked millet flour; GOPCMF: germination open-pan cooked millet flour; ECMF: extrusion-cooked millet flour. DPPH: 2,2-diphenyl-1-picrylhydrazyl; TE: trolox equivalent; GAE: gallic acid equivalent; CE: catechin equivalent; ND: Not detected.

In the phenolic profile**,** the extracts obtained from the millet flours were analyzed by UPLC-PDA-MS to tentatively identify potential candidates in each extract. Considering previous reports in the literature about the metabolites already identified in *Pennisetum glaucum*, it was possible to suggest the potential identifications related to three peaks (rt: 3.12, 3.24, and 3.37 min) detected in 254 nm and MS-negative mode, which resulted in the largest number of peaks. The first peak was tentatively identified as a flavonoid containing a m/z 468.34 and UV spectrum with λmax in 293.9, and 317.8. The second peak was related to diosmin, containing a m/z of 269.31 and UV spectrum with λmax in 266.5 and 348.9. Finally, the third peak was tentatively identified as a cyanidin 3-O-rutinoside betaine, with a m/z of 593.34 and UV spectrum with λmax in 268.9 and 327.3 nm **(Figure 2)**. Considering the quantification of each peak, the first was detected in the three extracts (NGOPCMF: 4.85 ± 0.67; GOPCMF: 7.93 ± 4.73, and ECMF: 2.60 ± 1.84 ug/g), but with no difference among them. The second and third peaks were only quantified in the ECMF: 0.59±0.16 and 1.95±0.35 ug/g, respectively. Additionally, other smaller peaks were analyzed in the extracts. However, the identification of each one was not accomplished, as it was not possible to compare their UV spectrum and m/z with metabolites already identified in millet **(Figure 2)**.

3.2. Iron Bioavailability by In Vitro Assay (Caco-2 Cell Ferritin Formation)

Ferritin concentrations were higher in cell exposed to germinated cooked millet flour and extruded millet flour compared to non-germinated cooked millet flour **(Table 6)**. Furthermore, germinated cooked millet flour and extruded millet flour reduced the phytate/iron molar ratio, compared to non-germinated cooked millet flour **(Table 6)**.

Table 6. Ferritin concentration in Caco-2 cells and phytate/iron molar ration of pre-cooked millet flours submitted to different processes.

Tested Sample	Non-germinated cooked millet flour	Germinated cooked millet flour	Extruded millet flour
Ferritin (ng/mg of protein)	1.82 ± 0.54^{b}	2.93 ± 0.21^{a}	3.21 ± 0.20^{a}
Phytate/iron molar ration	14.60 ± 0.24^{a}	12.81 ± 0.15^{b}	11.52 ± 0.18^{c}

The cells were exposed only to MEM (minimum essential media), without the addition of digested food and Fe. All samples were run in the same experiment. Values expressed as mean ± SD. Different letters indicate significant differences by the Tukey's test, at 5% probability.

4. DISCUSSION

The present study aimed to analyze the effect of extrusion and open-pan cooking on whole germinated and non-germinated grains of pearl millet (*Pennisetum glaucum* L. R. Br.), on its chemical-nutritional composition and in vitro iron bioavailability. Furthermore, it is worth highlighting that no studies were found in the literature that compared the nutritional and chemical characteristics of this millet subjected to different processing. Thus, this study can direct future research works tackling the best process to apply to the grain, based on the main interest of the study. In addition, the grains used in the present study belong to the same harvest; they were all grown under the same conditions. Therefore, we can confirm that the differences in chemical-nutritional composition observed among the flours are attributed to the processing applied to the grain. Furthermore, our results refer to cooked millet, which is the form of human consumption.

In this study, we observed that both germination open-pan cooked millet flour (GOPCMF) and extrusion-cooked millet flour (ECMF) preserved good chemical and nutritional composition and iron bioavailability. However, the germination open-pan cooked millet flour stands out for its increased amount of lysine and vitamin C. However, it presented reduction in phytochemicals, such as phytate, lipids, total phenolic compounds, total vitamin E and riboflavin, compared to the other flours. In addition, it maintained the content of dietary fiber and resistant starch and improved in vitro iron bioavailability. Besides, pre-cooked millet flours presented higher concentration of flavonoids, diosmin and cyanidin.

Although the protein levels were similar among the treatment, changes were observed in the amino acid profile. It demonstrated that both germination open-pan cooked millet flour and extrusion-cooked millet flour increased the concentration of essential amino acids and non-essential amino acids. The glutamine increases in both processes is beneficial, since glutamic acid is known as a precursor of γ-aminobutyric acid (GABA), which works as an inhibitory neurotransmitter in the brain and spinal cord. Furthermore, it is an insulin secretion, which prevents diabetes and helps regulate blood pressure, heart rate, and pain relief (DIAS-MARTINS et al., 2018; SHARMA; SAXENA; RIAR, 2018). In addition, germination open-pan cooked millet flour increased the lysine concentration. Germination is known to increase the lysine content in cereals (Obilana et al. (2018), which makes this processing interesting for pearl millet, as cereals are generally poor in lysine, possibly leading to nutritional limitations in regions that use it as a staple food. Then, germination favors amino acid profile improvement, without

changing the total protein concentration, since this process can hydrolyze high molecular weight proteins into smaller ones, such as peptides and amino acids, as observed by Xu et al. (2017).

Furthermore, the extrusion process consists of a combination of thermal and mechanical treatments that increase pressure inside the barrel, under conditions of low flour humidity, high screw speed, and narrow die output for a short period (Salazar-López et al., 2018), which promotes carbohydrate increase and lipid reduction, as observed in the present study. During this process, the starch structure is broken into small size molecules by the action of heat and humidity (PESSANHA et al., 2023). Besides, the reduction of lipids is probably due to the formation of complexes with other compounds present in the food matrix, such as starch, which may be related to its reduced availability (OBILANA; ODHAV; JIDEANI, 2018; SIMONS et al., 2015).

On the other hand, it was observed that germination reduced only lipid and ash, while carbohydrate, protein, dietary fiber, resistant starch and moisture were not changed. During the germination, lipids may be used as an energy source (LI et al., 2020b), while ash content may be reduced due to the loss of some minerals, such as potassium and phosphorus, as observed in our study. The non-alteration of the other nutrient may be explained by the germination time used in the present study (24h), which may have been insufficient to affect their content.

The lipid contents may be affected by the changes in the concentration of some fatty acids. In general, germination increased the total polyunsaturated fatty acids and decreased the total saturated and monounsaturated fatty acids. The increase in polyunsaturated fatty acid content may be due to glyceride hydrolysis, while the decrease in saturated and monounsaturated fatty acids may be due to β-oxidation, to provide energy for the germination of the grain (Li et al., 2020). These changes can improve health, since the increased consumption of polyunsaturated fatty acids and decreased consumption of saturated fatty acids are associated with benefits to cardiovascular health, type 2 diabetes, obesity, inflammation, and intestinal microbiota (DE MELLO et al., 2019).

On the other hand, extrusion-cooked millet flour increased the total saturated fatty acids and decreased the total polyunsaturated and monounsaturated fatty acids. According to Bertipaglia et al. (2008) and Obilana et al. (2018), the reduction of some fatty acids during extrusion may be explained by lipid reduction, as observed in our study. Further, the heat of the lipids, in the presence of oxygen in the extrusion process, may induce oxidation and the degradation of fatty acids. Besides, the extrusion process decreased the

total dietary fiber, which may be associated with the production of low molecular weight components that ethanol is not capable to precipitate (GAJULA et al., 2008). Then, since the method to analyze dietary fiber is gravimetric, it is not able to measure compounds below 10-12 polymeric degrees. In addition, resistant starch can become digestible after high shear extrusion, mainly when puffed extrudates are produced (ALBARRACÍN et al., 2019), thus decreasing its content and affecting the total dietary fiber content, as observed in the present study. Furthermore, the extrusion process reduced the moisture content, which may reduce the amount of resistant starch, since less water will result in higher friction and damage to the starch during the process (Simons et al. 2015).

Further, the reduction of total vitamin E in extrusion and germination processes possibly occurred due to lipid reduction, since the stability and sensitivity of fat-soluble vitamins are affected by lipid degradation (ARRIBAS et al., 2019). Alternatively, vitamin C increase in germination may have occurred due to *de novo* synthesis, since most seeds before germination have a very low or undetectable ascorbic acid content, as observed in the non-germinated process. In addition, the germination probably increased the activity of L-galactone-γ-lactone dehydrogenase (GLDH), an enzyme involved in the biosynthesis of vitamin C, consequently increasing the content of this vitamin (Gan et al., 2017).

Riboflavin decreased in germination and thiamine remained similar among the processes and treatment used in our study. Germination has demonstrated to increase B vitamins but, when this food mixture was exposed to heat treatment by autoclave, the thiamine content was reduced (Arora et al., 2011). Therefore, since germination can improve vitamin content, the kind of heating process applied after germination may affect its content.

Regarding minerals, the extrusion processes increase the iron and manganese concentration. This fact was not expected, but this increase can be explained by contamination during this process. Besides, germination process reduced phosphorus, potassium, zinc and magnesium, which can be due to the use of these minerals in the metabolic pathway, since these minerals can be used for energy production, or due to leaching into the soaking water (DE MIRANDA, 2006; NANJE GOWDA et al., 2022).

The concentration of phytic acid decreased in germination open-pan cooked millet flour, which reduced the phytate/iron molar ratio. This may be due to germination, which probably activated endogenous phytases that degraded phytic acid (ALBARRACÍN et

al., 2019; SARITA; EKTA SINGH, 2016). Phytate/iron molar ratio reduction may have affected the *in vitro* tests, which present improved iron availability by germination open-pan cooked and extrusion-cooked millet flours. These samples increase the production of ferritin by Caco-2 cells, thus demonstrating that both processes present a greater amount of iron bioavailable in the flours, compared to non-germination open-pan cooked millet flour. Futhermore, phytic acid reduction and vitamin C increase after germination helped to improve iron reduction and solubility, thus improving the bioavailability of this mineral in germination open-pan cooked millet flour.

The extrusion and non-germination process did not change the antioxidant capacity and total phenolic compounds. However, Salazar-López et al. (2018) observed that the extrusion process increased the antioxidant capacity and total phenolic content of other cereals, including sorghum. The results different from our findings can be due to the type of matrix and the high temperature used (around 180 ºC), which enables the breakdown of molecular structures and increases the extractability of such compounds. Further, the germination process decreased the total phenolic compounds content, which can be due to reduced phytic acid concentration.

Furthermore, the flavonoids were detected in the flours. This compound is one of the largest groups of secondary metabolites found in fruits and vegetables and has potent antioxidant properties (JUBAIDI et al., 2021). Diosmin, detected in extrusion-cooked millet flour is one of the flavones commonly found in vegetables and has antioxidant activities, with the capacity to eliminate reactive species, in addition to exhibiting potent anti-inflammatory properties through the regulation of toxicity, by reducing the expression of pro-inflammatory proteins (JIANG; DOSEFF; GROTEWOLD, 2016; JUBAIDI et al., 2021). Cyanidin, also detected in extrusion-cooked millet flour, is one of the most studied anthocyanins.A It also presents a beneficial effect on reducing oxidative stress, as it contains a positively charged oxygen atom in its molecule, which makes it a potent antioxidant and a distinct hydrogen donor (BUENO et al., 2012; JUBAIDI et al., 2021). These compounds presented antioxidant and anti-inflammatory effects, as observed in animal studies (THEODORO et al., 2021a, 2022).

It is essential to highlight that millet is an underexplored plant in terms of its metabolite profile. To the best of our knowledge, no extensive metabolomics study has focused on the bioactive compounds of this plant. In this context, suggesting the identification of its metabolites presents a challenging approach. Therefore, the present study can contribute to filling the gap in knowledge regarding this subject and encourage

other studies, using high-resolution MS equipment for more conclusive identification regarding such compounds in millet.

Furthermore, other studies have already investigated the effects of these processes on the technological properties of the grain, with improvements, for example, in texture parameters, viscosity, solubility and water absorption in millet-based products, also demonstrating that such processes are necessary to improve safety food and increase the commercial use of millet (Dias Martins, 2019; Pessanha et al., 2023). Therefore, both processes can be applied to the development of millet-based products, and the human consumption of this grain should be encouraged as it has good chemical and nutritional characteristics.

5. CONCLUSION

The germination open-pan cooked grains and extrusion-cooking changed the concentration of nutrients and non-nutrients in the millet flours, while keeping the chemical and nutritional characteristics of pearl millet and improving its in vitro iron bioavailability. The germination open-pan cooked millet flour stands out for presenting increase in lysine and vitamin C, reduction in the phytochemicals, such as phytate, and maintaining the content of dietary fiber and resistant starch. Therefore, although all flours have good nutritional composition, germination and extruded processes proved to be nutritionally more suitable for the development of millet-based products.

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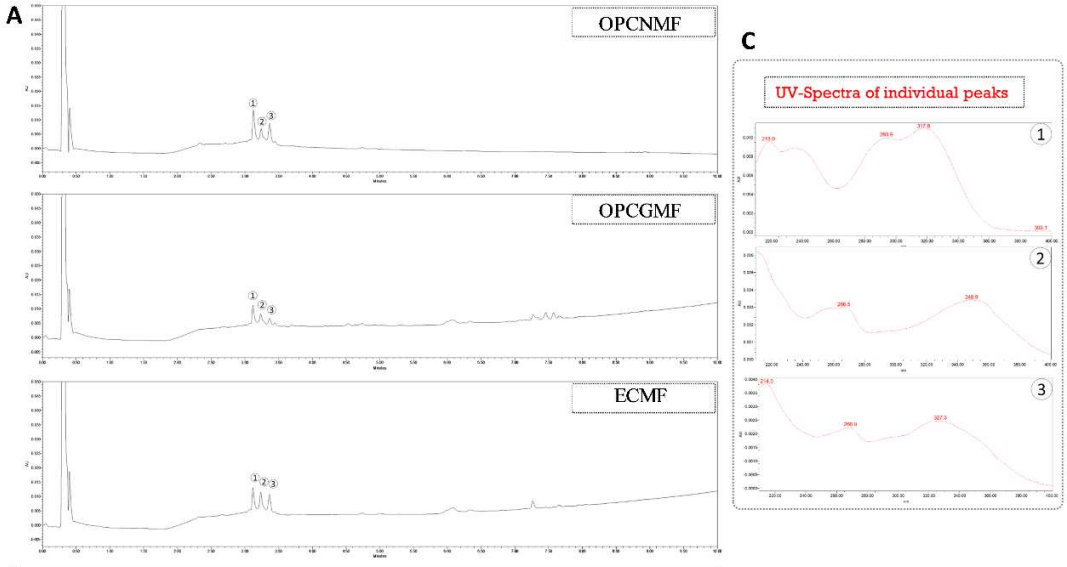
Paper 2

Supplementary Material



B

Peak Number	Retention time (min)	UV - λmax	*m/z*	Potential classification
1	3.12	293.9, 317.8	$[M-H]^-$= 468.34	Flavonoid*
2	3.24	266.5, 348.9	$[M-H]^-$= 269.31	Diosmin
3	3.37	268.9, 327.3	$[M-H]^-$= 593.34	Cyanidin 3-O-rutinoside betaine

Figure S1. Chromatogram of vitamins in pre-cooked millet flours submitted to different processes. Vitamin B2 in OPCNMF (A); Vitamin B2 in OPCGMF (B); Vitamin B2 in ECMF (C); Vitamin B1 in OPCNMF (D); Vitamin B1 in OPCGMF (E); Vitamin B1 in ECMF (F); vitamin E components in OPCNMF (G); vitamin E components in OPCGMF (H); vitamin E components in ECMF (I); vitamin C in OPCNMF (J); vitamin C in OPCGMF (K); vitamin C in ECMF (L). OPCNMF: open-pan cooked non-germinated millet flour; OPCGMF: open-pan cooked germinated millet flour; ECMF: extrusion-cooked millet flour.

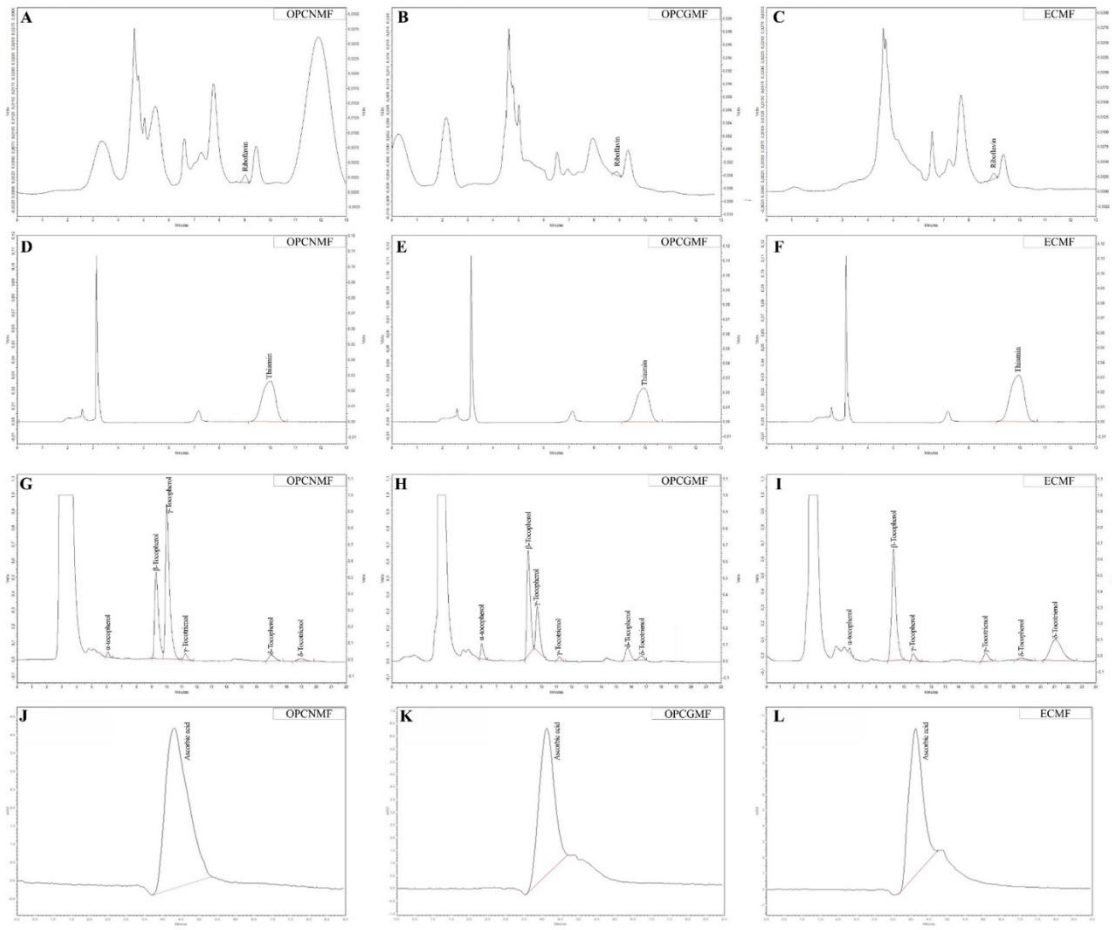


Figure S2. Chromatogram of the phenolic profile in pre-cooked millet flours extracts submitted to different processes, the most significant peaks, their respective retention times, UV spectra, *m/z* values, and the potential classification of each one. OPCNMF: open-pan cooked non-germinated millet flour; OPCGMF: open-pan cooked germinated millet flour; ECMF: extrusion-cooked millet flour.

8.3. Paper 3: Conventional and germinated pearl millet flour (*Pennisetum glaucum* (L.) R. Br.) improves iron metabolism and antioxidant capacity in Wistar rats

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Conventional and germinated pearl millet flour (*Pennisetum glaucum* (L.) R. Br.) improves iron metabolism and antioxidant capacity in *Wistar* rats

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ABSTRACT

The study evaluated the effect of the consumption of pearl millet whole grain pre-cooked conventional, germinated and extruded flours on iron metabolism and antioxidant capacity in rats. The animals received iron-free standard diet (SD iron-free) for 28 days and subsequently, were separated into 4 groups: ferrous sulfate (SD + FS), conventional millet flour (SD + CM), germinated millet flour (SD + GM), and extruded millet flour (SD + EM), for 21 days. The SD iron free group presented increased ferroportin, hephaestin and ferritin gene expression and MDA concentration compared to the SD + FS group. The SD + GM group increased DcytB, ferroportin and hephaestin gene expression. The SD + EM group increased the concentration of transferrin compared to the SD + FS group and serum iron, compared to the other groups, while reducing the liver's total antioxidant capacity (TAC). The SD + CM group increased ferritin expression. All test groups increased the gene expression of DMT1 and hephaestin, as well as serum iron and reduced transferrin, Nrf2 and TNF gene expression, and MDA, NO, SOD and CAT levels in relation to the SD + FS group. In view of this, millet provides promising bioavailability of iron. Besides, germination stands out for improving iron metabolism in relation to extrusion and is similar to conventional millet in antioxidant capacity.

1.INTRODUCTION

Iron is one of the most abundant elements in nature (SILVA; MURA, 2011). However, its ability to form highly insoluble oxides when in contact with oxygen reduces its bioavailability (GONZÁLEZ-DOMÍNGUEZ et al., 2020). Thus, iron deficiency is a nutritional health problem, which can lead to iron deficiency anemia, a disease that affects more than 2 billion people in low, medium and high-income populations worldwide, which increases morbidity and mortality (DRAKESMITH et al., 2021; SIHAG et al., 2016).

In developing countries, the diet is mainly composed of staple foods, as cereals and legumes, which have low iron content and/or bioavailability, thus making it difficult to achieve sufficient intake of this mineral. The population main food sources of iron are cereals and legumes, due to limited access to animal products, which provide a high

concentration of iron. Therefore, it is important to diversify staple foods, including food crops naturally rich in iron, and study processing techniques to improve the nutritional quality of these foods (ANITHA et al., 2021; DIAS-MARTINS et al., 2018; VETRIVENTHAN et al., 2021), including cereals, such as millet.

Millet is the sixth most cultivated cereal in the world. It is environmentally sustainable and its nutritional composition consists of proteins, dietary fiber, resistant starch, bioactive compounds and minerals, such as iron (DIAS-MARTINS et al., 2018; FAOSTAT, 2020). However, the presence of phytochemicals, such as phytates, in this food matrix can interfere with the biological use of these nutrients and the bioavailability of some minerals. Yet, the use of certain types of processing can reduce these components and improve the bioavailability of nutrients (DIAS-MARTINS et al., 2018).

Germination is a simple and inexpensive technique that activates phytases by hydrolyzing phytate and degrading bioactive compounds, into simpler structures, thus improving nutrient bioavailability and grain functionality (ELKHALIFA; BERNHARDT, 2010; SIHAG et al., 2016). Extrusion is a mechanical treatment, under high pressure and temperature, which can reduce components that form insoluble complexes with iron and other divalent cations and improve digestibility (DIAS-MARTINS et al., 2018; GULATI; ROSE, 2018).

The literature highlights the benefits of germination and extrusion for the bioavailability of nutrients and nutritional quality of millets in vitro (Balasubramanian et al., 2014; Owheruo et al., 2019). However, the effects of such processing on iron metabolism in vivo have not been evaluated yet. In addition, the main treatment used during iron deficiency is oral therapy with iron, but, the literature highlights that oral iron supplementation can lead to inflammation and oxidative stress, since this mineral has the ability to generate free radicals, which can form reactive oxygen species (MALESZA et al., 2022).

In view of this, our hypothesis was that the germination and extrusion of millet grains can result in improvements in the availability of iron, providing better use of this mineral in vivo. Besides this, the flours can improve the oxidative stress and inflammation in rats induced iron deficiency. Once our hypothesis about millet submitted to different processes is confirmed, it could help to encourage the increase of human consumption of this grain. Furthermore, this study will offer options to maximize access to nutrient and health benefits to reduce the risk of iron deficiency. Therefore, the objective of the present study was to evaluate the effect of the consumption of pearl millet whole grain pre-cooked

conventional, germinated and extruded flours on iron metabolism and antioxidant capacity in *Wistar* rats induced to iron deficiency.

2.MATERIAL AND METHODS

2.1. Process Applied to Millet Grain

The pearl millet grains (*Pennisetum glaucum* (L.) R. Br.), hybrid ADRg 9070, were harvested in the municipality of Itiquira (MT-Brazil), 2nd Crop/2021. Grain germination occurred in an adapted bread fermentation chamber (National Mfg. Co., Lincoln, USA), under controlled conditions of temperature at 30° ± 2 °C and 90% humidity for 24 h, with subsequent drying at 50 °C, in a fan oven DMS-G-EG (Macanuda Hauber, Joinville, Brazil) for 4 h, according to (Theodoro et al., 2021). Extrusion cooking was carried out in a Clextral Evolum HT25 twin-screw co-rotating extruder (Clextral Inc., Firminy, France) with a screw diameter of 25 mm, length/diameter ratio of 40:1 and ten temperature zones (30, 60, 90, 100, 100, 100, 110, 110, 120, 120 °C). The resulting puffed extrudates were dried in a fan oven 400/6ND (Nova Ética®, São Paulo, Brazil) at 60 °C for 2 h.

The whole pearl millet grains, named conventional and germinated, were previously cooked in an open pan at 180 °C, for 40 min (grain/water proportions of 1:4 for conventional and 1:5 for germinated) and then dried in fan oven 400/6ND (Nova Ética®, São Paulo, Brazil) at 50 °C, for 24 h (DIAS-MARTINS et al., 2018). The extruded product did not go through the same cooking process, since the combination of mechanical shearing and heat applied during the thermoplastic extrusion is a well-known cooking process. Subsequently, conventional, germinated and extruded whole grain pearl millet were ground in a stainless-steel combined hammer-knife mill, equipped with a 1 mm sieve (TREU, Rio de Janeiro, Brazil), at 500 kg/h, to obtain the flours, denominated CM, GM and EM, respectively.

2.2. Iron Determination

The level of iron in flours and diets was determined by coupled plasma atomic emission spectrometry, Optima 3300 DV model (Perkin Elmer, Waltham, USA), according to the methodology proposed by Gomes; Silva; Silva, (1996). The centesimal composition was evaluated using the methodologies proposed by the Association of

official analytical chemistry (AOAC, 2016). All analyses were carried out in triplicate, and the data were used to determine the composition of the experimental diets.

2.3. Animals and Diet

Forty 21-day old male *Wistar* rats (*Rattus norvegicus*) were distributed in individual stainless-steel cages, at controlled temperature (22 ± 2 °C) and a 12 h photoperiod. The hemoglobin depletion/repletion method was used, to assess iron bioavailability.

In the depletion phase, which lasted 28 days, the animals received a modified standard AIN-93G diet (SD) (REEVES; NIELSEN; FAHEY, 1993), using iron-free mineral mix and deionized water, *ad libitum,* for the induction of iron deficiency. The animals' weight gain and food consumption were evaluated weekly. At the end of this period, a drop of blood was collected by caudal puncture, and hemoglobin was measured in all animals using commercial colorimetric kits (Labtest®, MG, Brazil). A group of eight animals was euthanized to determine the effects of iron deficiency on these animals (SD iron-free group). The remaining animals were redistributed into four groups so that the average levels of hemoglobin and weight were homogeneous among the groups.

In the repletion phase, ferrous sulfate and millet flour were used as iron sources. Millet flours were provided in different amount because the objective of our study was to control iron concentration in the diet (13.29ppm for each treatment) (DA SILVA et al., 2016a) to show iron bioavailability in each food matrix. The approximate composition of millet flours was analyzed according to Association of official analytical chemistry (AOAC, 2016), and iron concentration was analyzed according to Gomes et al. (1996) (**Supplementary Table 1)**. The chemical composition of the flours was used to calculate the ingredients added to the experimental diet, balancing iron concentration, macronutrients (carbohydrate, lipids, proteins) and dietary fiber **(Supplementary Table 2)**.

The four experimental groups used were: standard diet with ferrous sulfate (SD+FS, n=8), standard diet with cooked conventional millet flour (SD+CM, n=8), standard diet with cooked germinated millet flour (SD+ GM, n=8) and standard diet with extruded millet flour (SD+EM, n=8). The animals received deionized water *ad libitum* and a controlled and weighed diet daily, for a period of 21 days. In this phase, weight gain and food consumption were also monitored to calculate food efficiency ratio (FER [weight gain (g)/food consumption (g)] x 100) and weekly hemoglobin level. On the 50th

day, after a 12 h fast, the animals were anesthetized with isoflurane (Isoforine, Cristália®) and euthanized by cardiac puncture. Blood was collected and centrifuged at 1008 *g* for 15 min at 4 °C, for serum separation. Serum iron, ferritin and transferrin concentration were performed. Hemoglobin was analyzed in whole blood. Liver and intestine were collected to assess iron bioavailability, oxidative stress, intestinal morphology and fecal pH (**Supplementary Figure 1)**.

All experimental procedures with the animals were carried out in accordance with the ethical principles for animal experiments. The study was approved by the Ethics in the Use of Animals Committee of the Federal University of Viçosa (CEUA/UFV; process nº 05/2022).

2.4. Hematological Tests: Hemoglobin, Serum Iron, Ferritin and Transferrin

Serum hemoglobin was measured using the methemoglobin cyanide method, using a Labtest kit (Ref 43 hemoglobin and Ref 47 hemoglobin standard) for in vitro colorimetric diagnosis. To calculate the hemoglobin concentration of the blood samples, the reading value of the absorbance of a standard hemoglobin solution was used as reference. Serum iron, ferritin and transferrin analyses were performed using a specific kit, as recommended by the manufacturer (Bioclin®, Brazil).

2.5. Iron Concentration in the Liver of the Animals

The iron concentration in the liver of the animals was determined by atomic absorption spectrophotometry, according to Gomes; Silva; Silva, (1996).

2.6. Iron Bioavailability

Hemoglobin regeneration efficiency (HRE %) was determined using the formula: HRE% = [(final mg Fe Hb − initial mg Fe Hb)/100]/mg Fe consumed. The iron content in hemoglobin was estimated by: [body weight (g) × Hb (g L−1) × 0.335 × 6.7]/1000. This variable was calculated assuming that the average iron content in hemoglobin is 0.335 and the total blood volume is equal to 6.7% of the rats' body weight. RBV HRE was determined by the formula: HRE% / average HRE% of the group. Iron use was calculated as: [HRE% × % dietary iron]/100, according to Hernández et al., (2003).

2.7. Gene Expression of Proteins Involved in Iron Metabolism, Oxidative Stress and Inflammation

The expression of mRNA levels in the duodenal mucosa and the liver of proteins involved in iron metabolism, oxidative stress, and inflammation were analyzed by the real-time polymerase chain reaction (RT-qPCR) technique, using the Fast SYBER Green Master Mix from Applied Biosystems (Agilent Technologies Stratagene Products Division, Foster City, USA). Sense and antisense oligonucleotides (Sigma-Aldrich Ltda, Sao Paulo, Brazil) for divalent metal transporter protein (DMT1), duodenal cytochrome B (DcytB), ferroportin, and hephaestin in the duodenum and for hepatic proteins, such as ferritin, transferrin, superoxide dismutase (SOD), nuclear factor erythroid 2 (Nrf2) and tumor necrosis factor (TNF) were used. The relative expression of mRNA levels was normalized by the endogenous control β-actin (**Supplementary Table 3)**. Quantification was performed on the StepOnePlus™ Real-Time PCR System equipment (Thermo Fisher Scientific), using the SYBR-Green fluorescence quantification system and the Primer Express software system (Applied Biosystems, Foster City, CA).

2.8. Fecal pH

Approximately 0.4 g of the cecum content was homogenized in 4 mL of distilled water, using a vortex, for fecal pH analysis. Subsequently, the glass electrode of the pH meter (Bel Engineering®) was inserted to measure the fecal pH (GRANCIERI et al., 2017).

2.9. Histomorphometry Analysis of Duodenum Tissue

Semi-serial histological fragments of the duodenum, 3 μm thick, were obtained using an automated rotary microtome (Reichert-Jung®, Genossen, Germany) and stained using the hematoxylin and eosin technique. Twenty random fields were selected per animal to measure villus depth, villus thickness, goblet cells, and circular and longitudinal muscle layer thickness. The slides were examined under an Olympus BX43 light microscope, with a 4x objective (GOMES et al., 2022), and the images were processed with the ImagePro-Plus® software system, version 4.5 (Media Cybernetics, Rockville, USA).

2.10. Antioxidant Potential and Oxidative Stress

2.10.1. Total Antioxidant Capacity and Antioxidant Enzymes

A commercial antioxidant assay kit (Sigma-Aldrich®, St. Louis, Missouri, USA) was used to verify the liver's total antioxidant capacity (TAC), according to the manufacturer's specifications. The absorbance reading was performed at 405 nm, in a Multiskan™ GO spectrophotometer (Thermo Fisher Scientifics; Waltham, USA).

Superoxide dismutase (SOD) was determined using the methodology described by Marklund (1985). The homogenates were added to the reagents and incubated for 5 minutes, at 37ºC. The absorbance was read at 570 nm in a Multiskan™ GO spectrophotometer (Thermo Fisher Scientifics, Waltham, USA). The SOD enzymatic activity unit was defined as the amount of enzyme required for 50% inhibition of pyrogallol autoxidation, under test conditions.

The activity of the enzyme catalase (CAT) was determined based on its ability to cleave hydrogen peroxide (H_2O_2) into water and molecular oxygen, according to the description made by Aebi (1984). A blank was prepared for each sample. The absorbances were determined at 0, 30 and 60 s at 240 nm in a spectrophotometer (Model T70 + UV / VIS - PG Instruments Ltd). One unit (U) of catalase corresponds to the hydrolysis of 1 mol of H_2O_2 (ε=39.4 $L.mol^{-1}.cm^{-1}$) per minute. To calculate the CAT, the delta obtained from the initial absorbance (time 0) minus the final absorbance (60 s) was used and the result obtained was multiplied by the constant 2.361.

2.10.2. Malondialdehyde

Lipid peroxidation was determined by means of the thiobarbituric acid reactive substances (TBARS) assay using the method described by Buege and Aust (1978). The liver homogenate was added with a TBARS solution (15% trichloroacetic acid, 0.075% thiobarbituric acid and 0.25 M HCl), the mixtures were shaken and incubated in a water bath for 40 min at 90° C. After cooling, n-butanol was added and the tubes were shaken and centrifuged. The supernatant was collected and transferred to an ELISA plate where the absorbance was determined at 532 nm in a Multiskan™ GO spectrophotometer (Thermo Fisher Scientifics; Waltham, MA, USA). The final values were calculated using the coefficient of molar absorptivity (1.56x105).

2.10.3. Nitric oxide

NO concentration was determined using the Griess method (GREEN et al., 1982). The liver homogenate was mixed with solutions A (1% sulfanilamide in orthophosphoric acid (2.5% H3PO4)) and B (0.1% naphthyl-1ethylenediamide dihydrochloride in 2.5% H3PO4 (1:1)) and incubated in the dark for 10 min. The reading was at 570 nm in a Multiskan™ GO spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA). The final concentration was calculated according to a standard curve ranging from 100 to 0 μM of a solution of sodium nitrite (NaNO2) and phosphate buffer (pH 7.4) and the results were expressed in μmol.

2.11. Statistical Analyzes

The results of the biological assay were initially submitted to the Shapiro-Wilk and Kolmogorov-Smirnov normality tests and, later, the one-way ANOVA analysis of variance was applied, followed by the *post-hoc* Newman-Keuls test for parametric variables. In addition, the Student's t test was applied for comparisons between the conventional flour and the processing (germinated or extruded flour). The analyses were performed using the GraphPad Prism software system (GraphPad Software, San Diego, CA), version 9.0. The significance level established for all tests was 5%.

3. RESULTS

3.1. Effect of an Iron-deficient Diet on Animal Metabolism

The animals fed with iron-free diet (SD iron-free) presented increased ($p<0.05$) ferroportin and hephaestin gene expression **(Figure 1C and D)** in the intestine and ferritin gene expression **(Figure 1F)** in the liver and reduced ($p<0.05$) transferrin gene expression **(Figure 1E)** in the liver, compared to the animals fed with ferrous sulfate (SD+FS). The DCYTB and DMT1 gene expression **(Figure 1A and B)** did not differ ($p>0.05$) between the groups.

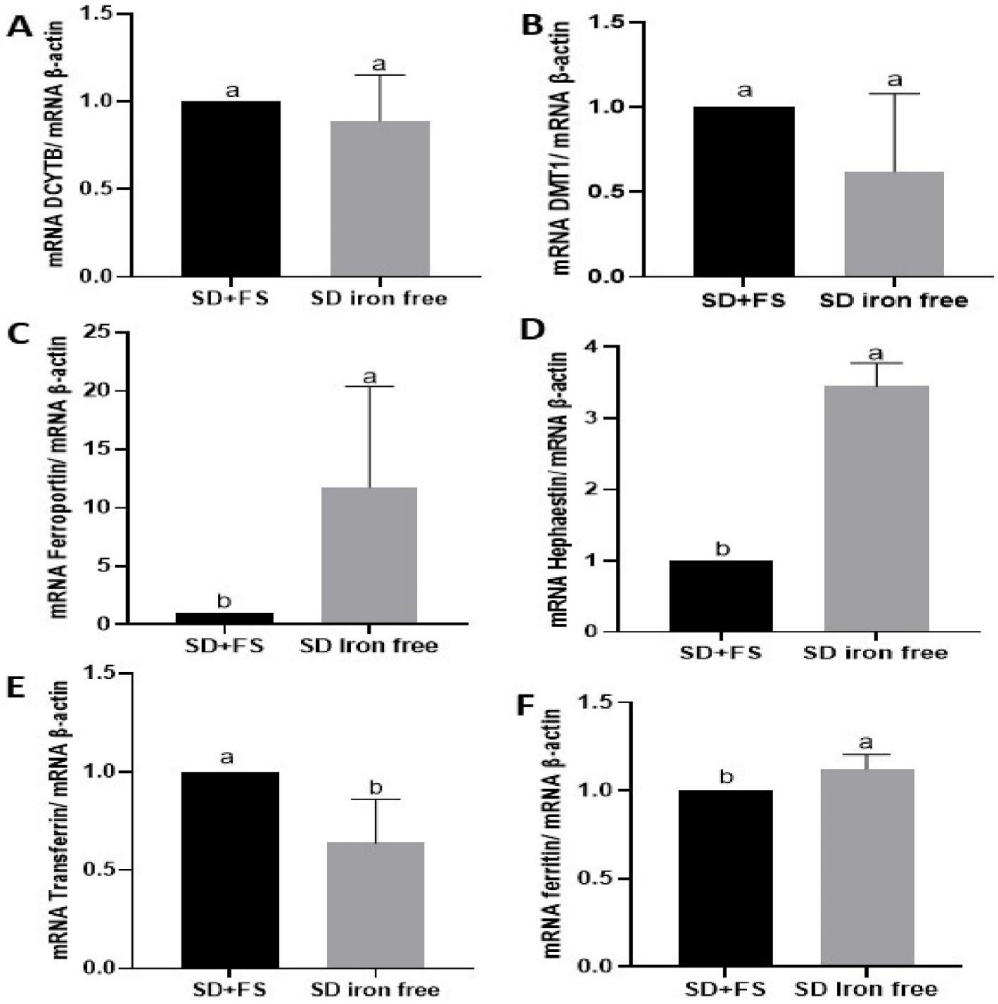

Fig. 1. Gene expression of proteins involved in iron metabolism in animals induced to iron deficiency and treated with ferrous sulfate. DCYTB: duodenal cytochrome B (A); DMT1: divalent metal transporter protein (B); ferroportin (C); hephaestin (D); transferrin (E) and ferritin (F). The mRNA of DCYTB, DMT1, ferroportin, and hephaestin were extracted from the intestine. The mRNA of transferrin and ferritin were extracted from the liver of rats and all of them were analyzed by RT-qPCR. Values expressed as mean and standard deviation (n = 5/group). Different letters indicate significant differences by the student's *t*-test, at 5% probability. SD + FS: standard diet + ferrous sulfate; SD iron free: standard diet without iron.

In addition, the animals in the SD iron free group also presented higher ($p<0.05$) concentration of MDA **(Figure 2A)** compared to the SD+FS group, while NO concentration, SOD and CAT activity, and liver TAC **(Figure 2B, C, D, and E)** did not differ ($p>0.05$) between the groups.

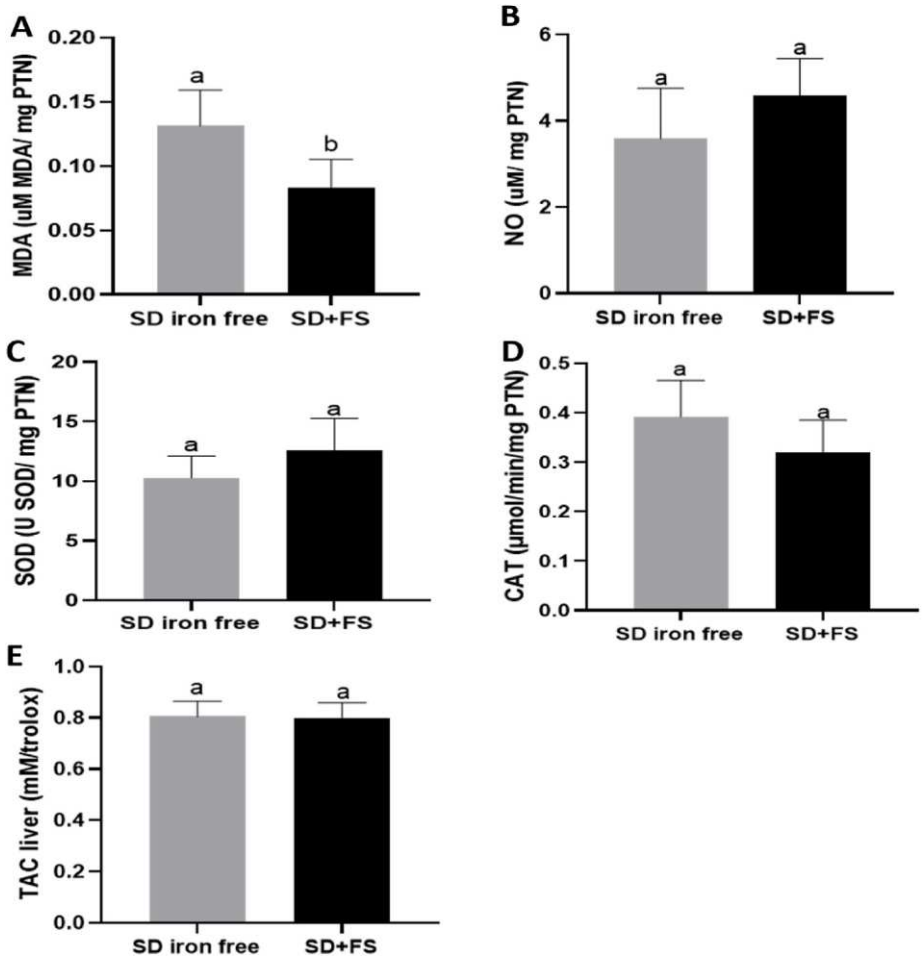

Fig. 2. Oxidative stress in animals induced to iron deficiency and treated with ferrous sulfate. MDA: malondialdehyde (A); NO: nitric oxide (B); SOD: superoxide dismutase (C); CAT: catalase (D); TAC liver: total antioxidant capacity of liver (E). Values expressed as mean and standard deviation (n = 8/group). Different letters indicate significant differences by the student's *t*-test, at 5% probability. SD iron free: standard diet without iron; SD + FS: standard diet + ferrous sulfate.

3.2. Effect of Pre-cooked Whole Grain Pearl Millet Flours on Food Consumption, Murinometric Variables and Hematological Tests

There was no difference ($p>0.05$) in total food consumption, iron intake, final body weight, FER or iron bioavailability, through HRE% and RBV HRE, among the experimental groups (**Supplementary Table 4)**. Regarding the hematological tests, the animals fed with millet flour (SD+CM; SD+GM and SD+EM) presented increased ($p<0.05$) serum iron concentration in relation to the control (SD+ FS). The animals fed with extruded millet presented the highest concentration ($p<0.05$) (**Figure 3D)**. Transferrin was higher ($p<0.05$) in the SD+EM group compared to the control group (SD+FS), but no significant difference was observed among the other groups (**Figure**

3E). Initial hemoglobin, final hemoglobin, liver iron and ferritin concentration did not differ among the groups **(Figure 3A, B, C and F)**.

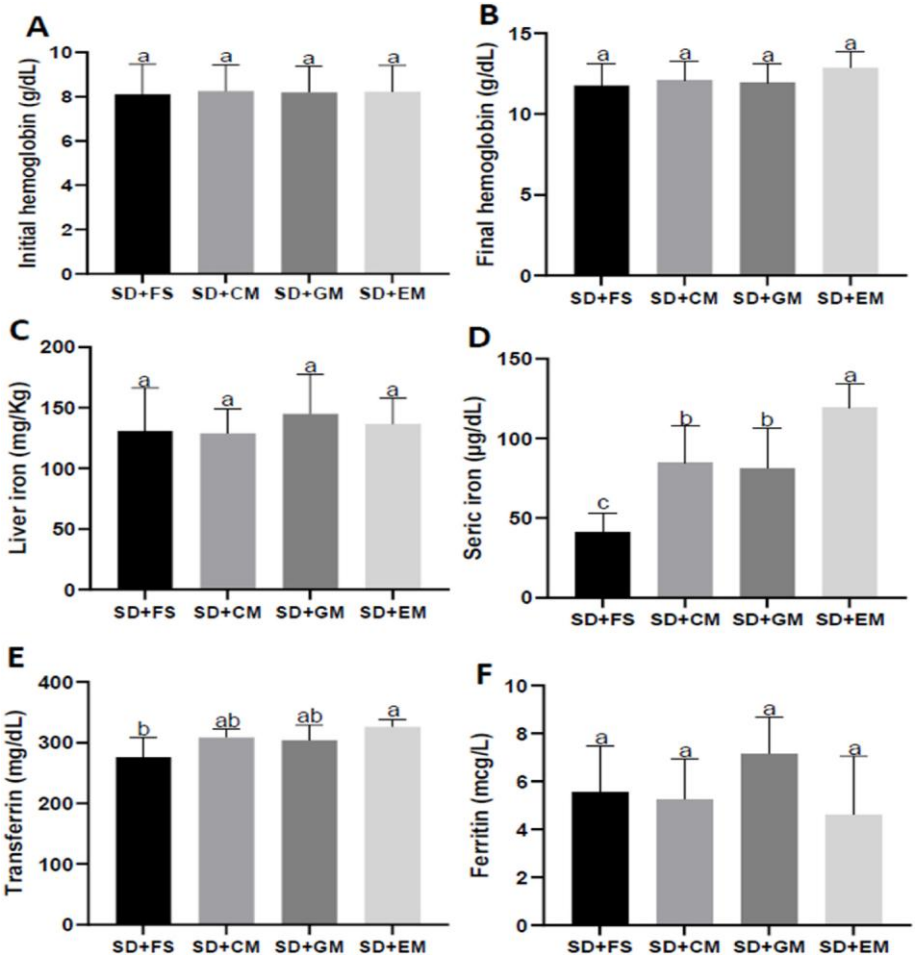

Fig. 3. Effect of millet flours submitted to different processing methods on hematological tests and liver iron, after 3 weeks of treatment. Initial hemoglobin (A); final hemoglobin (B); liver iron (C); seric iron (D); transferrin (E) and ferritin (F). Values expressed as mean and standard deviation (n = 8/group). Different letters indicate significant differences by the Newman-Keuls test, at 5% probability. SD + FS: standard diet + ferrous sulfate; SD + CM: standard diet + conventional millet flour; SD + GM: standard diet + Germinated millet flour; SD + EM: standard diet + extruded millet flour.

3.3. Effect of Pre-cooked Whole Grain Pearl Millet Flours on Gene Expression of Proteins Involved in Iron Metabolism

Regarding the gene expression of proteins involved in iron metabolism, the SD+GM group improved the efficiency of iron absorption by increasing ($p<0.05$) the gene expression of DcytB **(Figure 4A)**, ferroportin **(Figure 4C)** and hephastin **(Figure 4D)**

gene expression. SD+EM kept ($p>0.05$) the gene expression of DcytB and ferroportin **(Figure 4A and C)** and reduced ($p<0.05$) the expression of transferrin and ferritin **(Figure 4E and F)**, in relation to the SD+FS control group. The SD+CM group increased ($p<0.05$) the gene expression of ferritin **(Figure 4F)** and reduced ($p<0.05$) gene expression of ferroportin **(Figure 4C)** in relation to the other groups and kept ($p>0.05$) gene expression of DcytB **(Figure 4A)**, in relation to the SD+FS group. All test groups (SD+CM, SD+GM and SD+EM) increased ($p<0.05$) DMT1 and hephastin gene expression **(Figure 4B and D)** and reduced ($p<0.05$) transferrin gene expression **(Figure 4E)**, compared to the animals fed with ferrous sulfate.

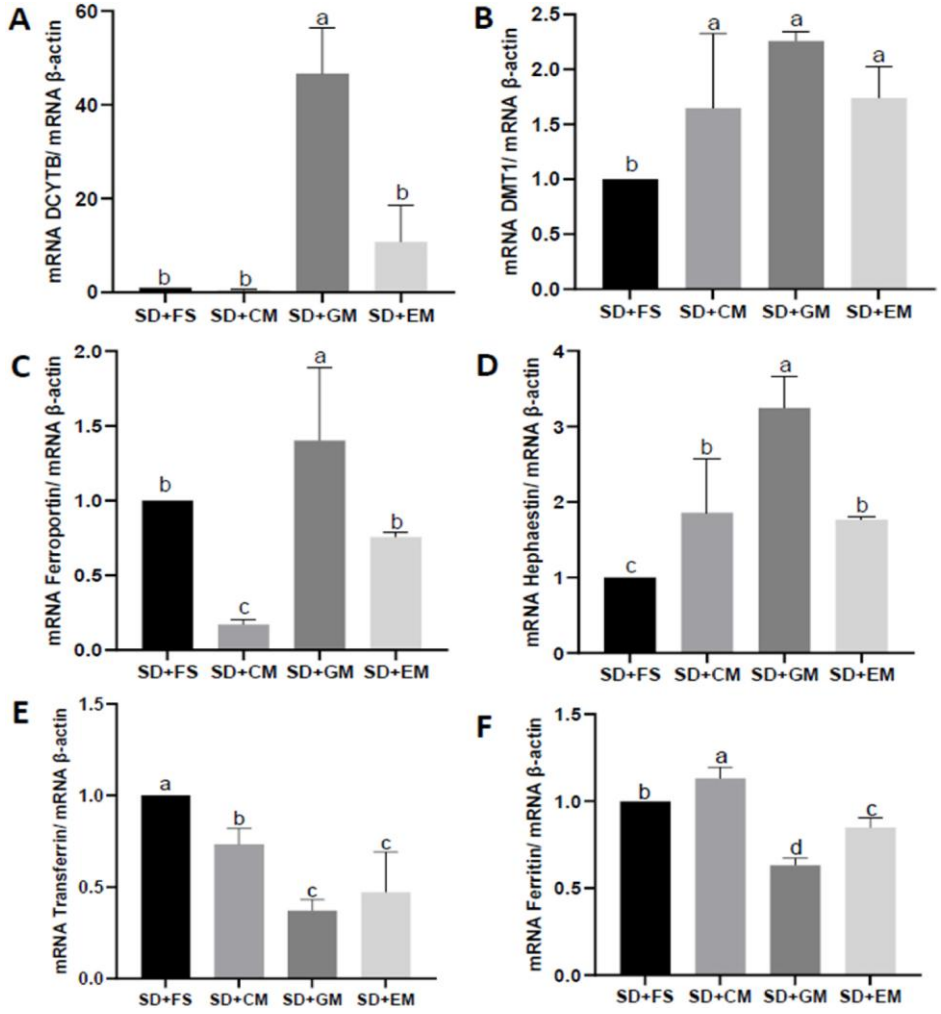

Fig. 4. Gene expression of proteins involved in iron metabolism in animals, after 3 weeks of treatment. DCYTB: duodenal cytochrome B (A); DMT1: divalent metal transporter protein (B); ferroportin (C); hephaestin (D); transferrin (E) and ferritin (F). The mRNA of DCYTB, DMT1, ferroportin, and hephaestin were extracted from the intestine and the mRNA of transferrin and ferritin were extracted from the liver of rats and all of them were analyzed by RT-qPCR. Values expressed as mean and standard deviation (n = 5/group). Different letters indicate significant

differences by the Newman-Keuls test, at 5% probability. SD + FS: standard diet + ferrous sulfate; SD + CM: standard diet + conventional millet flour; SD + GM: standard diet + Germinated millet flour; SD + EM: standard diet + extruded millet flour.

3.4. Effect of Pre-cooked Whole Grain Pearl Millet Flours on Histomorphometry Parameters and Fecal pH

Villus depth was higher ($p<0.05$) in the SD+CM group, compared to the SD+EM group (**Supplementary Table 5 and Supplementary figure 2)**. As for other variables, such as the thickness of circular and longitudinal muscle layers, goblet cell number and villus thickness, no significant difference ($p>0.05$) was observed among the groups (**Supplementary Table 5 and Supplementary figure 2)**.

The animals fed with the conventional and germinated flours (SD+CM and SD+GM) presented reduced ($p<0.05$) on fecal pH in relation to the animals fed with ferrous sulfate (SD+FS). This reduction was greater in the SD+GM group than in the other groups (**Supplementary Table 5)**. On the other hand, the group fed with extruded flour (SD+EM) had a higher ($p<0.05$) fecal pH compared to the SD+CM and SD+GM groups and was equal ($p>0.05$) to the control group (SD+FS) (**Supplementary Table 5)**.

3.5. Effect of Pre-cooked Whole Grain Pearl Millet Flours on Oxidative Stress and Inflammation

As for the gene expression of the proteins involved in oxidative stress and inflammation, Nrf2 and TNF were less expressed ($p<0.05$) in the groups fed with millet flours (SD+CM; SD+GM and SD+EM), compared to the control group (SD+FS) **(Figure 5A and C)**. SOD gene expression was higher ($p<0.05$) in the SD+CM group compared to the other groups **(Figure 5B)**.

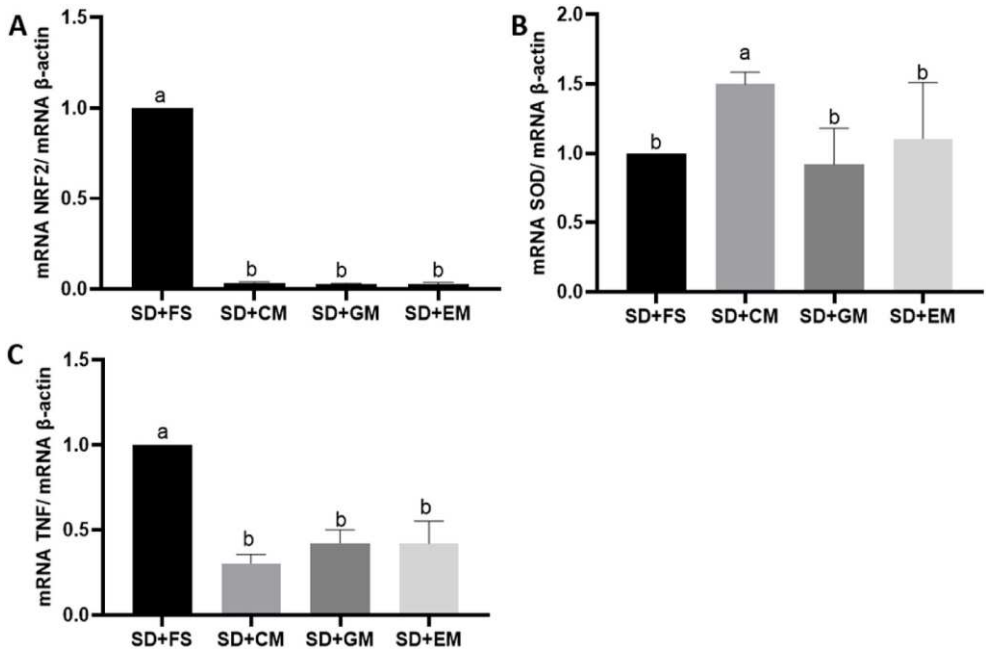

Fig. 5. Gene expression of proteins involved in oxidative stress and inflammation, after 3 weeks of treatment. Nrf2: nuclear factor erythroid 2; SOD: superoxide dismutase; TNF: tumor necrosis factor. Values expressed as mean and standard deviation (n = 5/group). Different letters indicate significant differences by the Newman-Keuls test, at 5% probability. SD + FS: standard diet + ferrous sulfate; SD + CM: standard diet + conventional millet flour; SD + GM: standard diet + Germinated millet flour; SD + EM: standard diet + extruded millet flour.

The concentration of MDA and NO were higher ($p<0.05$) in the control group (SD+FS), and the groups fed with millet flour (SD+CM; SD+GM and SD+EM) were able to reduce these markers of oxidative stress **(Figure 6A and B)**. The antioxidant enzymes SOD and CAT presented greater activity ($p<0.05$) in the control group (SD+FS), but were reduced in the groups fed with millet flours (SD+CM; SD+GM and SD+EM) **(Figure 6C and D).** The SD+CM group presented higher SOD expression **(Figure 5B)**, but it did not interfere with the quantification of this enzyme, which remained lower in relation to the control group (SD+FS) **(Figures 6C)**. Conventional and germinated millet maintained ($p>0.05$) the total antioxidant capacity of the liver. However, extruded millet reduced it ($p<0.05$), compared to the control group **(Figure 6E)**.

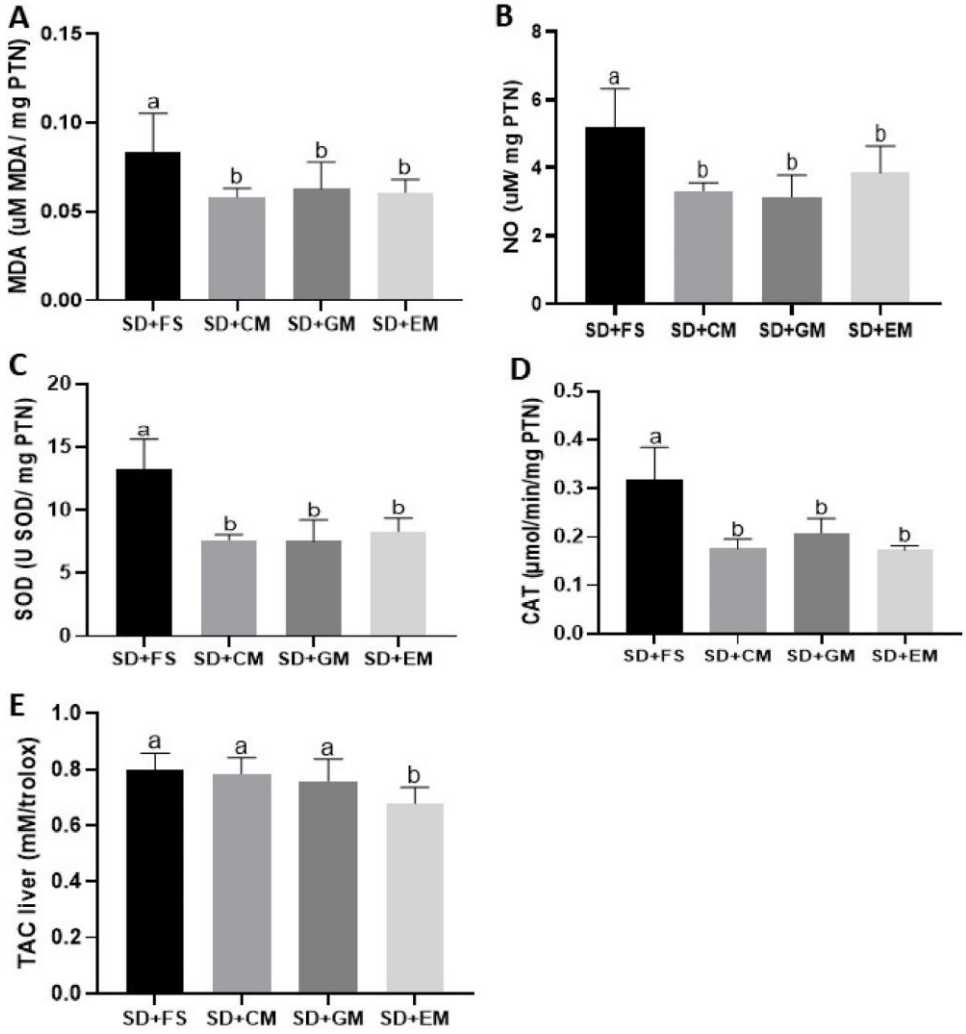

Fig. 6. Effect of pearl millet flour submitted to different processes on oxidative stress after 3 weeks of treatment. MDA: malondialdehyde (A); NO: nitric oxide (B); SOD: superoxide dismutase (C); CAT: catalase (D); TAC liver: total antioxidant capacity of liver (E). Values expressed as mean and standard deviation (n = 8/group). Different letters indicate significant differences by the Newman-Keuls test, at 5% probability. SD + FS: standard diet + ferrous sulfate; SD + CM: standard diet + conventional millet flour; SD + GM: standard diet + Germinated millet flour; SD + EM: standard diet + extruded millet flour.

4. DISCUSSION

The present study aimed to evaluate the effect of the consumption of whole grain cooked pearl millet flour submitted to different processes, namely, conventional, germinated and extruded, on markers of iron metabolism, morphometric parameters, antioxidant capacity and inflammation in *Wistar* rats induced to iron deficiency. In view of this, the results indicate that pearl millet flours have good iron bioavailability. However, germination stood out for improving the absorptive efficiency of iron, through the higher gene expression of DcytB, ferroportin and hephaestin. In addition, germination was able to maintain the serum ferritin concentration similar to that of the other groups

and reduce inflammation, compared to ferrous sulfate, and improve the antioxidant potential in relation to extrusion. These results may encourage the increase of human consumption of millet and provides options to maximize access to nutrients and health benefits to reduce the risk of iron deficiency.

The depletion phase (28 days) was enough to cause iron deficiency in the animals. The average hemoglobin content of the groups was 8.19 g/dL. The body regulates iron concentrations by controlling both the threat of inadequate supply and uncontrolled excess iron (NEMETH; GANZ, 2021). Therefore, in the depletion phase, the animals fed with iron-free diet (SD iron-free) increased the gene expression of ferroportin and hephaestin proteins in an attempt to improve iron absorption. On the other hand, the higher expression of ferritin may indicate inflammation, while the increase of MDA in this group may point to oxidative stress in these animals. In this case, it seems that ferritin levels are elevated in the body. This can occur either directly, with ferritin acting as a positive acute phase protein that sequesters and stores iron intracellularly to prevent iron excess availability in the body, or indirectly, when ferritin synthesis occurs secondarily through immune stimulation by inflammatory cytokines (MAHROUM et al., 2022).

The experimental diets of the repletion phase presented approximately 13.29 mg of Fe per kg. After this phase (21 days), the total diet consumed, iron intake and murinometric variables did not differ among the groups. The groups fed with millet flours (SD+CM, SD+GM and SD+EM) presented hemoglobin gain, hemoglobin regeneration efficiency and biological value related to the hemoglobin regeneration efficiency similar to that of the control group (SD+FS). It indicates good bioavailability of iron in millet flours, which was able to keep normal metabolism of animals.

In addition, the animals in the SD+GM group reduced the fecal pH, which despite not being an acidic pH, may have contributed to improve the intestinal microbiome, improving the brush bord membrane (BBM) functionality (RUSU et al., 2020), since our results showed higher expression of apical and basolateral membrane proteins of the duodenum, DcytB, ferroportin, and hephaestin, increasing the reduction and solubility of iron, as well as its absorptive efficiency. However, the expression of transport proteins, such as transferrin, and storage proteins, such as ferritin, decreased in animals fed with germinated flour, although, the quantification of these proteins did not differ from that of the animals fed with ferrous sulfate and conventional millet, which reveals similarity to the controls. Germination reduced phytic acid (0.63 ± 0.01) compared to the conventional flour (0.77 ± 0.01) and extruded flour (0.77 ± 0.01). This phytochemical is an inhibitor of

iron bioavailability (Sihag et al., 2016). Thus, this processing made the iron in the SD+GM group more bioavailable, which may have improved the gene expression of the proteins involved in the absorption of this mineral. On the other hand, hepatic iron concentration, as well hemoglobin and ferritin serum levels did not differ among groups. It may be due to the experiment time, which was not enough to detect differences among the flours, since these proteins are the last to be changed during iron metabolism (GAO et al., 2019).

Despite their reduced gene expression of ferritin and hepatic transferrin, the animals fed with extruded millet (SD+EM) presented higher levels of iron and transferrin in the blood. The extrusion process reduced the total dietary fiber (EM: 7.96% compared to CM: 11.88% and GM: 11.91%) and resistant starch (EM: 0.32% compared to CM: 3.38% and GM: 3.53%) in the millet flour, which may reduce iron- link-interaction compounds in the food matrix (KIEWLICZ; RYBICKA, 2020), make it more available, increasing the iron uptake (DMT1) and transportation (transferrin), reflecting in improvement in iron serum status.

Iron from conventional millet flour was possibly used primarily for storage in the liver in the form of ferritin. This can be suggested by the higher gene expression of ferritin in the liver and lower ferroportin in the enterocytes of animals fed with this flour (SD+CM). Free iron, when not used in metabolic processes, can be incorporated into ferritin, which is an iron storage protein that functions as the main cellular storage of iron in the body (PENG; UPRICHARD, 2017). In addition, the conventional millet flour increased the gene expression of transferrin in the liver. The quantification of transferrin and ferritin in the blood was similar to that in the control group (SD+FS), which demonstrates that the transport and serum storage of iron from this flour are equal to ferrous sulfate (GAO et al., 2019; PENG; UPRICHARD, 2017).

The higher depth of villi in the SD+CM group compared to the SD+EM group may be due to the greater amount of soluble dietary fiber in the conventional millet flour matrix (CM: 1.43%; GM: 0.49%; EM:1.14%) , which leads to higher consumption (0.45 ± 0.01 g), compared to extruded millet (0.36 ± 0.00 g), also working as a prebiotic and helping intestinal function (GOMES et al., 2021). In addition, these variables are related to improved iron bioavailability, since they increase the absorption surface in the intestine by stimulating the proliferation of epithelial cells (RUSU et al., 2020). Thus, it is possible to infer that the SD+CM group increased villus depth to improve iron absorption.

The animals fed with millet flour (SD+CM, SD+GM and SD+EM) increased the expression of DMT1, the protein that captures iron into enterocytes, which reveals that iron from millet was more absorbed than ferrous sulfate. This is corroborated by the results of MDA, NO and TNF, which presented higher oxidative stress and inflammation in the SD+FS group. In addition, the higher Nrf2 gene expression and SOD and CAT activity in this group indicate, up regulated pathway to synthetize antioxidant endogenous proteins, which reveals greater need for the activation of these proteins in this group. In addition, unabsorbed iron can accumulate in the intestine, and lead to the formation of reactive oxygen species, since it is highly reactive, thus favoring oxidative stress and inflammation (MALESZA et al., 2022).

The groups fed with millet flour (conventional, germinated and extruded) presented reduced MDA and NO concentration and TNF gene expression, which indicates that the flours of pearl millet were able to reduce oxidative stress and inflammation. The lower gene expression of Nrf2 and SOD and CAT activity in millet flour groups may be attributed to the presence of bioactive compounds, such as phenolic compounds present in these flours, mainly flavonoids and phenolic acids such as ferulic and p-coumaric acids (DIAS-MARTINS et al., 2018), without the need to activate the endogenous defense in animals through antioxidant enzymes (THEODORO et al., 2021a). These compounds can regulate immunity by interfering with the regulation of immune cells, reducing the synthesis of pro-inflammatory cytokines and inhibiting the enzymes that generate reactive species, such as NADPH oxidase (NOX) and xanthine oxidase, and improving antioxidant activity in cells (YAHFOUFI et al., 2018). In addition, regarding the processing, germination stands out once again, since extrusion cooking reduced liver TAC, possibly due to lower consumption of total phenolic compounds (0.58 ± 0.00 in the SD+EM group, compared to 0.83 ± 0.00 in the SD+GM group).

In addition, the germinated flour increased the gene expression of ferroportin, decreased the gene expression of TNF, which possibly reduces the hepcidin hormone, also regulating iron uptake. The inflammation increase hepcidin, block the ferroportin, the protein involved in iron uptake in basolateral membrane of enterocyte, which causes its internalization and degradation consequently reducing the transfer of iron to the bloodstream and promoting a protective inflammation effect on the body (NEMETH; GANZ, 2021). Thus, the results of the present study possibly indicate that, by improving the antioxidant effect and inflammation, germinated millet flour increased the expression of DcytB proteins, allowing a better reduction of Fe^{3+} to Fe^{2+} and favoring its capture by

enterocytes by DMT1, which transports iron through the enterocyte, and of ferroportin, which acts to export Fe^{2+} from enterocytes through the basolateral membrane (GAO et al., 2019). These increased proteins by GM reduced the harmful effects of iron deficiency, hence improving iron metabolism.

Then, regardless of the processing, pearl millet provides good iron bioavailability in rats, equal to or even better than ferrous sulfate. Besides improving oxidative stress and inflammation, millet flours demonstrated trophic effect on the enterocyte apical and basolateral membrane, which improved its intestinal functionality in relation to ferrous sulfate.

Regarding the processing, germination stands out, since, in addition to improving the efficiency of iron uptake in the apical and basolateral membrane of enterocyte, this cooked germinated pearl millet flour improved the total antioxidant capacity in the liver in animals, compared to extruded flour.

This study highlights as a strong point the use of experimental diets with the same iron concentration, demonstrating that the results are linked to differences in iron bioavailability in each matrix. Furthermore, this is the first study to evaluate the effect of cooked millet grains and their different types of processing (germination and extrusion) on iron metabolism in a living organism, becoming it a good option for using millet grain with health benefits. On the other hand, our study design was in a controlled environment and diet, which did not reflect iron metabolism in free-living individuals.

5. CONCLUSION

Pre-cooked whole grain pearl millet flours presented good iron bioavailability. In the processing, germination stands out, as it improves iron metabolism in relation to extrusion and is similar to conventional millet in terms of antioxidant capacity. In addition, millet flours improved inflammation and oxidative stress relative to ferrous sulfate, thus promoting iron uptake and intestinal functionality.

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

CRediT authorship contribution statement: Jaqueline Maciel Vieira Theodoro: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. B´arbara Pereira da Silva: Writing – review & editing, Methodology. Renata Celi Lopes Toledo: Formal analysis, Data curation. Mariana Grancieri: Writing – review & editing. Pietra Vidal Cardoso do Prado: Formal analysis. Izabela Maria Montezano de Carvalho: Writing – review & editing. Carlos Wanderlei Piler Carvalho: Supervision, Resources. H´ercia Stampini Duarte Martino: Writing – review & editing, Writing – original draft, Supervision, Project administration, Conceptualization.

Declaration of competing interest: 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.

Data availability: Data will be made available on request.

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Appendix A. Supplementary data: Supplementary data to this article can be found online at https://doi. org/10.1016/j.jcs.2023.103840.

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Paper 3

Supplementary material

Tables:

Table 1. Chemical composition of millet flour (g/100 g^{-1}).

Variables**	Conventional millet	Germinated millet	Extruded millet
Carbohydrate	68.24	67.68	74.87
Protein	11.16	11.46	10.97
Total fibers	11.88	11.91	7.96
Insoluble fiber	10.45	11.43	6.82
Soluble fiber	1.43	0.49	1.14
Lipids	5.58	5.03	2.82
Iron	0.45	0.42	0.56

**Values expressed in wet matter. Samples analyzed according to the methodology of (AOAC, 2016).

Table 2. Composition of the experimental diets.

	Depletion phase	Repletion phase			
Ingredients (g kg^{-1})	**SD iron free**	**SD + FS**	**SD + Conventional millet**	**SD + Germinated millet**	**SD + Extruded millet**
Ferrous sulphate	-	0.04	-	-	-
Conventional millet	-	-	267.8	-	-
Germinated millet	-	-	-	287.8	-
Extruded millet	-	-	-	-	212.8
Albumin*	254.4	254.4	216.3	212.4	224.7
Maize starch	346.1	346.1	163.1	149.0	186.0
Dextrinized starch	132.0	132.0	132.0	132.0	132.0
Sucrose	100.0	100.0	100.0	100.0	100.0
Soy oil	70.0	70.0	55.1	55.5	64,0
Cellulose	50.0	50.0	18.2	15.7	33.1
Iron free mineral mix	35.0	35.0	35.0	35.0	35.0
Vitamin mix	10.0	10.0	10.0	10.0	10.0
Choline bitartrate	2.5	2.5	2.5	2.5	2.5
Phytate/iron ration			14.5	12.8	11.5
Composition of diets					
Carbohydrate (%)	61.8	61.8	61.8	61.7	61.8
Protein (%)	21.4	21.4	21.4	21.4	21.4
Lipids (%)	7.0	7.0	7.0	7.0	7.0
Caloric density (kcal	3.7	3.7	3.7	3.7	3.7

Composition of the experimental diets based on the standard rodent diet (AIN-93G) (Reeves et al., 1993). SD: standard diet; SD + FS: standard diet (AIN-93G) + ferrous sulfate; SD + Conventional millet: standard diet + cooked conventional millet flour; SD

+ Germinated millet: standard diet + cooked germinated millet flour; SD + Extruded millet: standard diet + extruded millet flour. *Albumin based on 78.63% protein content.

Table 3: Sequence of oligonucleotides used for RT-qPCR.

Gene	Sense	Antisense
DMT-1	CTGATTTACAGTCTGGAGCAG	CACTTCAGCAAGGTGCAA
DcytB	TGCAGACGCAGAGTTAAGCA	CCGTGAAGTATACCGGCTCC
Ferroportin	TTCCGCACTTTTCGAGATGG	TACAGTCGAAGCCCAGGACCGT
Hephaestin	GGCACAGTTACAGGGCAGAT	AGTAACGTGGCAGTGCATCA
Ferritin	CAGCCGCCCTTACAAGTCCTCT	ATGGAGCTAACCGCGAAGAC
Transferrin	AGCTGCCACCTGAGAACATC	CGCACGCCCTTTATTCATGG
SOD	GAGCAGAAGGCAAGCGGT	CCACATTGCCCAGGTCTC
Nrf2	CACATCCAGACAGACACCAGT	CTACAAATGGGAATGTCTCTGC
TNF	TCAGTTCCATGGCCCAGAC	GTTGTCTTTGAGATCCATGCCATT
β-actin	GTCGTACCACTGGCATTGTG	TCAGCTGTGGTGGTGAA

DMT-1: divalent metal transporter protein; DcytB: duodenal cytochrome B; SOD: superoxide dismutase; Nrf2: nuclear factor erythroid 2; TNF: tumor necrosis factor.

Table 4: Effect of millet flours submitted to different processing methods on food consumption and murinometric variables, after 3 weeks of treatment.

Variables	SD+FS	SD+CM	SD+GM	SD+EM
Total food consumption (g)	319.5 ± 3.7^a	319.2 ± 6.7^a	319.8 ± 3.6^a	316.6 ± 3.7^a
Iron intake (g)	4.2 ± 0.1^a	4.2 ± 0.1^a	4.2 ± 0.0^a	4.2 ± 0.1^a
Final body weight (g)	284.0 ± 19.0^a	284.0 ± 12.5^a	286.7 ± 11.1^a	289.8 ± 21.1^a
FER %	0.1 ± 0.0^a	0.1 ± 0.0^a	0.1 ± 0.0^a	0.1 ± 0.0^a
HRE %	64.8 ± 7.7^a	67.4 ± 16.2^a	67.9 ± 13.4^a	77.8 ± 19.4^a
RBV HRE	1.0 ± 0.2^a	1.1 ± 0.3^a	1.0 ± 0.3^a	1.3 ± 0.4^a

Values expressed as mean and standard deviation (n=8/group). Different letters on the lines indicate significant differences by the Newman-Keuls test, at 5% probability. FER: feed efficiency ratio; HRE: hemoglobin regeneration efficiency; RBV HRE: biological value of hemoglobin regeneration efficiency. SD+FS: standard diet + ferrous sulfate;

SD+CM: standard diet + conventional millet flour; SD+GM: standard diet + Germinated millet flour; SD+ EM: standard diet + extruded millet flour.

Table 5. Effect of millet flour submitted to a different process on the histomorphometry of the duodenum and fecal pH, after 3 weeks of treatment.

Variables (µM)	**SD+FS**	**SD+CM**	**SD+GM**	**SD+EM**
Circular muscle layer thickness	109.9 ± 19.5^{a}	120.6 ± 16.2^{a}	103.6 ± 12.3^{a}	122.8 ± 16.6^{a}
Longitudinal muscle layer thickness	77.3 ± 19.6^{a}	75.0 ± 12.4^{a}	70.7 ± 10.0^{a}	72.6 ± 6.2^{a}
Villus depth	342.9 ± 48.5^{a}	384.3 ± 10.6^{a*}	370.5 ± 32.5^{a}	327.4 ± 31.2^{a*}
Villus thickness	57.1 ± 8.3^{a}	53.1 ± 5.0^{a}	50.7 ± 4.7^{a}	51.2 ± 3.2^{a}
Number of goblet cells	7.0 ± 0.9^{a}	8.1 ± 1.7^{a}	8.0 ± 1.9^{a}	8.5 ± 0.6^{a}
Fecal pH	8.6 ± 0.2^{a}	8.1 ± 0.3^{b}	7.7 ± 0.2^{c}	8.4 ± 0.1^{a}

Values expressed as mean and standard deviation (n=6/group). Different letters on the lines indicate significant differences by the Newman-Keuls test, while asterisks indicate significant difference by the t-test, at 5% probability. SD+FS: standard diet + ferrous sulfate; SD+CM: standard diet + conventional millet flour; SD+GM: standard diet + germinated millet flour; SD+ EM: standard diet + extruded millet flour.

Figures:

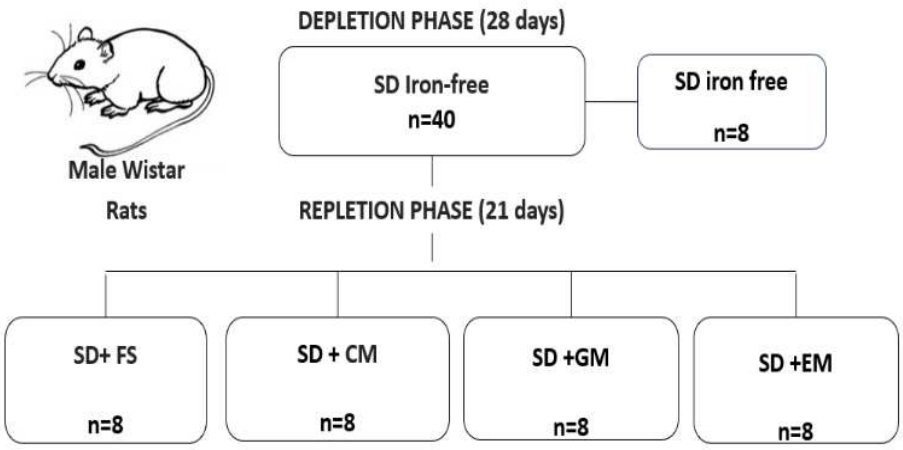

Figure 1. Experimental design. Depletion phase (28 days): the animals received iron-free modified standard diet (SD), for the induction of iron deficiency. Eight animals were euthanized to determine the effects of iron deficiency (SD iron free group). Repletion phase (21 days): the animals were separated into 4 experimental groups (n = 8/group) receiving 13.29 ppm of Fe/kg of diet: SD+FS: standard diet + ferrous sulfate (control diet); SD+CM: standard diet + conventional millet flour; SD+GM: standard diet + Germinated millet flour; SD+ EM: standard diet + extruded millet flour test diets.

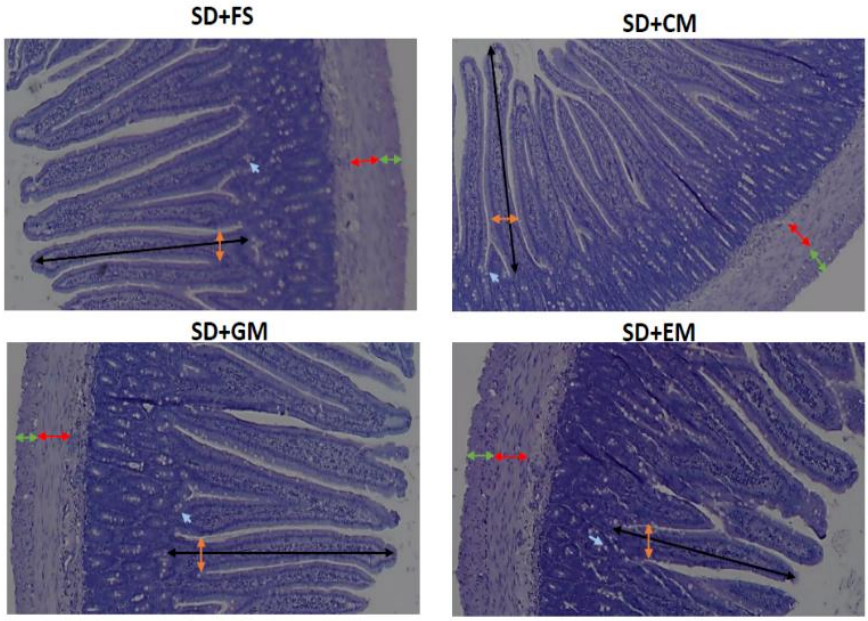

Figure 2. Effect of millet flours on the histomorphometry of the duodenum. Black arrows refer to villus depth, orange arrows refer to villus thickness, red arrows refer to circular muscle, green arrows refer to longitudinal muscle and blue arrows refer to the number of goblet cells, after 3 weeks of treatment. SD+FS: standard diet + ferrous sulfate; SD+CM: standard diet + conventional millet flour; SD+GM: standard diet + Germinated millet flour; SD+ EM: standard diet + extruded millet flour. Staining was carried out with hematoxylin and eosin. Objective magnitude: 4x.

8.4. Paper 4: Processed pearl millet improves the morphology and gut microbiota in Wistar rats

foods

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Article

Processed Pearl Millet Improves the Morphology and Gut Microbiota in Wistar Rats

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Abstract

This study evaluated the effect of pearl millet subjected to different processing on the intestinal health of rats. The animals were fed a standard iron-free diet (28 days) (hemoglobin depletion: 8.65 + 1.40 g/dL of hemoglobin). Subsequently, they were divided into four groups for hemoglobin repletion (21 days): standard diet + ferrous sulfate (SD + FS); standard diet + non-germinated open-pan cooked millet flour (SD + NGOPCMF); standard diet + germinated open-pan cooked millet flour (SD + GOPCMF); and standard diet + extrusion-cooked millet flour (SD + ECMF). Hemoglobin level did not differ among groups. The SD + NGOPCMF, SD + GOPCMF and SD + ECMF groups demonstrated a higher Chao index in the microbiome and a higher number and area of goblet cells and longitudinal muscle layer width. The SD + NGOPCMF and SD + GOPCMF groups demonstrated increased cecum weight, crypt depth, crypt thickness, and circular muscle layer width; lower fecal pH; and a higher relative abundance of Bacteroidota, while the SD + FS group showed the highest abundance of Actinobacteriota. The SD + GOPCMF group stood out for showing the lowest fecal pH, better α-diversity (Chao and Shannon index), and the highest width of the longitudinal muscle layer. In conclusion, pearl millet subjected to different processing, mainly germination, has the potential to improve the composition of the intestinal microbiota and the intestinal morphology in rats induced to iron deficiency.

Keywords: extrusion cooking; germination; hemoglobin depletion/repletion; intestinal health

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1. INTRODUCTION

Iron is an essential nutrient for both the host and the microbiome (PARMANAND et al., 2019). Iron deficiency can inhibit important metabolic processes, impair bacterial growth, and disrupt the organism's homeostasis (BOTTA et al., 2021; PARMANAND et al., 2019). On the other hand, excessive iron levels can induce oxidative stress and

promote the proliferation of pathogenic microorganisms (PHIPPS et al., 2020). Therefore, maintaining iron levels without toxicity is vital for optimal health.

One of the strategies to combat iron deficiency or anemia is the use of iron salts with high bioavailability. However, this approach is often associated with adverse gastrointestinal effects, including constipation, gastric irritation, nausea, and metallic taste (AUERBACH; SCHRIER, 2017). Moreover, the intake of iron salts may exacerbate inflammation and oxidative stress, since iron can generate free radicals that form reactive oxygen species (ROS), and also serves as an essential growth factor for potentially pathogenic bacteria, which can lead to dysbiosis and damage to intestinal health (MALESZA et al., 2022; TOLKIEN et al., 2015). In developing countries, access to animal-derived products (meat, fish and eggs) is limited, making cereals and legumes the primary dietary sources of iron. Therefore, diversifying staple foods by incorporating crops that are natural sources of iron, such as millet, is essential, in addition to being an option for vegans, vegetarians and flexitarians (ANITHA et al., 2021; VETRIVENTHAN et al., 2021). Millet ranks sixth among the most produced crops in the world, with an area of 30 million hectares in the arid and semiarid tropical regions of Asia and Africa (SATYAVATHI et al., 2021). Although it is a good source of iron, the availability of this mineral in millet is influenced by the presence of non-heme iron, which is less easily absorbed compared to the heme iron found in animal products (GANNON; GLAHN; MEHTA, 2021). On the other hand, in addition to minerals, especially calcium (300 mg/kg), zinc (43 mg/kg) and iron (18 mg/kg), pearl millet (*Pennisetum glaucum* (L.) R. Br.) is a valuable source of protein (8 to 11%), dietary fiber (7 to 12%), resistant starch (average con- tent of 3%), and other bioactive compounds, such as polyphenols (Diosmin and Cyanidin) and fatty acids (mainly polyunsaturated), which confer several health benefits, including promoting intestinal health, which can favor nutrient absorption (DIAS-MARTINS et al., 2018; THEODORO et al., 2024).

The inclusion of pearl millet as a strategy to mitigate metabolic changes that are harmful to the body's homeostasis has been investigated, and research has demonstrated its potential antioxidant and anti-inflammatory properties, as well as its benefits for intestinal health in animals (THEODORO et al., 2021a, 2022, 2024b), in addition to the glucose-lowering effect in individuals with impaired glucose tolerance (REN et al., 2018). Furthermore, studies have also demonstrated that certain processing techniques, such as germination and extrusion cooking, can enhance the nutritional quality of this cereal (OWHERUO; IFESAN; KOLAWOLE, 2019;

PESSANHA et al., 2023; THEODORO et al., 2024) and its effect on glycemic and insulinemic control, food intake and appetite sensation in eutrophic adults (MAGALHÃES et al., 2024). However, pearl millet processed by germination and extrusion acting on intestinal health has not yet been evaluated. Therefore, the present study aimed to evaluate the effect of pearl millet subjected to different processing methods on the intestinal health of iron-deficient rats, using the hemoglobin depletion/repletion model. The hypothesis is that non-germinated, germinated and extruded pre-cooked millet flours, as a source of iron and bioactive compounds, would improve the composition of the intestinal microbiota and the morphology of the intestine in rats induced to iron deficiency.

2. MATERIAL AND METHODS

Processing of millet grains and flour preparation

Pearl millet grains (*Pennisetum glaucum* (L.) R. Br.), hybrid ADRg 9070, kindly donated by Atto Sementes (Rondonopolis, Brazil), harvested during the second growing season of 2021 in Itiquira, Mato Grosso, Brazil, were used.

The grains were germinated under controlled conditions of temperature (30° ± 2 °C) and humidity (90%) for 24 h (National Mfg. Co., Lincoln, USA), and subsequently dried at 50 °C for 4 h (Macanuda Hauber, Joinville, Brazil) (THEODORO et al., 2021a). The extrusion process was performed in an Evolum HT25 (Clextral Inc., Firminy, France) twin-screw co-rotating extruder with a screw diameter of 25 mm and length-to-diameter ratio of 40:1. The following temperature profile of the ten heating zones were used, from feeding to the output: 25, 40, 50, 90, 100, 110, 110, 110, 120, 120 °C and feed rate and moisture were kept constants at 14% (injection water pump) and 10 kg/h (gravimetric feeder), respectively. The puffed extrudates were then dried at 60 °C for 2 h in a 400/6ND ventilated oven (Nova Ética®, São Paulo, Brazil) (THEODORO et al., 2024a).

The heat treatment of the ungerminated and germinated millet grains took place in a pan (180 ◦C/40 min) and then they were dried (50 ◦C/24 h) (Nova Ética®, São Paulo, Brazil) (DIAS-MARTINS et al., 2018; THEODORO et al., 2024). To produce the flours, the pre-cooked whole grains were ground in a mill equipped with a 1 mm sieve (TREU, Rio de Janeiro, Brazil), operating at a capacity of 500 kg/h. The flours were then stored in a sealed plastic bag at −20 ◦C ± 1 ◦C until analysis.

The flours were designated as follows: non-germinated open-pan cooked millet flour (NGOPCMF: 68.24% carbohydrates, 11.16% protein, 11.88% total dietary fiber, 10.45% insoluble fiber, 1.43% soluble fiber, 3.38 g/100 g resistant starch, 5.58% lipids, and 4.49 mg/100 g iron), germinated open-pan cooked millet flour (GOPCMF: 67.68% carbohydrates, 11.46% protein, 11.91% total dietary fiber, 11.43% insoluble fiber, 0.49% soluble fiber, 3.53 g/100 g resistant starch, 5.03% lipids, and 4.18 mg/100 g iron) and extrusion-cooked millet flour (ECMF: 74.87% carbohydrates, 10.97% protein, 7.96% total dietary fiber, 6.82% insoluble fiber. 1.14% soluble fiber, 0.32 g/100 g resistant starch, 2.82% lipids and 5.64 mg/100 g iron).

Experimental Design

Thirty-two male *Wistar* rats (*Rattus norvegicus*), 21 days old, were distributed in individual stainless-steel cages, with controlled temperature conditions (22 ± 2 °C) and a 12 h photoperiod. The rats were induced to iron deficiency through the hemoglobin depletion/repletion method. The animals were fed an AIN-93G standard diet (SD) (REEVES; NIELSEN; FAHEY, 1993), containing an iron-free mineral mix, to deplete hemoglobin levels and induce iron deficiency (28 days). After this phase, the hemoglobin concentration was evaluated by the colorimetric assay kit (Labtest®, Lagoa Santa, MG, Brazil). Subsequently, during the repletion phase, the animals were randomly assigned to four groups for treatment groups, ensuring homogeneity based on mean hemoglobin levels (8.65 $\pm$ 1.40 g/ dL) and body weight (263.53 $\pm$ 23.05 g). During treatment, iron was provided in the form of ferrous sulfate or available on millet flours, at a standardized concentration of 13.29 ppm per treatment (DA SILVA et al., 2016a).

Four experimental groups were used (n=8): standard diet with ferrous sulfate (SD+FS), standard diet with non-germinated millet flour cooked in an open pan (SD+NGOPCMF), standard diet with germinated millet flour cooked in an open pan (SD+GOPCMF), and standard diet with millet flour processed by extrusion (SD+ECMF). Animals received deionized water ad libitum and a controlled diet, that were weighed daily over a 21-day period. Weight gain and hemoglobin levels were monitored weekly. On day 50, the animals were anesthetized with isoflurane (Isoforine, Cristália®) and euthanized via cardiac puncture. The proximal colon was collected, washed with phosphate-buffered solution (PBS), and kept in 10% formaldehyde at room temperature.

The cecum was weighed, and its contents were collected and immediately stored at -80 ºC.

The study was carried out in accordance with the ethical principles for animal experimentation and was approved by the Animal Use Ethics Committee of the Federal University of Viçosa (CEUA/UFV; process no. 05/2022).

Hemoglobin measurement

Hemoglobin levels were determined using a Labtest colorimetric kit (Ref 43 - hemoglobin, and Ref 47- standard hemoglobin) (Labtest®, Lagoa Santa, MG, Brazil). using the absorbance reading of a standard hemoglobin solution as a reference. The reading was performed on a Multiskan™ GO spectrophotometer (Thermo Fisher Scientifics; Waltham, MA, USA), at a wavelength of 540 nm.

Bristol scale and feces color

The Bristol Stool Form Scale (BSFS) was used to obtain information about intestinal transit and bowel function, as described by Martinez and Azevedo (2012). Analysis of feces color was performed by macroscopic observation, adapting the classification proposed by Silveira Júnior (1988).

Fecal pH

A 0.4 g aliquot of cecum content and 4 mL of distilled water were mixed using a vortex for 20 s. The pH was then measured by inserting a glass electrode (Bel Engineering®) directly into the suspension (GRANCIERI et al., 2017).

DNA extraction, sequencing and data analysis

Genomic DNA was extracted and purified from cecal content samples using QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol.

Extracted DNA samples were submitted to NovoGene Co., Ltd (Beijing, China) for amplification and paired-end sequencing of the V3-V4 region of the 16S rRNA gene (forward: 341 F: 50-CCTACG-GGNGGCWGCAG-30 and reverse: 805 R: 50-GACTACHVGG-GTATCTAATCC-30) on an Illumina MiSeq platform (Illumina Inc.,

San Diego, CA, USA). The resulting libraries were demultiplexed and paired in QIIME2 (version 2023.7) (BOLYEN et al., 2019). Paired-end reads were passed to DADA2 for trimming, dereplication, denoising, and filtering (CALLAHAN et al., 2016). The quality threshold was set at 30, and chimeric sequences were removed. A total of 1,654,918 reads were generated with a median of 51,716 reads per sample while 174 sequences were detected. After removing singletons and sequences present in fewer than 10% of the sample, 117 unique sequences remained. Taxonomy was assigned with a naïve-Bayes classifier, trained with the 341F/805R amplicons against the Silva 138.1 nr99 SSU database (QUAST et al., 2013).

Microbiome analysis and visualization were performed using MicrobiomeAnalyst (Xia Lab, McGill University, Montréal, QC, Canada) (DHARIWAL et al., 2017). Alpha diversity was assessed using the Chao 1, Shannon, and Simpson indices, while β diversity among groups was evaluated based on the Bray–Curtis dissimilarity index and tested using PERMANOVA. The linear discriminant analysis effect size (LEfSe) method was used to investigate differences in gut microbiota and identify significant bacterial biomarkers among groups (SEGATA et al., 2011).

Short-chain fatty acids (SCFA) concentration

The concentration of short-chain fatty acids (SCFAs) in the cecum content was analyzed using the methodology proposed by Siegfried and Ruckemann (1984). SCFA quantification was determined by high-performance liquid chromatography (HPLC), using a Dionex Ultimate 3000 Dual detector HPLC system (Dionex Corporation, Sunnyvale, CA, USA), as described by Gomes et al. (2022). Acetic, propionic, butyric and valeric acids were used as standards for the calibration curve.

Histomorphometry analysis of the colon

Histological sections (3 μm thick) of the proximal colon were stained with hematoxylin and eosin using a rotary microtome (Reichert-Jung®, Genossen, Germany) and examined under an Olympus BX43 optical microscope with a 4x objective (GOMES et al., 2022). Crypt depth, crypt thickness, circular and longitudinal muscle layer thickness, and the number and area of goblet cells were measured in twenty fields per animal, using Image-Pro-Plus® software, version 4.5 (Media Cybernetics, Rockville, MD, USA).

Correlation analysis of gut microbiota and SCFA concentration with markers of inflammation and oxidative stress

To assess the relationship between changes in the relative abundance of intestinal microbiota and changes in markers of inflammation and oxidative stress, a Pearson correlation analysis was performed. The relative abundance data were log-transformed to account for compositionality of ASV variables prior to running your correlations. Markers of inflammation (tumor necrosis factor gene expression (mRNA TNF)) and oxidative stress (nuclear factor erythroid 2 gene expression (mRNA NRF2); malondialdehyde concentration (MDA); nitric oxide concentration (NO); superoxide dismutase activity (SOD) and catalase activity (CAT)) had been previously evaluated and published (THEODORO et al., 2024b). The expression of TNF and NRF2 mRNA levels in the liver was analyzed by real-time polymerase chain reaction (RT-qPCR) and quantification was performed on the StepOnePlus™ Real-Time PCR System, using the SYBR-Green fluorescence quantification system and Primer Express software, v3.0.1 (Applied Biosystems, Foster City, CA, USA). The concentration of MDA and NO and the activity of the antioxidant enzymes SOD and CAT were evaluated in the liver tissue homogenate, and the reading was performed on a Multiskan™ GO spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA) (THEODORO et al., 2024b).

In silico docking

Interactions of the phenolic compounds previously identified in millet flours (Diosmin and Cyanidin 3-O-rutinoside betaine) (THEODORO et al., 2024a) with the markers of inflammation and antioxidant potential (Nrf2 gene expression, SOD activity; TNF-alpha gene expression and catalase activity) were analyzed by *in silico* analysis. The 3D crystal structures were obtained from the Protein Data Bank (PDB) website (http://www.rcsb.org/pdb/home/home.do): Nrf2 (PDB: 4xmb), SOD (PDB: 1pu0); TNF-alpha (PDB: 2az5), and catalase (PDB: 1dgf). Diosmin and Cyanidin 3-O-rutinoside betaine were used as ligands, and their structures were recovered from the PubChem Compound database (https://pubchem.ncbi.nlm.nih.gov/, accessed on 1 April 2025).

Flexible torsions, charges, and grid size were carried out by AutoDock Tools, and docking calculations were performed using AutoDock Vina (TROTT; OLSON, 2010). The binding pose with the lowest binding energy (highest binding affinity) was selected

as a representative image to be visualized in the Discovery Studio 2016 Client (Dassault Systemes Biovia Corp®, San Diego, CA, USA).

Statistical Analyzes

The experiment was performed with a sample size of 8 animals per group, with each animal representing a repetition. The results were assessed for normality using the Shapiro–Wilk and Kolmogorov–Smirnov tests. Subsequently, the data were subjected to one-way ANOVA and the Newman–Keuls post hoc test. Differences in beta diversity were analyzed using pairwise PERMANOVA. In the taxonomic analysis, comparisons of relative abundance between groups were made at the phylum level by one-way ANOVA and the Newman–Keuls test. Pearson's correlation analysis was used to assess the relationship between comparative alterations in intestinal microbiota relative abundance and markers of inflammation and oxidative stress. The ASV variables were previously transformed into log ratios to take into account their compositional nature. All statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA), version 9.0. The significance level of 5% ($p < 0.05$) was established for all tests.

3. RESULTS

3.1. Effect of Processed Pearl Millet on food intake, body weight, hemoglobin levels, cecum weight and intestinal transit in rats

Throughout the three-weeks of the repletion period, no differences ($p > 0.05$) were observed in food intake, hemoglobin levels, or body weight among the experimental groups **(Figure 1A-C)**. However, at the end of the experiment, the SD+NGOPCMF and SD+GOPCMF groups showed higher cecum weight ($p < 0.05$) compared to other groups **(Figure 1D)**.

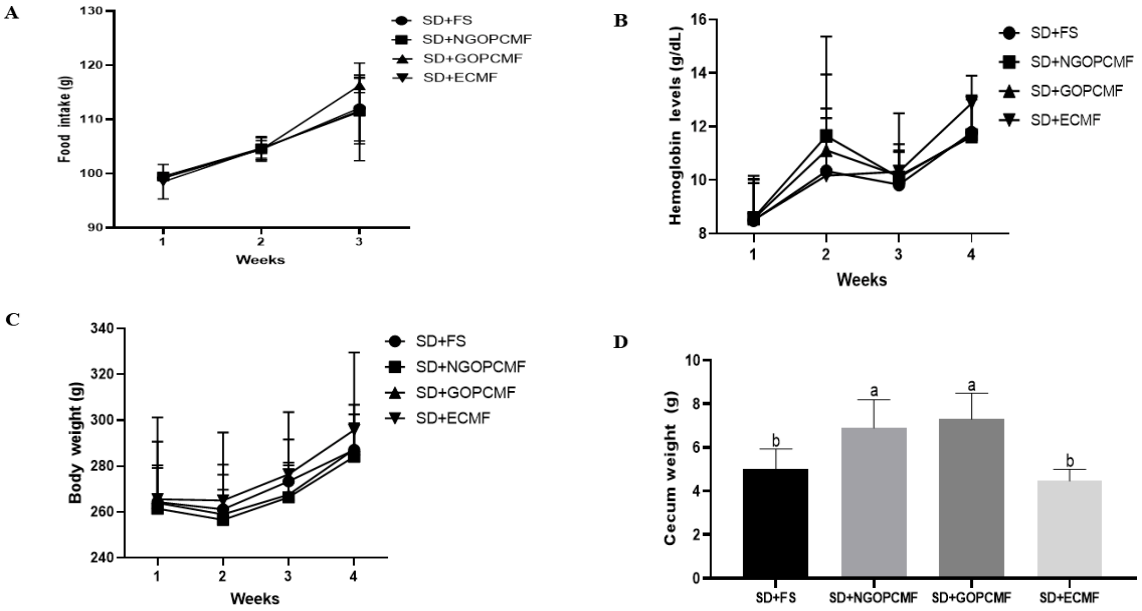


Figure 1. Effect of pearl millet subjected to different processing methods on food intake (A), hemoglobin levels (B), body weight (C), cecum weight (D) and Bristol scale and feces color (E) after three weeks of treatment. Values expressed as mean and standard deviation (n = 8/group). Means without letters indicate no difference and different letters indicate significant differences by the Newman-Keuls test, at 5% probability. SD + FS: standard diet + ferrous sulfate; SD + NGOPCMF: standard diet + non-germinated open-pan cooked millet flour; SD + GOPCMF: standard diet + germinated open-pan cooked millet flour; SD + ECMF: standard diet + extrusion-cooked millet flour.

According to the Bristol scale, the feces of the SD+NGOPCMF and SD+GOPCMF groups were darker in color in relation to the other groups (SD+ECMF and SD+FS) **(Table 1)**. In terms of consistency, the groups treated with pre-cooked millet flour presented smoother and softer feces compared to the group treated with ferrous sulfate. Nevertheless, all groups were classified as having a normal intestinal transit pattern **(Table 1)**.

Table 1. Effect of processed pearl millet on Bristol scale and feces color after three weeks of treatment.

Group	Feces Color	Feces Consistency
SD + FS	Light brown-colored feces	Similarly to a sausage, but with a crack on the surface
SD + NGOPCMF	Dark brown-colored feces	Like sausage or snake, smooth and soft
SD + GOPCMF	Dark brown-colored feces	Like sausage or snake, smooth and soft
SD + ECMF	Light brown-colored feces	Like sausage or snake, smooth and soft

SD + FS: standard diet + ferrous sulfate; SD + NGOPCMF: standard diet + non-germinated open-pan cooked millet flour; SD + GOPCMF: standard diet + germinated open-pan cooked millet flour; SD + ECMF: standard diet + extrusion-cooked millet flour.

3.2. Effect of Processed Pearl Millet on on fecal pH and short-chain fatty acids concentration

The SD+GOPCMF group showed the lowest fecal pH ($p < 0.05$), followed by the SD+NGOPCMF group. No difference ($p > 0.05$) was observed between the SD+ECMF and control (SD+FS) groups. Regarding short-chain fatty acids (SCFA) concentration, no differences ($p > 0.05$) were observed among the experimental groups after the three-week treatment period **(Table 2)**.

Table 2. Effect of processed pearl millet on fecal pH and short-chain fatty acids production after three weeks of treatment.

Variables	**SD+FS**	**SD+NGOPCMF**	**SD+GOPCMF**	**SD+ECMF**
Fecal pH	$8.57 + 0.18^{a}$	$8.07 + 0.35^{b}$	$7.71 + 0.24^{c}$	$8.37 + 0.11^{a}$
Short Chain Fatty Acids (mM)				
Acetic acid (mM)	2.78 ± 0.50^{a}	3.23 ± 0.84^{a}	3.24 ± 0.69^{a}	2.85 ± 0.43^{a}
Propionic acid (mM)	1.69 ± 0.76^{a}	1.73 ± 0.56^{a}	1.37 ± 0.29^{a}	1.12 ± 0.22^{a}
Butyric acid (mM)	0.77 ± 0.14^{a}	0.72 ± 0.14^{a}	0.75 ± 0.19^{a}	0.67 ± 0.08^{a}
Valeric acid (mM)	0.20 ± 0.05^{a}	0.21 ± 0.03^{a}	0.19 ± 0.00^{a}	0.18 ± 0.03^{a}

Means followed by different letters in the line differ by the Newman-Keuls test, at the 5% level of significance. Short-chain fatty acids were analyzed by HPLC. SD + FS: standard diet + ferrous sulfate; SD + NGOPCMF: standard diet + non-germinated open-pan cooked millet flour; SD + GOPCMF: standard diet + germinated open-pan cooked millet flour; SD + ECMF: standard diet + extrusion cooked millet flour.

3.3. Effects of processed pearl *millet on microbial community Diversity*

In the α diversity analysis, the SD+NGOPCMF, SD+GOPCMF and SD+ECMF groups demonstrated a higher ($p < 0.05$) Chao index compared to the control group (SD+FS) **(Figure 2A)**. The SD+GOPCMF and SD+ECMF groups demonstrated a higher Shannon index ($p < 0.05$), while SD+NGOPCMF did not differ ($p > 0.05$) from the other groups fed with millet (SD+GOPCMF and SD+ECMF), but also did not differ ($p > 0.05$) from the SD+FS group **(Figure 2B)**. The Simpson index did not differ ($p > 0.05$) among

the groups **(Figure 2C)**. In β-diversity, the PERMANOVA analysis demonstrated that the clustering differed among the experimental groups ($p < 0.001$), except for the SD+ECMF and SD+FS groups that did not differ from each other **(Figure 2D)**.

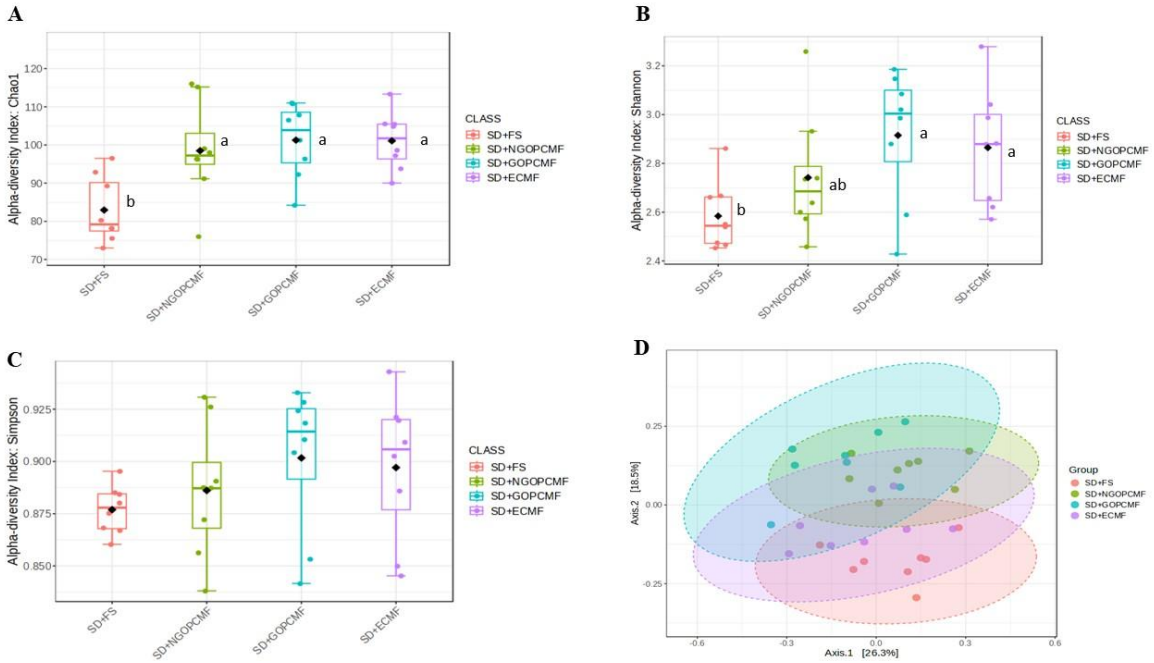


Figure 2. Microbial diversity of the cecal microbiome of different experimental groups after three weeks of the treatment. The α-diversity by: (A) Chao Index; (B) Shannon Index; (C) Simpson Index. ANOVA $p<0.05$. The β-diversity is shown through the Bray–Curtis dissimilarity distances separated by two principal components (PCoA) (D). PCoA analyzed by the PERMANOVA test, $p < 0.001$. Each dot refers to one animal (n = 8/group). SD + FS: standard diet + ferrous sulfate; SD + NGOPCMF: standard diet + non-germinated open-pan cooked millet flour; SD + GOPCMF: standard diet + germinated open-pan cooked millet flour; SD + ECMF: standard diet + extrusion-cooked millet flour. Different letters indicate significance by the Newman–Keuls test, at 5% probability.

In the taxonomic classification, the phyla Actinobacteriota, Bacteroidota, and Firmicutes were the most predominant in all experimental groups **(Figure 3A, B)**. The SD+NGOPCMF and SD+GOPCMF groups demonstrated higher ($p < 0.05$) relative abundance of Bacteroidota compared to the SD+FS group, while the SD+ECF group did not differ from the other groups. On the other hand, SD+FS group showed the highest ($p < 0.05$) relative abundance of Actinobacteriota. No differences ($p > 0.05$) were observed in the relative abundance of Firmicutes among the experimental groups **(Figure 3A, B)**.

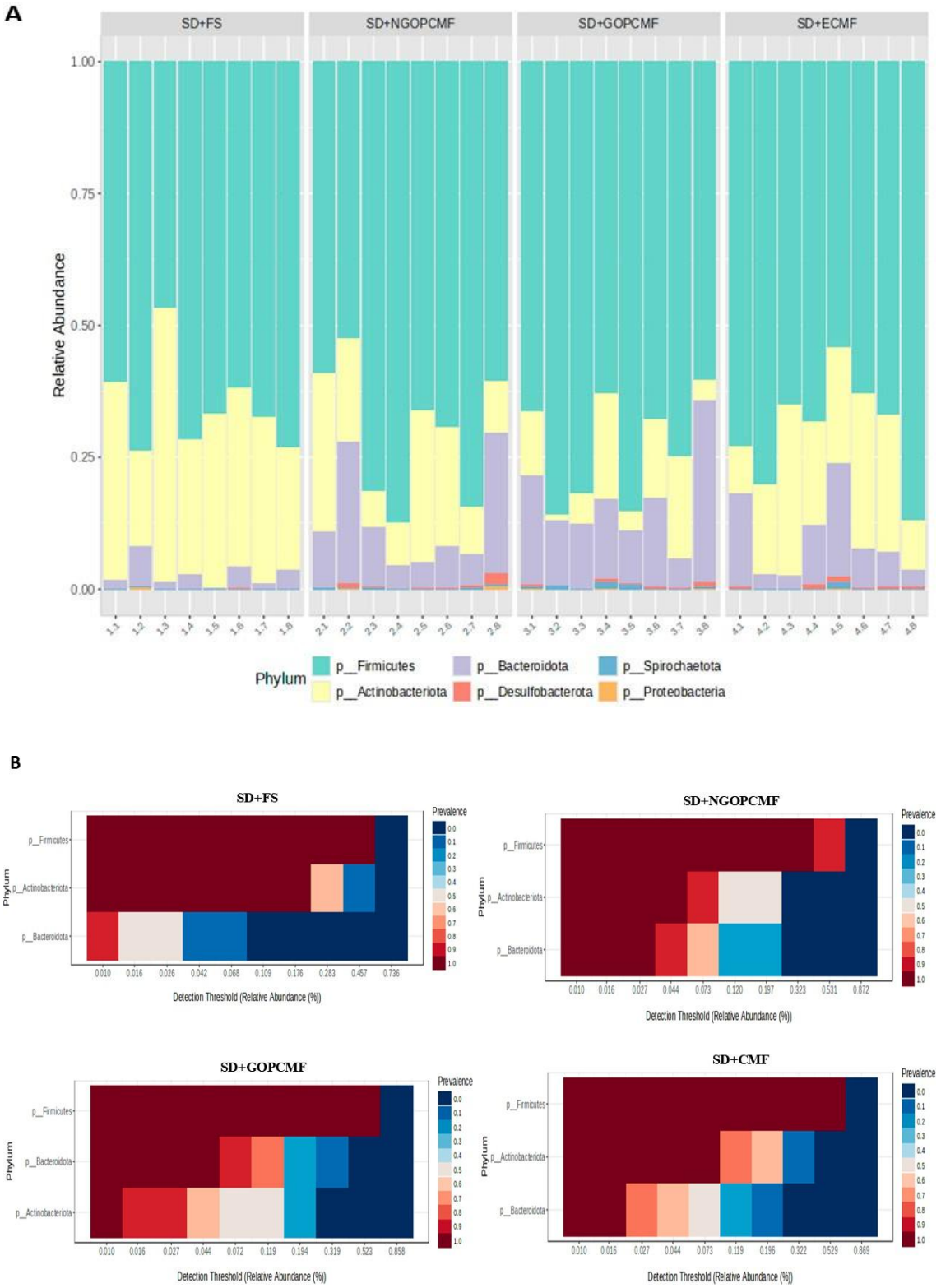
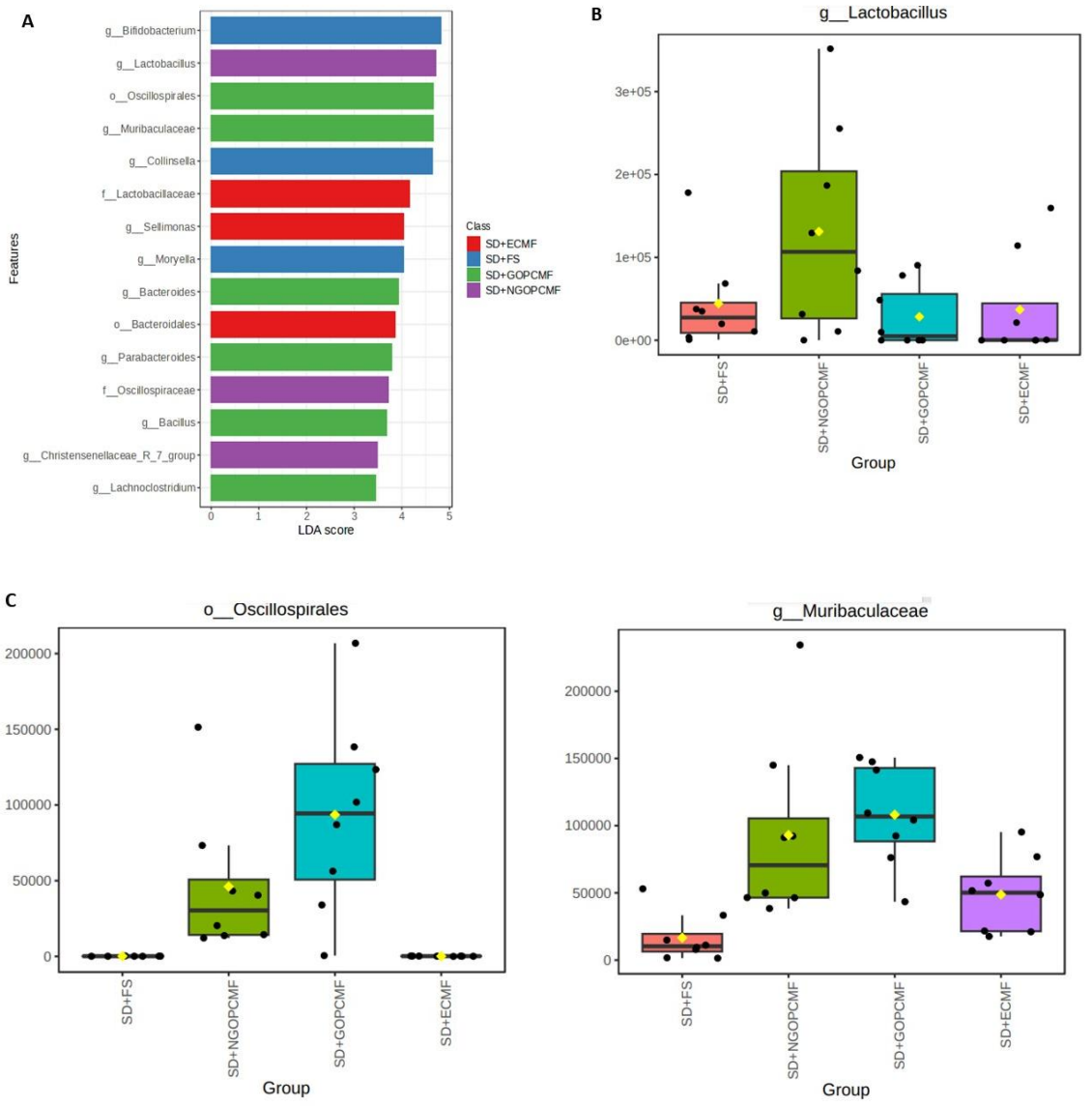


Figure 3. Changes in the composition of the intestinal microbiota after three weeks of treatment. Relative abundance (A) and core microbiome (B) at the phylum level (n = 8/group). SD + FS: standard diet + ferrous sulfate; SD + NGOPCMF: standard diet + non-germinated open-pan cooked millet flour; SD + GOPCMF: standard diet + germinated open-pan cooked millet flour; SD + ECMF: standard diet + extrusion-cooked millet flour. Taxa with counts <10 were merged.

Regarding the LEfSe analysis, in the groups treated with millet flour, the genus *Lactobacillus*, belonging to the phylum Firmicutes, was relatively enriched ($p < 0.05$) in the SD + NGOPCMF group **(Figure 4A, B)**. The order Oscillospirales, belonging to the phylum Firmicutes, and the family *Muribaculacea*, belonging to the phylum Bacteroidota, were relatively enriched ($p < 0.05$) in the SD+GOPCMF group **(Figure 4A, C)**. The family Lactobacillaceae, followed by the genus *Sellimonas*, both belonging to the phylum Firmicutes, were relatively enriched ($p < 0.05$) in the SD+ECMF group **(Figure 4A, D)**. In the control group (SD + FS), the genus *Bifidobacterium*, belonging to the phylum Actinobacteriota, was relatively enriched ($p < 0.05$) **(Figure 4A, E)**.



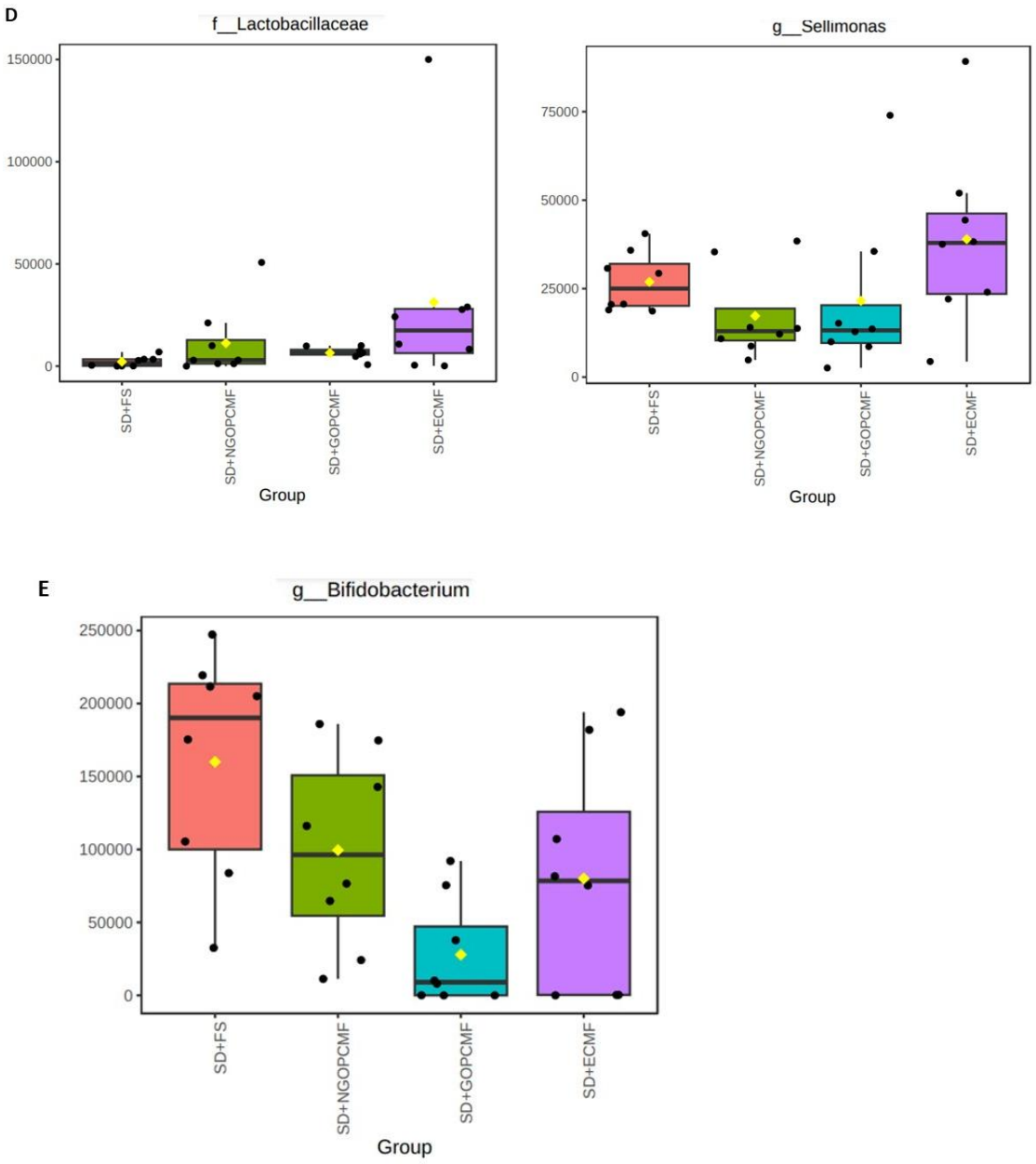


Figure 4. Linear Discriminant Analysis Effect Size (LEfSe) (**A**). Filtered count of significant features of relative enrichment by group: SD+NGOPCMF (**B**), SD+GOPCMF (**C**), SD+ECMF (**D**) and SD+FS (**E**). (n = 8/group). SD + FS: standard diet + ferrous sulfate; SD + NGOPCMF: standard diet + non-germinated open-pan cooked millet flour; SD + GOPCMF: standard diet + germinated open-pan cooked millet flour; SD + ECMF: standard diet + extrusion-cooked millet flour.

3.4. Effects of processed pearl millet on intestinal morphology

The number and area of goblet cells were higher ($p < 0.05$) in the SD+NGOPCMF, SD+GOPCMF and SD+ECMF groups compared to the SD+FS group. Crypt depth and crypt thickness were also higher ($p < 0.05$) in the SD+NGOPCMF and SD+GOPCMF

groups. For crypt thickness, the SD+ECMF group did not differ from the other millet flour groups, while the ferrous sulfate group showed lower values **(Table 3 and Figure 5)**.

Table 3. Effect of processed pearl millet on morphology health, after three weeks of treatment.

Variables	SD + FS	SD + NGOPCMF	SD + GOPCMF	SD + ECMF
Number of goblet cells	9.12 ±0.86 ^{b}	12.03 ±1.47 ^{a}	12.32 ±1.62 ^{a}	10.96 ±0.74 ^{a}
Goblet cell area	101.40 ±3.64 ^{c}	143.70 ±0.86 ^{a}	146.40 ±3.87 ^{a}	113.30 ±3.65 ^{b}
Crypts depth (µM)	121.40 ±1.91 ^{b}	131.10 ±1.75 ^{a}	132.80 ±1.77 ^{a}	121.50 ±1.03 ^{b}
Crypts thickness (µM)	22.54 ±0.93 ^{b}	24.67 ±0.93 ^{a}	24.52 ±0.77 ^{a}	23.56 ±0.76 ^{ab}
Longitudinal mucus layer width ((µM)	38.56 ±6.68 ^{c}	50.55 ±7.45 ^{b}	73.71 ±4.39 ^{a}	48.28 ±5.94 ^{b}
Circular mucus layer width ((µM)	124.10± 14.46 ^{b}	147.40 ±16.48 ^{a}	161.30 ±13.32 ^{a}	126.10 ±16.89 ^{b}

Means followed by different letters in the line differ by the Newman–Keuls test at the 5% level of significance. SD + FS: standard diet + ferrous sulfate; SD + NGOPCMF: standard diet + non-germinated open-pan cooked millet flour; SD + GOPCMF: standard diet + germinated open-pan cooked millet flour; SD + ECMF: standard diet + extrusion-cooked millet flour.

The width of the longitudinal muscle layer was higher ($p < 0.05$) in animals treated with pre-cooked millet flour (SD+NGOPCMF, SD+GOPCMF, and SD+ECMF), with germinated flour group (SD+GOPCMF) presenting the highest values ($p < 0.05$). The width of the circular muscle layer was also higher ($p < 0.05$) in the SD+NGOPCMF and SD+GOPCMF groups in relation to the SD+ECMF and SD+FS groups **(Table 3 and Figure 5)**.

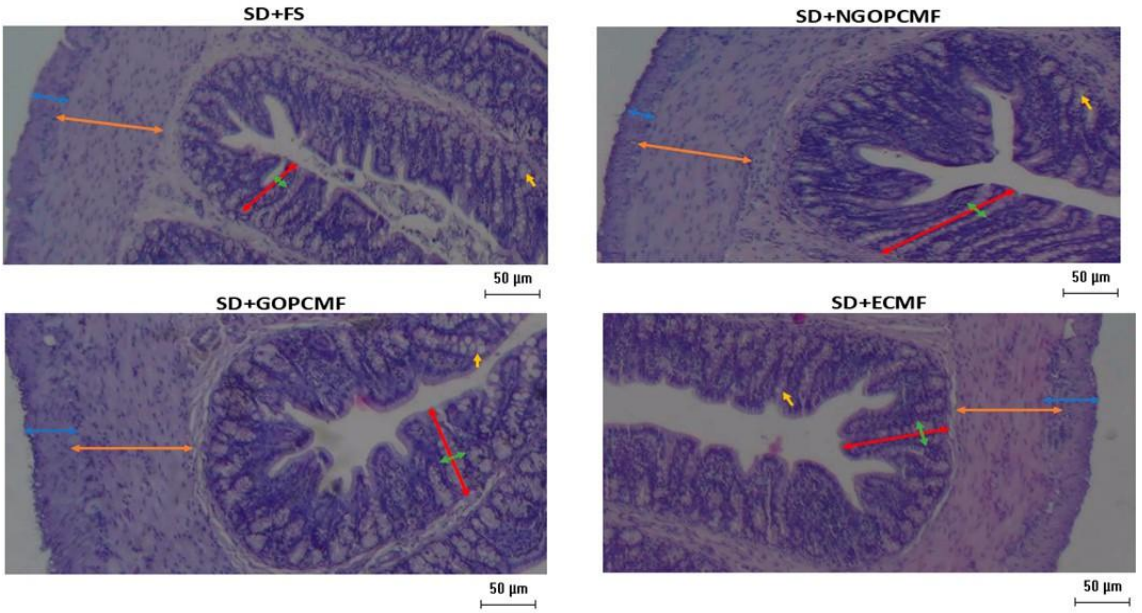


Figure 5. Effect of pearl millet subjected to different processing on the histomorphometry of the colon after three weeks of treatment. Yellow arrows refer to the goblet cells, red arrows refer to crypt depth, green arrows refer to crypt thickness, orange arrows refer to circular mucus layer width and blue arrows refer to longitudinal mucus layer width. SD + FS: standard diet + ferrous sulfate; SD + NGOPCMF: standard diet + non-germinated open-pan cooked millet flour; SD + GOPCMF: standard diet + germinated open-pan cooked millet flour; SD + ECMF: standard diet + extrusion cooked millet flour. Staining was carried out with hematoxylin and eosin. Bar: 50 μm. Objective magnitude: 4x.

3.5. Correlation of gut microbiota and SCFA concentration with inflammation and oxidative stress markers

When evaluating the correlation between the comparative alterations in intestinal microbiota relative abundance with markers of inflammation and oxidative stress, the phylum Actinobacteriota was positively correlated with the gene expression of TNF ($rs = 0.434$; $p < 0.05$) and Nrf2 ($rs = 0.474$; $p < 0.05$). On the other hand, the phylum Bacteroidota was negatively correlated with the gene expression of TNF ($rs = -0.588$; $p < 0.05$) and Nrf2 ($rs = -0.682$; $p < 0.05$), as well as Desulfobacterota (TNF ($rs = -0.472$; $p < 0.05$) and Nrf2 (-0.588; $p < 0.05$)). Furthermore, Actinobacteriota was also positively correlated with fecal pH ($rs = 0.376$; $p < 0.05$) and negatively correlated with Bacteroidota ($rs = -0.500$; $p < 0.05$), Firmicutes ($rs = -0.608$; $p < 0.05$), and Spirochaetota ($rs = -0.466$; $p < 0.05$). The phylum Bacteroidota was positively correlated with Desulfobacterota ($rs = 0.764$; $p < 0.05$), Proteobacteria ($rs = 0.638$; $p < 0.05$) and Spirochaetota ($rs = 0.688$; $p < 0.05$). The phy- lum Desulfobacterota was positively correlated with Proteobacteria ($rs = 0.619$; $p < 0.05$) and Spirochaetota ($rs = 0.460$; $p < 0.05$). Proteobacteria was positively correlated with Spirochaetota ($rs = 0.544$; $p < 0.05$). In addition, markers of inflammation and oxidative stress were correlated with each other ($p < 0.05$) **(Figure 6)**.

Regarding SCFA, propionic acid was positively correlated with Nrf2 gene expression ($rs = 0.422$; $p < 0.05$). Acetic acid showed negative correlations with nitric oxide ($rs = -0.351$; $p < 0.05$) and SOD ($rs = -0.390$; $p < 0.05$), while butyric acid was also negatively correlated with SOD ($rs = -0.362$; $p < 0.05$). Additionally, positive correlations ($p < 0.05$) were observed among the SCFAs themselves **(Figure 6)**.

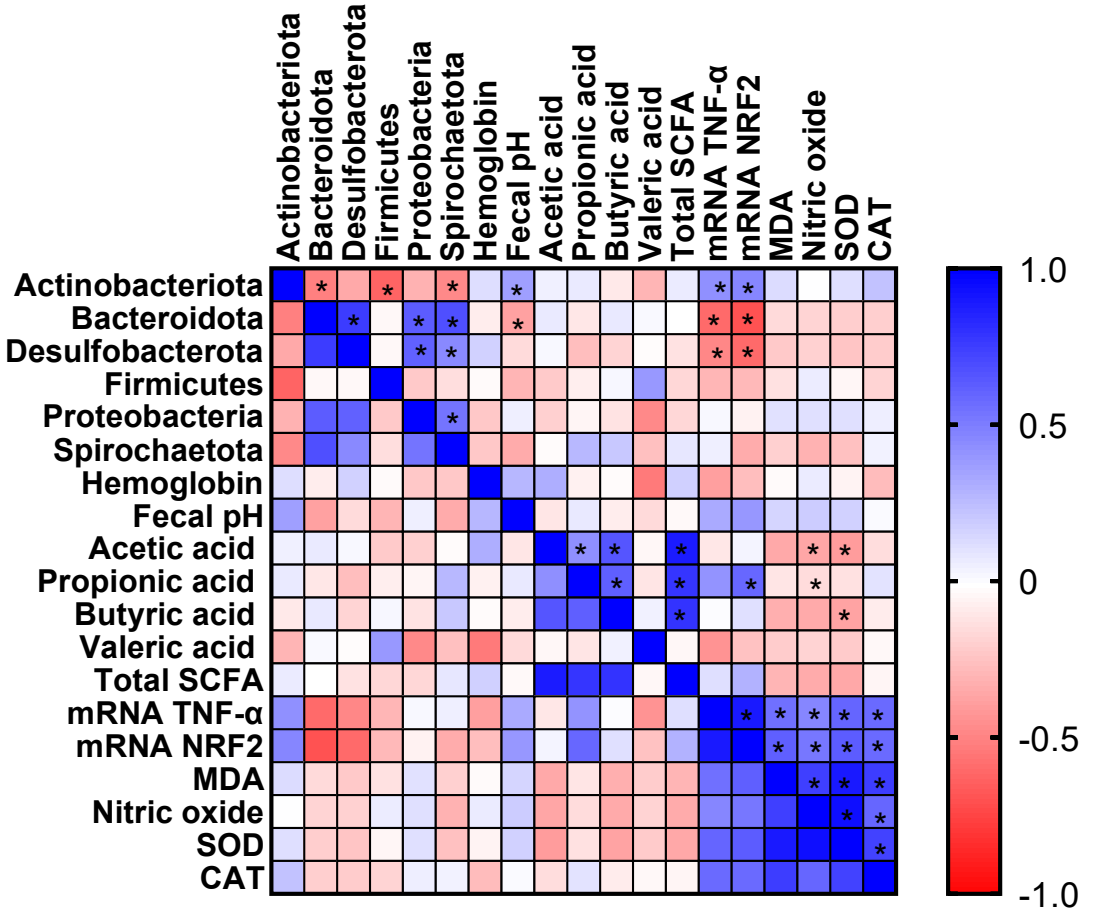


Figure 6. Heat map of Pearson's correlations between alterations in intestinal microbiota relative abundance and markers of inflammation and oxidative stress after three weeks of treatment with millet subjected to different processing. Short chain fatty acid (SCFA); tumor necrosis factor (TNF); nuclear factor erythroid 2 (Nrf2); Malondialdehyde (MDA); Superoxide dismutase (SOD); Catalase (CAT); n = 8/group. * $p < 0.05$. Data from markers of inflammation and oxidative stress were previously published (THEODORO et al., 2024b).

3.6. In silico docking

Diosmin and Cyanidin 3-O-rutinoside betaine showed a good interaction with the analyzed markers Nrf2, SOD; TNF-alpha and catalase, since all of them presented low binding energy **(Table 4)**. However, Diosmin showed the greatest interaction with Nrf2 (EFE: -11.4), SOD (EFE: -7.9), and catalase (EFE: -9.2) and Cyanidin 3-O-rutinoside betaine had the best interaction with TNF-alpha (EFE: -9.6) (**Table 4 and Figure 7**). Furthermore, it was observed that the main bonds between the ligands (compounds) and the markers were conventional hydrogen bonds and Pi-sigma bonds (**Figure 7**).

Table 4. Estimated free energy binding (EFE) and chemical interactions among the phenolic compounds presents in millet with the markers Nrf2, SOD, TNF-alpha, and catalase.

Compounds	Diosmin		Cyanidin 3-O-rutinoside betaine	
	EFE	Interacting AA residues	EFE	Interacting AA residues
Nrf2	**-11.4**	VAL A: 467; VAL A: 420; VAL A: 369; ALA A: 607; CYS A: 368; VAL A: 606; GLY A: 367; ILE A: 559; VAL A: 465; VAL A: 512; GLY A: 423.	-9.9	SER A: 363; SER A: 602; ARG A:415; GLY A:509; ALA A:556; VAL A: 604
SOD	**-7.9**	HIS A: 110; SER A: 107; ARG A: 69; VAL A: 103; GLU A: 77	-7.4	SER A: 102; SER A: 105; GLN A: 22; SER A: 25; HIS A: 110; VAL A: 103; LEU A:67
TNF-alpha	-7.9	LEU A: 36; TYR A: 59; TYR A: 151	**-9.6**	ARG A:82; ASN B:92; ASN D: 92; GLN B: 125; ARG D: 82; VAL B:91; VAL D: 91
Catalase	**-9.2**	GLN A: 455; SER A: 201; TYR A: 215; TRP A: 303; ARG A: 203; PHE A: 446; PHE A: 198; VAL A: 450; HIS A: 305	-9.0	ARG A: 203; TYR A: 215; PRO A: 151; PHE A: 198; ASN A: 149

Docking calculation were carried out using AutoDock Vina. Negative values mean spontaneous reaction. The most potent interaction between compounds and receptor are in bold. EFE: Estimated free energy binding; AA: amino acids; Nrf2: nuclear factor erythroid 2; SOD: superoxide dismutase; TNF-alpha: tumor necrosis factor-alpha.

Docking calculation were carried out using AutoDock Vina. Negative values mean spontaneous reaction. The most potent interaction between compounds and receptor are in bold. EFE: Estimated free energy binding; AA: amino acids; Nrf2: nuclear factor erythroid 2; SOD: superoxide dismutase; TNF-alpha: tumor necrosis factor-alpha.

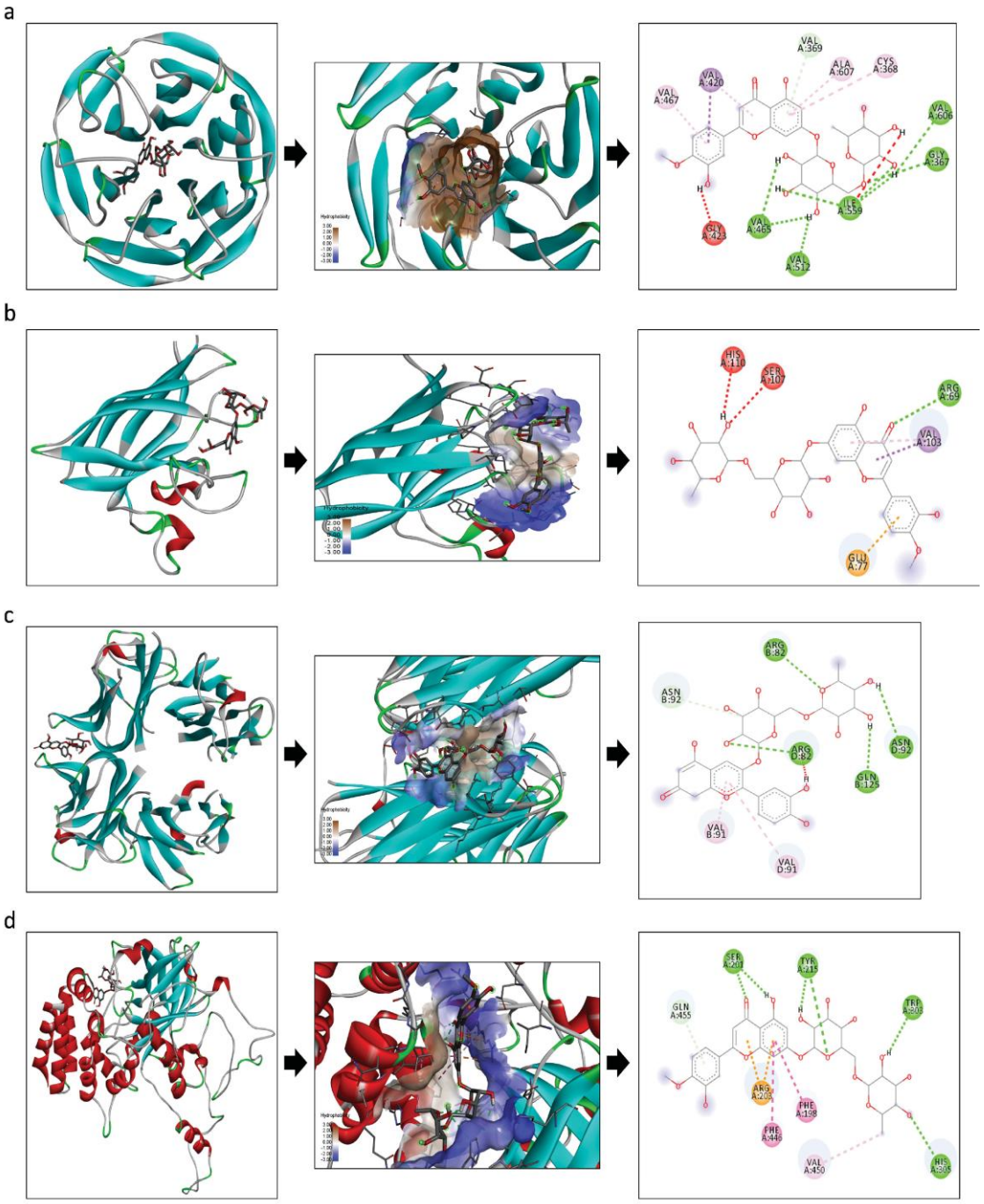


Figure 7. The in silico interaction of the identified phenolic compounds present in pearl millet with inflammation and oxidative markers. (a) Interaction between Nrf2 and Diosmin; (b) Interaction between SOD and Diosmin; (c) Interaction between TNF-alpha and Cyanidin 3-O-rutinoside betaine; (d) Interaction between catalase and Diosmin. Dates analyzed by AutoDock Vina® and visualized using Discovery Studio 2016 Client®.

4. DISCUSSION

This study evaluated the effect of pearl millet subjected to different processing methods on intestinal health in rats, using the hemoglobin depletion/repletion model, with millet flour being the only source of iron for the test groups, and ferrous sulfate for the

control group. The results demonstrated that pre-cooked millet flours, regardless of the processing used, have the potential to improve intestinal morphology and modulate the composition of the gut microbiota. After three weeks of treatment, rats receiving millet flours showed enhanced Chao index, increased number and area of goblet cells and width of the longitudinal muscle layer. Furthermore, the SD+NGOPCMF and SD+GOPCMF groups demonstrated reduced fecal pH, different clustering in β-diversity, higher relative abundance of Bacteroidota, increased cecum weight, higher crypt depth and thickness, and wider circular muscle layer width. The SD+GOPCMF group stood out for showing the lowest fecal pH, better α-diversity (Chao and Shannon indices), and highest width of the longitudinal muscle layer. The amount of millet flour used in this study is equivalent to approximately one teacup of flour in human dietary terms. This quantity can be easily incorporated into the daily diet through common culinary preparations such as cakes, cookies and pasta, as reported in previous studies (THEODORO et al., 2021a, 2021b).

During the treatment period, food intake, body weight and hemoglobin levels were similar among all groups, demonstrating that millet flours were as effective as ferrous sulfate in restoring iron deficiency. These findings suggest good iron bioavailability of pearl millet flours (NGOPCMF = 4.49 mg iron/100 g flour; GOPCMF 4.18 mg iron/100 g flour; ECMF = 5.64 mg iron/100 g flour), since the hemoglobin regeneration efficiency (HRE%) was similar among the animals treated with millet flour and the control group (SD + FS = 64.8 ± 7.7a; SD + NGOPCMF = 67.4 ± 16.2a; SD + GOPCMF = 67.9 ± 13.4a; SD + ECMF = 77.8 ± 19.4a).

The high cecum weight observed in animals belong to SD+NGOPCMF and SD+GOPCMF groups can be due the type of dietary fiber and the amount of resistant starch present in the non-germinated and germinated pearl millet flours. Although all experimental diets contained the same total amount of dietary fiber, predominantly insoluble fiber (provided from pearl millet flours in the test groups and cellulose in the control group), the SD+NGOPCMF and SD+GOPCMF groups also consumed soluble fiber and resistant starch inherent to the millet food matrix. These components likely contributed to the increased cecum weight. In contrast, the similarity between the SD+ECMF and SD+FS groups may be explained by the extrusion process. Resistant starch can become digestible following high-shear extrusion, thus decreasing its content and altering the dietary fiber profile (ALBARRACÍN et al., 2019; THEODORO et al., 2024a). This may have compromised the availability and physiological effect of these components in the animals' diet.

Furthermore, the improvement in fecal consistency observed in the SD + NGOPCMF, SD + GOPCMF and SD + ECMF groups can also be attributed to the presence of soluble fiber in the food matrix. Although no changes were observed in intestinal transit time, soluble dietary fiber contributed to the formation of smoother and softer stools, as classified by the Bristol scale. Thus, dietary fiber influenced fecal solubility, potentially enhancing nutrient absorption (GOMES et al., 2021), including iron and other bioactive compounds, such as phenolic compounds, promoting improved intestinal health (RINNINELLA et al., 2019). The darker color of the feces of the SD + NGOPCMF and SD + GOPCMF groups is probably due to the higher pigmentation of the flours compared to the SD + ECMF and SD + FS groups.

Dietary fiber and resistant starch may have localized impacts on the intestinal mucosa, stimulating the growth of epithelial cells. They may also interact with other nutrients present in the food matrix, such as proteins and fatty acids, to promote growth and improve muscle structure and function (GAO et al., 2020; HAN et al., 2023). The higher concentration of bioactive compounds, such as polyunsaturated fatty acids, in the non-germinated and germinated pearl millet flours (THEODORO et al., 2024a) probably facilitated nutrients interaction, leading to increased crypt depth and width of the circular muscle layer in the SD+NGOPCMF and SD+GOPCMF groups.

Furthermore, studies show that the consumption of dietary fiber, resistant starch and polyphenols is directly linked to an increase in the number and diameter of goblet cells and higher mucus production, providing higher protection for the membrane, in addition to an increase in the surface area, higher depth of the crypts and improvement in the integrity of the barrier, improving intestinal functionality and nutrient absorption (DINGEO et al., 2020; GAO et al., 2020; HAN et al., 2023). These functional and morphological effects appear to occur due to increased motility of the digestive tract, leading to cell hyperplasia and/or hypertrophy (DA SILVA et al., 2016; GAO et al., 2020), as observed in the groups treated with millet flour.

Dietary fiber and resistant starch also acts as prebiotic and, upon fermentation in the intestine, promote the production of SCFA, leading to a reduction in fecal pH (GOMES et al., 2021; THEODORO et al., 2022). In the present study, the SD+GOPCMF group stood out for showing a highest reduction in fecal pH, followed by the SD+NGOPCMF group. Although fecal pH did not reach acidic levels, probably explaining the absence of differences in SCFA production, there were notable changes in the gut microbiota composition in the groups treated with millet flour.

The prebiotics and phenolic compounds in pre-cooked pearl millet flours improved the α and β diversity of the gut microbiota (KASPRZAK-DROZD et al., 2021), promoting an increased prevalence of Bacteroidota and contributing to immune regulation by stimulating goblet cells proliferation. This likely resulted in greater mucin production, thereby strengthening the intestinal barrier, enhancing nutrient absorption, and improving overall gut health (HAN et al., 2023; MACIEL-FIUZA et al., 2023).

In addition, increased mucin production may influence the release of growth factors and the activation of signaling pathways involved in muscle development, contributing to the increase width of the longitudinal muscle layer (GAO et al., 2020; HAN et al., 2023). The thickened muscle layer observed in animals fed millet flour, with emphasis on the SD+GOPCMF group that showed highest width of the longitudinal muscle layer, is associated with enhanced intestinal motility, which may facilitate more efficient transport and absorption of nutrients. This finding is consistent with a previous study, where higher expression of divalent metal transporter 1 (DMT1) was reported in animals receiving pre-cooked pearl millet flours, thus promoting better iron absorption at the enterocyte level (THEODORO et al., 2024b).

Furthermore, the higher number and area of goblet cells, in addition to improving immune defense, possibly contributed to a higher Chao index in the animals fed millet flour in the present study. Glycine-rich mucin can be cleaved, generating energy for the growth of microorganisms, benefiting the microbiome and bacterial colonization (WANG; SHEN, 2024).

Pearl millet presents low-specificity (soluble) fibers, easily accessible and utilized by many bacteria, and high-specificity (insoluble) fibers, accessible and fermentable by a limited number of bacteria. Thus, the dietary fiber composition of pearl millet may result in both competitive pressures for nutrient utilization, favoring the growth of diverse bacteria, and less competitive structural resources, accessible to few bacteria, favoring the growth of specific taxa (CANTU-JUNGLES et al., 2021; CANTU-JUNGLES; HAMAKER, 2020). This implies increased diversity and differential composition of the microbial community, as demonstrated in the groups treated with millet flours. The reduction in competitive pressures not only means less competition and similarity in responses between communities, but also that more substrate will be available for fewer target bacteria, which can grow efficiently and significantly alter the gut microbial structure (CANTU-JUNGLES et al., 2021). This is in line with the findings of the present study, which demonstrated an increase in Chao index and different Bray-Curtis clustering

in the groups treated with millet flours, suggesting a more diverse microbial community compared to the control group, which received only cellulose in the diet.

The SD + GOPCMF group stands out for the best α-diversity, for Chao (species richness) and Shannon (species diversity) indices, in addition to showing enrichment of Muribaculaceae, which exerts a probiotic effect and is associated with the production of SCFA via endogenous (mucin) and exogenous (dietary fiber) polysaccharides (ZHU et al., 2024).

Accompanying increased microbial diversity was a higher relative abundance of Bacteroidota in the groups treated with millet flours compared to controls. On the other hand, the group treated with ferrous sulfate showed a higher relative abundance of Actinobacteriota and a reduction in Bacteroidota. The predominance of the phylum Bacteroidota, with enrichment of the genera *Lactobacillus* and *Sellimonas* and Muribaculaceae in the groups fed with millet flour is associated with immune system regulation (LI et al., 2024; MUÑOZ et al., 2020; ZHU et al., 2024), since these may exert probiotic and anti-inflammatory effects and reduce oxidative stress by neutralizing free radicals and other reactive oxygen species (MACIEL-FIUZA et al., 2023; MOSTAFAVI ABDOLMALEKY; ZHOU, 2024). However, the increased abundance of Actinobacteriota may lead to an exacerbated inflammatory response, since some members of this phylum are capable of produce lipopolysaccharides (LPS) and other pro-inflammatory compounds. Moreover, these bacteria can also contribute to oxidative stress by generating free radicals and other reactive oxygen species (MOSTAFAVI ABDOLMALEKY; ZHOU, 2024).

The intake of iron salts increases unabsorbed iron in the large intestine, which alters the balance between potentially beneficial and potentially pathogenic bacteria, likely leading to intestinal dysbiosis, increased inflammation, and oxidative stress (PAGANINI; ZIMMERMANN, 2017; PHIPPS et al., 2020). In the present study, the Actinobacteriota phylum was positively correlated with the gene expression of TNF-alpha and Nrf2, corroborating previous findings that animals treated with ferrous sulfate demonstrated higher gene expression of these markers, along with increased levels of malondialdehyde and nitric oxide (THEODORO et al., 2024b).

On the other hand, a negative correlation between Bacteroidota and Desulfobacterota abundance and the inflammatory and antioxidant potential markers, TNF and Nrf2, respectively, was also demonstrated. Furthermore, in silico docking analysis demonstrated interaction between the phenolic compounds (Diosmin and

Cyanidin 3-O-rutinoside betaine) and markers of antioxidant potential and inflammation (Nrf2, SOD, CAT and TNF). According to the other results found, it is possible to assume that Cyanidin 3-O-rutinoside betaine acted as an anti-inflammatory compound, possibly reducing the gene expression of TNF, while Diosmin exerted an antioxidant effect, possibly reducing the gene expression of Nrf2, SOD and CAT not requiring endogenous defense, and favoring the homeostasis of the organism. This is in line with previous studies that reported microbiota modulation and anti-inflammatory and antioxidant effect of pearl millet consumption (OWHERUO; IFESAN; KOLAWOLE, 2019; THEODORO et al., 2021a, 2022, 2024b).

Therefore, animals fed with pearl millet flours demonstrated increased microbial diversity and improved intestinal function, promoting the absorption of nutrients such as iron. These effects contributed not only to the recovery of iron deficiency but also supported antioxidant and anti-inflammatory responses, highlighting the potential of processed pearl millet to enhance intestinal health.

This study is innovative in investigating the effects of the processed millet on intestinal health in an iron deficiency mode. Despite the promising results, a limitation of the study should be considered. The intestinal microbiota and other variables associated with intestinal health were not evaluated in animals at the end of the depletion phase, i.e., in iron deficiency. However, these results are encouraging for replication of the study evaluating intestinal health in both the depletion and repletion phases.

CONCLUSION

Non-germinated, germinated and extruded pre-cooked millet flours effectively restored blood iron concentration, increased microbial diversity, and improved intestinal morphology in rats induced to iron deficiency. The SD+GOPCMF group stood out for showing lowest fecal pH, better alpha-diversity (Chao and Shannon indices) and the highest width of the longitudinal muscle layer. These findings underscore the potential of pearl millet subjected to different processing methods, mainly germination, as a source of iron and bioactive compounds, promoting antioxidant and anti-inflammatory effects and favoring intestinal health in vivo.

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L.A.d.S., formal analysis; V.P.B.d.S.J., formal analysis; N.B.W., formal analysis; R.C.L.T., formal analysis, data curation; M.G., writing – review & editing, formal analysis; C.W.P.C., supervision, resources; J.F.P., formal analysis; B.P.d.S., writing – review & editing, supervision, methodology; H.S.D.M., writing – review & editing, writing – original draft, supervision, project administration, conceptualization. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

SD + FS: standard diet + ferrous sulfate
SD + NGOPCMF: standard diet + non-germinated open-pan cooked millet flour
SD + GOPCMF: standard diet + germinated open-pan cooked millet flour
SD + ECMF: standard diet + extrusion-cooked millet flour
SCFAs: short-chain fatty acids

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9. GENERAL CONCLUSION

The consumption of raw germinated pearl millet flour, associated with a diet rich in fat and fructose, improves the morphology, function and composition of the intestinal microbiota, increasing beta diversity, cecum weight, cecum/body weight ratio, crypt depth, crypt thickness and the number of goblet cells. In addition, it reduces fecal pH and urinary excretion of mannitol and increases the production of propionate. Therefore, it highlights the potential of raw germinated millet flour, replacing 43.6% of dietary fiber, to improve intestinal health in rats induced to metabolic alterations.

Extrusion cooking and germination followed by cooking in an open pan changes the concentration of nutrients and non-nutrients in millet flour, maintaining good chemical-nutritional composition and improving the bioavailability of iron in vitro, compared to non-germinated open-pan cooked millet. Extruded millet shows an increase in carbohydrate, iron, manganese, diosmin and cyanidin content, but a decrease in total dietary fiber, resistant starch, lipids and total vitamin E content, compared to non-germinated open-pan cooked millet. Germinated millet stands out for presenting an increase in lysine and vitamin C, a reduction in phytochemicals, such as phytate, whereas preserving of dietary fiber and resistant starch content. Therefore, although all cooked millet flours have good chemical-nutritional composition, the germination and extrusion processes, mainly germination, have proven to be nutritionally more suitable for the development of millet-based products.

Pre-cooked pearl millet flours (non-germinated, germinated and extruded) demonstrate good in vivo iron bioavailability, but germinated millet stands out showing a better effect on iron metabolism compared to extrusion, and being similar to non-germinated millet in terms of antioxidant capacity. Pearl millet flours also improved inflammation and oxidative stress, thus promoting good iron absorption and intestinal functionality in rats induced to iron deficiency. In addition, pre-cooked pearl millet flours (non-germinated, germinated and extruded), in addition to restoring blood iron concentration in a similar way to ferrous sulfate, also improve microbial diversity and intestinal morphology in rats induced to iron deficiency. Germinated pearl millet stands out for showing a greater reduction in fecal pH, improving alpha-diversity (Chao and Shannon indices) and increasing the width of the longitudinal muscle layer. These findings reinforce the potential of pearl millet subjected to different processing methods, mainly germination, as a source of iron and bioactive compounds, promoting antioxidant and anti-inflammatory effects and favoring intestinal health in vivo.

Our results demonstrate that the inclusion of pearl millet in the diet, regardless of processing, may be a promising option to beneficially modulate intestinal health and provide antioxidant and anti-inflammatory effects. Germination and extrusion processing may add more functional effect to the grain, improving iron bioavailability and favoring the body's homeostasis. However, germinated pearl millet stands out for favoring the chemical-nutritional composition and beneficial effects on the body, both added to a normal diet or to a high-fat diet.

10. CONCLUSÃO GERAL

O consumo de farinha de milheto pérola germinado cru, associado a uma dieta rica em gordura e frutose, melhora a morfologia, a função e a composição da microbiota intestinal, aumentando a diversidade beta, o peso do ceco, a relação ceco/peso corporal, a profundidade da cripta, a espessura da cripta e o número de células caliciformes. Além disso, reduz o pH fecal e a excreção urinária de manitol e aumenta a produção de propionato. Portanto, destaca-se o potencial da farinha de milheto germinado crua, substituindo 43,6% da fibra alimentar, para melhorar a saúde intestinal em ratos induzidos a alterações metabólicas.

O cozimento por extrusão e a germinação seguida de cozimento em panela aberta alteram a concentração de nutrientes e não nutrientes na farinha de milheto pérola, mantendo uma boa composição química-nutricional e melhorando a biodisponibilidade de ferro in vitro, em comparação ao milheto não germinado cozido. O milheto extrudado apresenta aumento nos teores de carboidratos, ferro, manganês, diosmina e cianidina, porém demonstra diminuição nos teores de fibra alimentar total, amido resistente, lipídios e vitamina E total, em comparação ao milheto não germinado cozido. O milheto germinado destaca-se por apresentar aumento nos teores de lisina e vitamina C, redução de fitoquímicos, como fitato, além de preservação dos teores de fibra alimentar e amido resistente. Portanto, embora todas as farinhas de milheto cozido apresentem boa composição químico-nutricional, os processos de germinação e extrusão, principalmente a germinação, têm se mostrado nutricionalmente mais adequados para o desenvolvimento de produtos à base de milheto.

As farinhas de milheto pérola pré-cozido (não germinado, germinado e extrudado) demonstram boa biodisponibilidade de ferro in vivo, mas o milheto germinado se destaca por apresentar melhor efeito no metabolismo do ferro em comparação à extrusão, sendo semelhante ao milheto não germinado em termos de capacidade antioxidante. As farinhas

de milheto pérola também melhoraram a inflamação e o estresse oxidativo, promovendo assim uma boa absorção de ferro e funcionalidade intestinal em ratos induzidos à deficiência de ferro. Além disso, as farinhas de milheto pérola pré-cozido (não germinadas, germinadas e extrusadas), além de restaurarem a concentração sanguínea de ferro de forma semelhante ao sulfato ferroso, também melhoram a diversidade microbiana e a morfologia intestinal em ratos induzidos à deficiência de ferro. O milheto pérola germinado destaca-se por apresentar maior redução do pH fecal, melhorando a alfa-diversidade (índices de Chao e Shannon) e aumentando a largura da camada muscular longitudinal. Esses achados reforçam o potencial do milheto pérola submetido a diferentes métodos de processamento, principalmente a germinação, como fonte de ferro e compostos bioativos, promovendo efeitos antioxidantes e anti-inflamatórios e favorecendo a saúde intestinal in vivo.

Nossos resultados demonstram que a inclusão de milheto pérola na dieta, independentemente do processamento, pode ser uma opção promissora para modular beneficamente a saúde intestinal e proporcionar efeitos antioxidantes e anti-inflamatórios. Os processos de germinação e extrusão podem adicionar mais efeito funcional ao grão, melhorando a biodisponibilidade do ferro e favorecendo a homeostase do organismo. No entanto, o milheto germinado se destaca por favorecer a composição químico-nutricional e os efeitos benéficos ao organismo, tanto adicionado a uma dieta normal quanto a uma dieta rica em gordura.

11. FINAL CONSIDERATIONS

Pearl millet is a cereal with good chemical and nutritional composition, being a natural source of prebiotics, bioactive compounds and iron. It has the potential to be environmentally sustainable and resistant, facilitating its production even in adverse climate conditions, in addition to being accessible to the poorest segments of the population and presenting a low incidence of mycotoxin contamination compared to other crops, such as wheat and corn. Some processing, such as extrusion and germination, can contribute to the good chemical and nutritional composition and better iron bioavailability of *Penninsetum glaucum*. In addition, germination increase the lysine content, which is naturally deficient in millet.

Open-pan cooking, germination or extrusion processes can be applied to millet, depending on the expected purpose of the final product. In addition, pearl millet can be used alone or combined with other foods, such as legumes, to develop products with

added nutritional value, being a good option to overcome situations of food and nutritional insecurity, in addition to being an alternative for develop products that appeal to health and sustainability, plant-based, meeting current market demand.

Our results demonstrate that the inclusion of millet in the diet, regardless of processing, can be a promising option to promote homeostasis in the body. However, our in vivo studies were performed in a controlled environment and diet, which may not adequately reflect the iron metabolism and effect on intestinal health in free-living individuals. Therefore, new studies using pearl millet, especially germinated millet flour, evaluating the intestinal health of individuals with metabolic alterations and individuals with iron deficiency are needed to investigate whether these effects persist in the human body. Studies using a shorter germination time (e.g. 8h) also could be carried out to verify whether the results are better with short germination.

12. APPENDIX

12.1. Appendix A - Approval of the Ethics Committee regarding biological test 1

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 39/2019, intitulado "**Efeitos da farinha de milheto germinado (*Pennnisetum glaucum* L. *R Br.*) em alterações metabólicas de ratos alimentados com dieta rica em gordura saturada 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 39/2019, named **"Effects of germinated millet flour (Pennnisetum glaucum L. R Br.) On metabolic alterations of rats fed a high saturated fat 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 03, 2019 valid for 12 months.

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

12.2. Appendix B- Approval of the Ethics Committee regarding biological test 2

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 05/2022, intitulado **"Efeito de farinhas de milheto (Pennisetum glaucum (L.) R. BR.) na biodisponibilidade de ferro e saúde intestinal *In vivo*"**, 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/MCTIC, 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/MCTIC, portanto sendo aprovado por esta Comissão em 13/04/2022, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 05/2022, named **"Effect of millet (Pennisetum glaucum (L.) R. BR.) flours on iron bioavailability and intestinal health In vivo"**, is in agreement with the a actual Brazilian legislation (Lei Nº 11.794, 2008, Normative Resolutions edited by CONCEA/MCTIC, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTIC therefore being approved by the Committee on April 13, 2022 valid for 12 months.

Profa. Mariella Bontempo Duca de Freitas
Coordenadora
Comissão de Ética no Uso de Animais – CEUA/UFV