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EXPLORING THE GENETIC AND PHENOTYPIC DIVERSITY OF PEARL MILLET [*Pennisetum Glaucum* (L.) R. Br.] VIA UNTARGETED METABOLOMICS

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Master's thesis submitted to the Graduate Program in Food and Nutrition at the Federal University of the State of Rio de Janeiro as a partial requirement for obtaining the degree of Master of Science in Food.

Supervisor: Mariana Simões Larraz Ferreira

Co-supervisor: Carlos Wanderlei Piler de Carvalho

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RESUMO

O milheto *[Pennisetum glaucum* (L.) R. Br.] é uma cultura que se adapta facilmente a diferentes ambientes e possui uma composição benéfica para a nutrição humana, embora ainda seja considerado um cereal negligenciado e subutilizado. A complexidade de metabólitos dos grãos de milheto pode variar de acordo com os genótipos e as condições ambientais, destacando a importância do uso de técnicas de metabolômica não direcionada para o mapeamento dessas variações. Este estudo teve como objetivo caracterizar de maneira abrangente os metabólitos de um grande conjunto de amostras de grãos de milheto e, subsequentemente, identificar metabólitos preditores associados com importantes características fenotípicas usando metabolômica baseada em espectrometria de massas e ferramentas avançadas de bioinformática. Grãos oriundos de 60 populações de polinização aberta e 203 diferentes genótipos da coleção nuclear do Banco Ativo de germoplasma de milheto do Brasil foram cedidos pela Embrapa Milho e Sorgo. Os grãos inteiros foram liofilizados, moídos e a extração dos metabólitos foi realizada de com etanol (80%) de forma automatizada. A metabolômica baseada em cromatografia líquida acoplada a espectrometria de massas foi usada para obter os perfis de metabólitos. A modelagem linear generalizada com penalização foi aplicada para explorar a correlação entre o metabolismo da coleção nuclear e 21 características fenotípicas. Dentre os principais achados, 263 metabolitos foram anotados, com predominância de lipídeos polares, seguidos por fenilpropanóides e policetídeos. No total, 97 compostos exibiram diferença de distribuição entre as populações, com destaque para os flavonoides. A modelagem linear generalizada foi capaz de predizer oito traços fenotípicos qualitativos a partir da coleção nuclear de milheto pérola, alcançando uma acurácia de 74% a 87%. Foram detectados 834 metabólitos preditores (575 anotados e 259 *unknowns*), majoritariamente carboidratos, aminoácidos, flavonoides e terpenos. Desses, 23 metabólitos preditores também foram encontrados na assinatura metabólica das populações de polinização aberta, podendo ser utilizados futuramente para validação do modelo. Essas descobertas ressaltam o valor do perfil de metabólitos do milheto, fornecendo informações para programas de melhoramento genético voltados para a seleção de cultivares com base em metabólitos de interesse para a alimentação humana e na predição de traços fenotípicos.

Palavras-chave: compostos bioativos, modelagem linear generalizada, aprendizado de máquina, metabólitos.

ABSTRACT

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is a crop that easily adapts to various environments and has a composition beneficial for human nutrition, although it is still considered a neglected and underutilized cereal. The complexity of millet grain metabolites varies with genotypes and environmental conditions, highlighting the importance of using non-targeted metabolomics techniques to map these variations. This study aimed to comprehensively characterize the metabolites of a large set of pearl millet grain samples and subsequently identify predictive metabolites associated with important phenotypic traits using mass spectrometry-based metabolomics and advanced bioinformatics tools. Whole grains from 60 open-pollinated populations and 203 different genotypes from the core collection of the Brazilian Millet Germplasm Active Bank were provided by Embrapa Corn and Sorghum. The whole grains were lyophilized, ground, and metabolite extraction was performed with 80% ethanol in an automated manner. Liquid chromatography coupled with mass spectrometry-based metabolomics was used to obtain metabolite profiles. Generalized linear modeling with penalty was applied to explore the correlation between the metabolism of the core collection and 21 phenotypic traits. Among the main findings, 263 metabolites were annotated, predominantly polar lipids, followed by phenylpropanoids and polyketides. In total, 97 compounds exhibited a distribution difference between populations, with flavonoids being particularly notable. Generalized linear modeling was able to predict eight qualitative phenotypic traits from the pearl millet core collection, achieving an accuracy of 74% to 87%. A total of 834 predictive metabolites (575 annotated and 259 unknowns) were detected, mostly carbohydrates, amino acids, flavonoids, and terpenes. Of these, 23 predictive metabolites were also found in the metabolic signature of the open-pollinated populations and could be used in the future for model validation. These findings underscore the value of the millet metabolite profile, providing information for breeding programs aimed at selecting cultivars based on metabolites of interest for human food and predicting phenotypic traits.

Keywords: bioactive compounds, generalized linear modeling, machine learning, metabolites.

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Abbreviations

ANR - Agence Nationale de la Recherche

CAPES - Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CE-MS - Capillary Electrophoresis-Mass Spectrometry

CSV - Comma-Separated Values

CNPq - Conselho Nacional de Desenvolvimento Científico e Tecnológico

CV - Coefficient of Variation

DTF - Days to Flowering

EMBRAPA - Brazilian Agricultural Research Corporation

ESI - Electrospray Ionization

FA - Fatty Acyls

FAO - Food and Agriculture Organization

FAOSTAT - FAO Statistical Database

FAPERJ - Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro

FBMN - Feature-Based Molecular Networking

GC-MS - Gas Chromatography-Mass Spectrometry

GLM - Generalized Linear Model

GLMNET - Generalized Linear Model Elastic Net

GNPS - Global Natural Products Social Molecular Networking

GP - Glycerophospholipids

HCA - Hierarchical Cluster Analysis

HRMS - High-Resolution Mass Spectrometry

ICRISAT - International Crops Research Institute for the Semi-Arid Tropics

INBS - Infrastructure Nationale en Biologie et Santé

INRAE - Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement

LASSO - Least Absolute Shrinkage and Selection Operator

LC-MS - Liquid Chromatography-Mass Spectrometry

LOWESS - Locally Weighted Regression Scatterplot Smoothing

LPC - Lysophosphatidylcholines

LPE - Lysophosphatidylethanolamines

MetaboHUB - French national infrastructure dedicated to metabolomics and fluxomics

MGF - Mascot Generic Format

MS - Mass Spectrometry

MS/MS - Tandem Mass Spectrometry

MS1 - Mass Spectrometry level 1 data

MS2 - Mass Spectrometry level 2 data

MSI - Metabolomics Standards Initiative

m/z - Mass-to-Charge Ratio

NCDs - Non-Communicable Diseases

NMR - Nuclear Magnetic Resonance

NUS - Neglected and Underutilized Species

OPPs - Open-Pollinated Populations

OPVs - Open-Pollinated Varieties

PC - Phenolic Compounds; Phosphatidylcholines; Principal Component

PCA - Principal Component Analysis

PG - Phosphatidylglycerols

PI - Phosphoinositides

PL - Prenol Lipids

PPGAN/UNIRIO - Graduate Program in Food and Nutrition at the Federal University of the State of Rio de Janeiro

QqQ-MS/MS - Triple Quadrupole Tandem Mass Spectrometry

QC - Quality Control

R^2 - Coefficient of Determination

RT - Retention Time

UHPLC - Ultra High-Performance Liquid Chromatography

UHPLC-HRMS - Ultra High-Performance Liquid Chromatography-High Resolution Mass Spectrometry

UHPLC-LTQ-Orbitrap - Ultra High-Performance Liquid Chromatography-Linear Trap Quadrupole-Orbitrap

UHPLC-ESI-QTOF - Ultra Performance Liquid Chromatography-Electrospray Ionization-Quadrupole Time of Flight

UNIRIO – Universidade Federal do Estado do Rio de Janeiro

SUMMARY

INTRODUCTION

The escalating frequency of droughts and floods in the 21st century, attributed to global climate change and the heightened impact of anthropogenic activities, poses a critical threat to cereal crop cycles, particularly those of wheat and rice (Feng et al., 2013; Ray et al., 2012). These factors have significantly impacted grain production and access, exacerbating the global food insecurity scenario (FAO, 2022).

In this context, the world has turned its attention to cereals considered a minority or even as neglected and underutilized species (NUS), especially millet crops. The term millet is designated to a diverse of minor cereals barnyard millet (*Echinochloa esculenta*), finger millet (*Eleusine coracana*), foxtail millet (*Setaria italica*), kodo millet (*Paspalum scrobiculatum*), little millet (*Panicum sumatrense*), pearl millet (*Pennisetum glaucum*) e proso millet (*Panicum miliaceum*) being the most representative.

Among the species, pearl millet [*Pennisetum glaucum* (L.) R. Br] is the most widely used for human food, fodder and animal feed, accounting for most of the world's millet production and trade (FAO, 2021). In Brazil, it is a crucial part of no-till systems in the *Cerrado* region and is cultivated before the second crop period (off-season). Historically, it has been used in the country for forage production and silage of cattle and sheep (Jat et al., 2013). However, the National Health Surveillance Agency (ANVISA) has recently authorized the use of pearl millet for human consumption, reinforcing its potential as a food source (Brasil, 2021).

Pearl millet has a rich diversity of genotypes, with ICRISAT hosting the most extensive collection globally, comprising 23,092 accessions from 52 countries (Upadhyaya et al., 2009). This highly diverse crop with a predominantly cross-pollinated breeding system, displays high degrees of heterosis for grain and fodder yield and other agronomic traits (Singh & Jauhar, 2006). It offers numerous agronomic advantages due to its resilience against extreme weather conditions and climate change, thriving in unpredictable weather patterns and nutrient-deficient soils, well adapted to semi-arid areas (D. Hunter et al., 2019).

The nutritional appeal of pearl millet relies on outstanding composition: it is high in slowly digestible and resistant starch that is structured into compact granules, which contributes to its low glycemic index (Lemgharbi et al., 2017; Mondal et al., 2022); the protein fraction is non-allergenic and well balanced in amino acids, except for lysine and threonine. It is also gluten-free, offering a dietary alternative for individuals with celiac disease (Hassan et al., 2021); Moreover, it contains higher lipids levels in comparison to other cereals, among them up to 0.5% bound to starch which slows starch hydrolysis and enhances its hypoglycemic properties (Annor et al., 2015, 2017; Pruthi & Bhatia, 1970). For a cereal, it is considered elevated in omega-3, linked to greater protection against the development of chronic non-communicable diseases (NCDs) such as cardiovascular disease, diabetes, metabolic syndrome, and cancer (Saleh et al., 2013). Moreover, it presents lower levels of mycotoxins when compared to maize (Wilson et al., 2006). Additionally, it is also rich in a range of phenolic compounds (PC), most notably ferulic, *p*-coumaric and cinnamic acids. These PCs are not only abundant antioxidants in the human diet but also exhibit numerous bioactivities. They play critical roles in biochemical and enzymatic reactions within the human metabolism, particularly in their interactions with the gut microbiota (Hanhineva et al., 2010; Rhowell Jr et al., 2022).

The complexity of pearl millet grain metabolites is intrinsically linked to genotype and growing conditions, reflecting the response of biological systems to genetic and environmental factors (Fiehn, 2002). Therefore, the application of a metabolomics approach to characterize the metabolite profile from a large set of genotypes is essential to evaluate and understand the genetic diversity of this crop. Untargeted metabolomics is particularly important in this context and, when integrated with predictive analytics and machine learning, enables the prediction of plant phenotypic traits based on metabolite data. These strategies, whether used independently or in combination, enhance the genetic basis for cultivar selection based on metabolites of interest for food and predictive biomarkers.

To advance research in this area, the present manuscript aimed to characterize the metabolite profile of grains from a large germplasm collection of pearl millet using metabolomic tools and to assess the capacity of metabolite data in predicting agronomically important traits. This work is guided by four major questions: (i) What is the metabolite signature of pearl millet grains? (ii) Is it possible to differentiate the germplasm based on metabolite data? (iii) What is the role of phenolic compounds? (iv) Which biomarkers can predict key phenotypic traits in pearl millet?

This Master thesis was conducted in the Food and Nutrition Graduate Program (*Programa de Pós-Graduação em Alimentos e Nutrição*-PPGAN) at UNIRIO following the research line “Processing, quality, valorization of food, coproducts and residues” in the framework of the research project “Metabolomics and proteomics of food”. This work is part of the research group of CNPq “Proteomics and metabolomics of bioactive compounds - Omics sciences applied to organisms of economic and biotechnological interest” and was carried out in the Laboratory of Bioactives of UNIRIO. This work is a result of an international collaboration between two science and technology institutions (EMBRAPA and INRAE), two universities (UNIRIO and *Université de Bordeaux*) and two countries (Brazil and France). The samples were produced by Embrapa, and the experimental analytical part was conducted at INRAE [UMR Biology of Fruit and Pathology] during a 6-month internship in Bordeaux, France.

This manuscript is divided into four chapters. The first chapter provides a brief literature review of the morphological characteristics of pearl millet grains, their chemical composition, and nutritional aspects, as well as the phenolic compounds present in pearl millet and the metabolomic tools used for compound characterization.

In the second chapter is presented an original research article currently in preparation, which will be submitted to the Journal of Agricultural and Food Chemistry. The article entitled “Pearl Millet Towards Human Consumption: Exploring Metabolite Diversity in Open-Pollinated Populations from the Brazilian *Cerrado* Biome,” profiles the metabolites of grains from sixty open-pollinated populations (OPPs) of pearl millet cultivated in the Brazilian *Cerrado* using UHPLC-HRMS. This work unveils the metabolite signature of the grains and identifies metabolites with significant variability from a nutritional perspective. It represents an initial step towards understanding the metabolite potential of these OPPs and lays the groundwork for developing cultivars selected based on metabolites of interest for food.

The third chapter also features an original research paper submitted to Metabolomics. The paper entitled “Predictive Metabolomics of Pearl Millet Phenotypic Traits Using a Germplasm Panel of Genetic Diversity” examines 203 genotypes from the core collection of the Brazilian Pearl Millet Germplasm Bank. This study utilizes a novel approach by integrating predictive metabolomics with machine learning to investigate the relationships between metabolic profiles and phenotypic traits. It successfully predicts eight qualitative phenotypic traits using the metabolic data and identifies 834 unique predictive biomarkers.

Finally, a concluding chapter synthesizes the major findings from all chapters, providing an overview of the manuscript, giving some perspectives

CHAPTER 1 – GENERAL LITERATURE REVIEW

1. AGRONOMIC CONCEPTS DEFINITIONS

Genotype is the total genetic constitution of an organism, determined by the set of genes arranged on chromosomes (Allem et al., 1996). Plant germplasm refers to the genetic information stored in seeds and represents the physical basis of the genetic assets that comprise the hereditary material of a species. Within this framework, a germplasm bank collection assembles genotypes from diverse geographical and environmental origins, providing essential and viable raw material for research and breeding programs (Allem et al., 1996). The nuclear collection represents, with minimal repetition, the genetic diversity of a cultivated species and its related species. For this purpose, 10% to 15% of the original germplasm collection is selected, representing 70% to 80% of the available genetic variability in the species of interest and its wild relatives (Allem et al., 1996).

A cultivar or variety is defined as a group of commercially cultivated genotypes that are distinguished by their morphological, physiological, cytological, biochemical, or other characteristics from related groups within the same species. These cultivars, when propagated retain their distinctive characteristics (Allem et al., 1996). The process of cultivar development involves several key steps: first, maintaining or developing sources of genetic variability; second, developing open-pollinated cultivars either for direct use or to derive breeding lines; and finally, developing lines specifically for the production of hybrids (de Oliveira et al., 2005).

Open-pollinated populations (OPPs) reproduce naturally without human intervention, with pollen distributed by environmental agents like wind and insects among individuals within the same population. This process allows OPPs to maintain high genetic variability within the population while also preserving genetic stability across generations, providing a valuable genetic base for breeders (CIMMYT, 1999). In addition, the term "open-pollinated varieties" (OPVs) refers to OPPs that have undergone the breeding process and have been commercially released, making them available on the market. Lines/ lineages, on the other hand, are groups of individuals that share a common ancestry and are maintained by self-fertilization and controlled crosses and have a high degree of genetic uniformity (Allem et al., 1996).

A composite is a population of plants derived from the uncontrolled inter-crossing of different lines and varieties of various origins, creating a genetically mixed group (de Oliveira et al., 2005). Conversely, a synthetic population is created by deliberately combining specific parental lines selected for their desirable traits, creating a genetically variable population in which individuals, although genetically distinct, share desirable traits (de Oliveira et al., 2005).

Hybrids are crosses between two different strains and lines or crop types, to maintain purity and heterosis (greater vigor than the parent) of the first filial generations of seed so that high performance is carried over to the commercial product (Sparks, 2018).

2. MORPHOLOGICAL CHARACTERISTICS OF PEARL MILLET GRAIN

The term 'millets' encompasses a wide variety of cereals belonging to the family *Poaceae* (*Graminiae*), traditionally cultivated in Africa and Asia (Geisen et al., 2021). Ten species are the most widely cultivated in the world for their grain production: Pearl millet (*Pennisetum glaucum*), Finger millet (*Eleusine coracana*), Foxtail millet (*Setaria italica*), Japanese barnyard millet (*Echinochloa esculenta*), Indian barnyard millet (*Echinochloa frumentacea*), Kodo millet (*Paspalum scrobiculatum*), Little millet (*Panicum sumatrense*), Proso millet (*Panicum miliaceum*), Tef (*Eragrostis tef*), Fonio (*Digitaria exilis* and *Digitaria iburua*) (Figure 1). Sometimes sorghum can be found or put together with millets in this presentation (Taylor, 2016). Despite belonging to the same family, there is significant diversity at the morphogenetic level, differing in genome size, ploidy levels, and breeding systems (Das et al., 2019).

Figure 1. Variety of different millet species.

Millets have several applications, including use as straw for grazing fodder, silage, and hay, as well as in human nutrition. From these, pearl millet [*Pennisetum Glaucum* (L.) R. Br.] is a highly diverse crop with a predominantly cross-pollinated breeding system and displays high degrees of heterosis for grain and fodder yield and other agronomic traits (Singh & Jauhar, 2006). Consequently, heterozygous hybrids derived from these parental lines are likely to demonstrate superior performance.

It is highly adapted to arid and semi-arid tropical regions due to its ability to grow in adverse conditions (e.g. low soil fertility, high soil pH, high soil Al^{3+} saturation, low soil moisture, high temperature, high soil salinity, and scanty rainfall) (Varshney et al., 2017). It has a C4 photosynthetic pathway, characterized by its ability to initially fix carbon dioxide into a four-carbon compound during photosynthesis, thereby reducing the energy used in photorespiration (Edwards & Huber, 1981). Moreover, it is fast-growing, with an annual summer cycle of between 75 and 120 days, reaching a height of 1.5 to 3 meters and a deep root system capable of reaching a depth of 3.60 meters (Skerman & Riveros, 1992). The panicles are similar in size and shape, being compact and cylindrical with a diameter of 2 to 3 cm, 15 to 45 cm long, and capable of producing around 500 to 2000 seeds (Durães et al., 2003).

The grains are spheric, measure about 3 to 4 mm in length, and the relative weight of a thousand grains is 3 to 15 g (Taylor, 2016). The structure is similar to other tropical cereals, such as corn and sorghum, but the germ represents about 16.5% of the grain, consequently, the endosperm is reduced to 75% and the pericarp comprises about 8.4%. Pericarp is composed of three layers: epicarp, mesocarp, and endocarp. In the mesocarp are found the starch granules, as in the sorghum grains. Below the pericarp is an integument, which can be pigmented, and below it is the aleurone layer, part of the endosperm. Finally, the grain is wrapped in a layer of waxy cutin, which protects it from the weather (Figure 2) (Taylor, 2016). The aleurone layer is characterized by the presence of primary lipids, proteins, phytins, and other pigments that affect the color of the grain (Rachie & Majmudar, 1980). The endosperm is divided into corneum (vitreous or hard component) and floury/farinaceous (soft). This differentiation occurs due to the presence of a continuous protein matrix, forming a network with no air space and few starch granules in the corneal fraction. As it is in the cells of the farinaceous endosperm, the conformation is rich in starch granules, aerated spaces, and lower protein content.

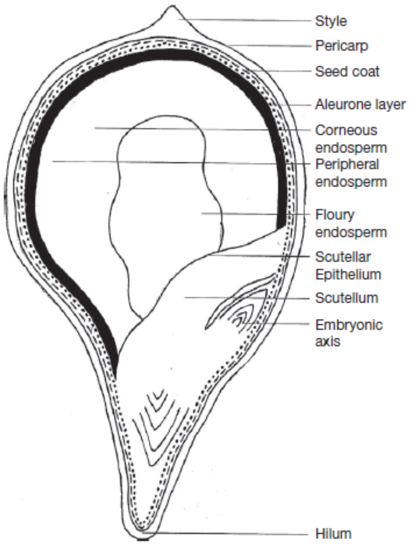

Figure 2. Structures of pearl millet grain tissues.

Source: Taylor (2016)

3. CHEMICAL COMPOSITION AND NUTRITIONAL ASPECTS

Pearl millet is rightly termed as “nutricereal” as it is a good source of energy, carbohydrate, protein, fat, ash, dietary fiber, iron and zinc (Table 1). Pearl millet is a rich source of energy (361 Kcal/100g) comparable with sorghum (349 Kcal/100g), wheat (346 Kcal/100g), rice (345Kcal/100g) and maize (325Kcal/100g) (Satyavathi et al., 2017). The differentiated structural distribution gives a unique chemical composition to the millet grain (Abdelrahman et al., 1984). The most abundant macronutrients are carbohydrates, especially starch (56-65%), with content like wheat (69%) and lower than corn (78%) and rice (85%) (Tomar et al., 2021). Free sugars range from 2.6 to 2.8% of the grain, with sucrose being the most important. In addition, about 20% of starch is in the form of amylose (Krishnan & Meera, 2018). The percentage of resistant starch is 2.8 to 5.1% and of dietary fiber or non-starch polysaccharides is 11.9 to 13.3%. This distribution gives millet a low glycemic index (55), favoring postprandial glycemic control and making it an important ally in reducing plasma cholesterol (Willett et al., 2002).

The second representative group is proteins, which correspond to an average of 11.8% of the composition of millet, higher than rice (8.6%), corn (9.2%) and close to sorghum (10.7%). The amino acid composition is relatively low in proline, cysteine, methionine and tryptophan, but high in lysine (2.8-3.2 g/100 g protein). In addition, this cereal is non-allergenic and gluten-free, making it an alternative for people with celiac disease and other sensitivities triggered by gluten consumption (Hassan et al., 2021).

In relation to the lipid fraction (6.4%), pearl millet has a substantially higher content when compared to other cereals, being twice the amount of corn (3.3%) and sorghum (3.4%), for example. Linoleic acid (C18:2) makes up about 39 to 45% of lipids, oleic acid (C18:1) 21 to 27%, and palmitic acid (C16:0) 20 to 21% (Annor et al., 2015). In millets, bound lipids, especially linked to starch, can represent 0.5% of total lipid content (Pruthi & Bhatia, 1970). As an advantage, this aids in hypoglycemic properties, as the amount and type of fatty acids, especially unsaturated ones, significantly decrease starch hydrolysis rates (Annor et al., 2015, 2017). On the other hand, it implies a challenge for the stability of pearl millet flour due to the susceptibility of unsaturated fatty acids to oxidation (Tiwari et al., 2014). Overall, pearl millet has mainly B vitamins and tocopherols (vitamin E), which are found in the aleurone layer and in the germ. Removing the outer layers by decortication or honing reduces niacin, riboflavin, and thiamine levels by about 50% in flour (Nambiar et al., 2011).

According to Dias-Martins et al, (2018), the ash content of pearl millet is similar to wheat, on average 1.8%, but lower than that of oats (2.5%) and higher than corn (1.3%) and rice (0.9%). It is also considered a good source of iron, copper, zinc, potassium, calcium, sodium, magnesium and phosphorus (Oshodi, 1999). These minerals perform several functions in the body, such as synthesis and structural stabilization of proteins, enzyme cofactors, and modulation of immune response (Yahia et al., 2019). However, they are mainly found in the pericarp, aleurone layer and germ, which can be harmed by grinding (Taylor, 2016).

Table 1. Comparison in the nutritive value of pearl millet with wheat and rice.

Nutrients	Constituents (per 100g)		Pearl Millet	Wheat	Rice
Macro nutrients	Carbohydrates	Sugars (g)	67.5	71.2	78.2
		Fiber (g)	1.2	1.2	0.2
	Proteins (g)		11.6	11.8	6.8
	Fats (g)		5.0	1.5	0.5
Micronutrients					
Minerals	Calcium (mg)		42.0	41.0	10.0
	Phosphorous (mg)		296.0	306.0	160.0
	Iron (mg)		8.0	5.3	0.7
	Zinc (mg)		3.1	2.7	1.4
	Sodium (mg)		10.9	17.1	-
	Magnesium (mg)		137.0	138.0	90.0
Vitamins	Vitamin A (mcg)		132.0	64.0	0.0
	Thiamine (mg)		0.3	0.5	0.1
	Riboflavin (mg)		0.3	0.2	0.1
	Niacin (mg)		2.3	5.5	1.9
	Folic acid (mcg)		45.5	36.6	8.0

Source: Satyavathi et al., (2017)

Despite having a rich nutritional composition, pearl millet also has some anti-nutritional factors, such as phytic acid, which has a chelating capacity and decreases the bioavailability of minerals, especially zinc, potassium, calcium, iron and magnesium (Boncompagni et al., 2018). In addition to having a higher content of oligosaccharides of the raffinose family (~8%) than other cereals (0.1 – 1.6%) and like legumes (e.g. pea 6.9%), these are also considered antinutritional factors as they have lower bioavailability and cause discomfort and flatulence (Henry & Saini, 1989; Jones et al., 1999; Mondal et al., 2022). However, there is increasing recognition of the health benefits associated with many anti-nutrients, especially in preventing diseases like cancer and heart disease (Champ, 2002). In addition, the presence of phenolic compounds, mainly of the flavonoid class such as condensed tannins and *C*-glucosyl flavones, known to be goitrogenic, also act as antinutritional factors in pearl millet.

In general, these antinutritional factors reduce the digestibility of proteins and carbohydrates, complex minerals by decreasing bioavailability, and impair the performance of proteolytic and amylolytic enzymes (Boncompagni et al., 2018; Nambiar et al., 2011). It should be noted that the adverse biological effects caused by these antinutritional factors, mainly related to thyroid metabolism, are variable and depend on the genotype, the composition of phenolic compounds (PC), and the type of processing applied (Boncompagni et al., 2018; Theodoro et al., 2021).

PC are the most abundant and numerous phytochemicals in plants (Yahia et al., 2019). In pearl millet grains, the main ones are cinnamic acid derivatives (hydroxycinnamic acid, coumaric acid, ferulic acid, sinapic acid) and in smaller concentrations benzoic acid derivatives (hydroxybenzoic acid, gallic acid, *p*-hydroxybenzoic acid, vanillic, syringic and protocatechuic) (Nani et al., 2015). Phenolic compounds have numerous bioactivities related to potential beneficial effects on health, mainly related to antioxidant and anti-inflammatory activity, demonstrating a preventive effect on several diseases such as diabetes, obesity, cancer, cardiovascular diseases, osteoporosis, neurodegenerative diseases, among others (de Araújo et al., 2021). A diet rich in cereal PC has even been associated with improved gut health (Rhowell Jr et al., 2022), due to its involvement in modulating the microbiota and gut immune response, however, these interaction mechanisms are not yet fully elucidated (Zhang et al., 2020).

PC are secondary plant metabolites that are synthesized in adverse situations to act as protective factors. In cereals, they have a protective role in conditions of extreme temperatures, drought and salinity, through the capacity of osmoregulation (Chalker-Scott, 1999). They also have other interesting properties such as color and flavor checking, synthesis of enzymes and vitamins, in addition to minimizing the effects of lipid peroxidation (Vuolo et al., 2019). However, its content in the plant matrix depends on several factors, including genetic variety of species, soil and climate conditions, growth conditions, and others (de la Rosa et al., 2019). In cereals, the phenolic profile can also vary between genotypes, aiding in the selection of varieties for greater retention of bioactive compounds, in addition to also varying during the grain maturation (Santos et al., 2019). In fact, the stage of maturation of the grain is decisive, since the formation of PC occurs from the amino acids tyrosine, phenylalanine and malonate, in the early stages of grain development (Zhen et al., 2016).

Chemically, PCs are substances composed of at least one aromatic core containing one or more hydroxyl groups. PCs are widely distributed in plant tissues and can be found in fruits, seeds, leaves, stems, and roots in soluble (free or conjugated esters) and insoluble (bound) forms. Soluble PCs are present in free forms within the vacuole of the plant cell or in conjugated form esterified to sugars or other low molecular weight compounds, whereas insoluble phenolics are covalently bound to plant cell wall components (Zhang et al., 2020).

There are more than 8000 PC already identified that are divided into two major groups: flavonoids and non-flavonoids (Figure 3). Flavonoids have in their chemical structure two aromatic rings connected by a heterocyclic ring (C6-C3-C6) and are most often found in their glycosylated form (Bravo, 1998). The class of flavonoids is divided into several subclasses, the main ones being: anthocyanins, flavanones, isoflavones, flavones, flavonols, and flavanols. Among the non-flavonoid compounds, there are stilbenes, lignans, coumarins and phenolic acids, which are of great interest for human consumption. Phenolic acids can be derived from hydroxycinnamic acid (C6-C3) (e.g., caffeic acid, *p*-coumaric acid) or hydroxybenzoic acid (C6-C6) (e.g., vanillic acid, gallic acid, ellagic acid) (Mazza & Brouillard, 1987).

PCs are distributed differently in the different tissues of cereal grains. About 60% of the PC of millet is found in the bound form and is concentrated in the outer layers of the grain (bran and aleurone layer) which represent about 12% of the grain mass (Devi et al., 2014). However, to date, the literature lacks and diverges information regarding the identification and quantification of these compounds in pearl millet.

The main PCs found in pearl millet are ferulic (trans and cis), *p*-coumaric and cinnamic acids (McDonough et al., 2000), and in pearl millet *p*-coumaric acid represents about 80%, and ferulic acid 12% of the total PC (Nani et al., 2015). In addition, a study evaluating seven varieties of pearl millet by paper chromatography identified the following PCs: vanillic, syringic, salicylic, benzoic p-OH, and melilotic acid (Nambiar et al., 2012). Also, ferulic acid was identified as the most abundant phenolic acid bound, while protocatechuic acid was the most abundant free phenolic acid in the finger millet species (Subba Rao & Muralikrishna, 2002).

Pearl millet contains a diversity of flavonoids including anthocyanidins, chalcones, flavanols, flavones and flavanones. In addition, some colored grain varieties harbor proanthocyanidins (condensed tannins), which contribute substantially to the reddish coloration (Dykes & Rooney, 2006; McDonough et al., 2000; Serna Saldivar, 2003). In pearl millet, flavonoids account for approximately 90% of the free PC content (Balli, Bellumori, Orlandini, et al., 2020; Balli, Bellumori, Pucci, et al., 2020). The presence of three *C*-glycosylated flavonoids (luteolin-(7-*O*-glucopyranosyl)-8-*C*-glucopyranoside, vicenin II and vitexin), one Flavonoid *O*-glucosylated (vitexin 2"-*O*-rhamnoside) and five cinnamic derivatives (Balli, Bellumori, Orlandini, et al., 2020; Balli, Bellumori, Pucci, et al., 2020) stand out

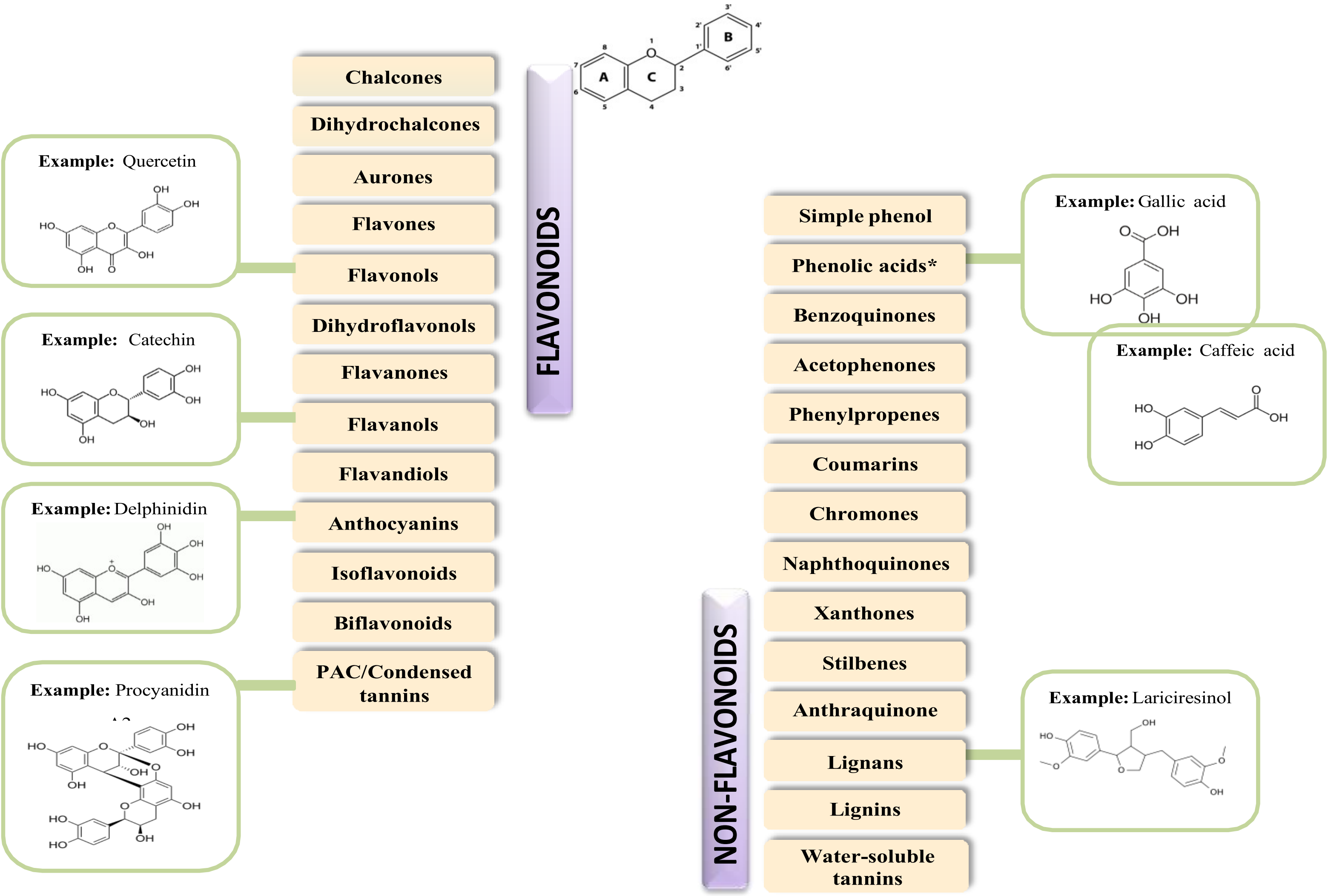

Figure 3. Major classes of phenolic compounds
Source: Rodríguez Pérez, 2016

4. METABOLOMICS TOOLS FOR METABOLITE SCREEN PROFILING AND CROP NUTRITIONAL ELUCIDATION

Metabolomics is a large-scale analytical approach used to characterize the set of low molecular weight molecules synthesized by living organisms (metabolome) (Jacobs et al., 2021). It can be divided into quantitative or semi-quantitative analyses of known metabolites (targeted) or a global analysis of metabolites in a qualitative and semi-quantitative manner (untargeted) (Sussulini, 2017). In a metabolomic experiment, the goal is to gather as much metabolic information as possible from an organism or biological system (Xu et al., 2014; Yi et al., 2016).

Different approaches are employed to assist in the characterization of the metabolome and can provide insight into metabolism. For example, metabolic footprinting, which evaluates the metabolites excreted by a cell/organism under controlled conditions; metabolic profiling, used to identify and quantify predefined metabolites generally related to a specific metabolic pathway; and metabolic fingerprinting, a global, high-throughput, and rapid analysis to provide sample classification, also used as a screening tool to discriminate samples from different biological states or origins (Astarita & Langridge, 2014; Ellis et al., 2007).

The most commonly used equipments include nuclear magnetic resonance (NMR) and mass spectrometry (MS). Generally, mass spectrometry is coupled with a separation technique, such as gas chromatography (GC-MS), liquid chromatography (LC-MS), or capillary electrophoresis (CE-MS), which increases selectivity, sensitivity, separation efficiency, and provides structural and molar mass information. These techniques is regarded as an indispensable and irreplaceable tool for the analysis of biomolecules and has been widely used to characterize and quantify metabolites of grain crops (Lima et al., 2024).

Metabolomics can bring insights through the characterization of health-relevant metabolites, particularly bioactive compounds. From a national perspective, Santos et al., (2019) elucidated the phenolic composition and regulation of metabolism in seven Brazilian wheat (*Triticum aestivum*) genotypes throughout grain maturation. The phenolic composition of wheat depends on the technological quality, being higher in flours with higher gluten content (Santos et al., 2022). Moreover, the first study to use an omics approach for the characterization of PC in pigmented rice bran identified black rice bran as potential for human nutrition (Santos et al., 2021). Additionally, it was seen that thermoplastic extrusion can significantly increase the content of free PC in sorghum (*Sorghum bicolor* (L.)) genotypes with condensed tannins (D'Almeida et al., 2021). In pearl millet, Chandrasekara & Shahidi (2011) performed the first reported metabolic profiling work applying LC-MS/MS demonstrating that bound PC are the most abundant for this cereal, but it should be noted that the composition of PC varies between different fractions, genotypes and environment conditions. Furthermore, the profile of phenolic compounds specifically accumulated in grains of hybrid millets was demonstrated (Li et al., 2018).

From another perspective, various studies have demonstrated the potential of metabolomics to assist plant breeding programs. For example, Dhawale (2022) identified key metabolites associated with drought tolerance in millet (*Panicum sumatrense L.),* contributing to the understanding of abiotic stress responses. Cao et al., (2022) revealed the molecular mechanism of drought-induced stress in the species *Panicum miliaceum*, demonstrating an important role in the expression of anthocyanins. Additionally, grains from four genotypes of durum wheat (*Triticum durum Desf.*) grown over three consecutive years unveiled major influences of genotype-environment interactions affecting levels of phytosterols, tocopherols and unsaturated fatty acids (Beleggia et al., 2013).

Untargeted metabolomics can also be used to discover biomarkers, which predict phenotypes before these characteristics become apparent, thereby optimizing the selection of crop populations (Steinfath et al., 2010). To address the challenge of the complexity of metabolic data (e.g. outnumbering features in the samples, high noise levels, batch effects, missing values), employing mathematical and machine learning (ML) tools has proven effective in supporting metabolomics data analysis (Liebal et al., 2020). In tartary buckwheat (*Fagopyrum tataricum*), three predictor biomarkers (kaempferol-3-O-hexoside, kaempferol-7-O-glucoside, and naringenin-O-hexoside) were established to estimate the total phenolic content and antioxidant capacity of seeds using a random forest model (Wang et al., 2023). Moreover, metabolite levels in the tyrosine pathway were effective predictors of yield when analyzing five hybrid rice varieties (Dan et al., 2021). One step further, predictive multi-omics successfully predicted 21 agronomic traits across 197 recombinant inbred lines of foxtail millet over three distinct years (Wei et al., 2023).

In this context, the present work proposed to apply untargeted metabolomics and predictive tools to initially characterize the metabolites in pearl millet grains and subsequently identify predictive biomarkers for important phenotypic trait

CHAPTER 4: MAIN CONCLUSION

Pearl millet is emerging prominently in a challenging global context where natural resources are being depleted, and climate change is impacting major cereal crops. Additionally, population growth and the urgent need to ensure global food security position it as a key player. Despite these advantages, it remains an underutilized crop with significant nutritional potential that needs to be fully leveraged. The comprehensive metabolomic analyses conducted on a large sample set of pearl millet have provided the background robustness of our findings. Our studies, exploring both the metabolite diversity in open-pollinated populations from the Brazilian Cerrado and the predictive metabolomics of phenotypic traits in a Brazilian germplasm core collection, collectively emphasize the power of untargeted metabolomics.

Metabolic profiling revealed what can be described as the unique metabolic signature of pearl millet, featuring a broad spectrum of lipid compounds and phenylpropanoids and polyketides. Eight phenolic compounds were annotated, gingerol, caffeate, *p*-coumaric acid and diosmin as level 1. Furthermore, flavonoids showed the highest variability in abundance among the samples. All these results highlight the bioactive potential of pearl millet grains. The integration of predictive metabolomics with machine learning has proven effective in predicting eight qualitative phenotypic traits. Moreover, this approach has successfully identified predictor biomarkers. Of these, 23 belong to the metabolic signature, and 15 are associated with high variability in abundance in the OPP samples. This study represents the first attempt to exploit a germplasm bank of pearl millet for metabolome characterization and predictive modeling of critical phenotypic traits, providing a valuable tool for future breeding programs to develop cultivars based on both agronomic attributes and metabolites of interest for food applications. From a future perspective, integrating the search for metabolites of interest and biomarkers with Genome-Wide Marker Association Studies could be instrumental in identifying genes and crucial biochemical pathways associated with these markers.

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