Millet starch as food-grade Pickering particles in emulsion stabilization: Fabrication, characterization, and application studies

By

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Navami M M

DEDICATION

To the experiences we never expected and the paths that were redirected

To my own family and friends and the ones I found along the way….

"When it looks impossible, look deeper. And then fight like you can win"

-Rost, Horizon Forbidden West

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SYMBOLS

β	Beta
α	Alpha
µ	Micro
µm	Micrometer
g	Gram
mg	Milligram
µg	Microgram
%	Percentage
°C	Degree Celsius
mL	Milliliter
µl	Microliters
h	Hour
min	Minutes
w/v	Weight by volume
nm	Nanometre
mV	Millivolt
rpm	Rates per minute
ppm	Parts per million
Min	Minutes
Kb	Kilobase
mL	Milliliter
ppb	Parts per billion

ABBREVIATIONS

RSC	Radical Scavenging Activity
DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	2, 2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
GAE	Gallic Acid Equivalent
QE	Quercetin Equivalent
WHC	Water Holding Capacity
OHC	Oil Holding Capacity
WRC	Water Retention Capacity
SC	Swelling Capacity
CFU	Colony Forming Units
PDCAAS	Protein Digestibility Corrected Amino Acid Score
DIAAS	Digestible Indispensable Amino Acid Score
HPLC	High Pressure Liquid Chromatography
LCMS/MS	Liquid Chromatography Mass Spectrometry
TPC	Total Phenol Content
TFC	Total Flavonoid Content
AMY	Amylose
AMP	Amylopectin
CE	Collision Energy

AAE	Ascorbic Acid Equivalent
SCFA	Short Chain Fatty Acids
MRS	De Man, Rogosa And Sharpe
GRAS	Generally Regarded As Safe
SDF	Soluble Dietary Fiber
IDF	Insoluble Dietary Fiber
MRM	Multiple Reaction Monitoring

PREFACE

Millets are collective group of small seeded annual grasses that are grown as grain crops, primarily on marginal land in dry areas of temperate, sub-tropical and tropical regions. They belong to the Poaceae family and are considered ancient grains. Millets are highly resilient and can grow in a variety of environmental conditions, particularly in semi-arid regions. They are a staple food source in many parts of Africa and Asia. There are several types of millets, including pearl millet, sorghum, finger millet, foxtail millet, proso millet, and barnyard millet, kodo millet and little millet among others. Each type has its own unique characteristics and nutritional profiles. These properties are altered when the millet grains are germinated. The major constituent of the millet is its starch which contributes about 60% of total millet grain and decides the quality of millet-based food products. The application of starch for various purposes is dependent upon its physicochemical, structural, and functional properties. Native form of starch has limitations in its functionality therefore modifying it by physical, chemical or enzymatic treatments alters its properties. Physical modification methods are more viable than chemical treatments as they are economical, non-toxic, and natural.

Millet starch finds application as a gluten-free thickening agent and stabilizer in the food industry, enhancing the texture and stability of a variety of products, from soups and sauces to gluten-free baked goods. Additionally, it has potential in non-food industries, such as pharmaceuticals and textiles, where starch-based materials can serve as binders, coatings, and sizing agents. However, the domain for the utilization of millet starch as Pickering particles remains largely uncharted. Pickering particles are solid particles that are employed to stabilize emulsions. Unlike traditional emulsifiers, Pickering particles are characterized by their ability to adsorb at the interface between two immiscible phases, such as oil and water, forming a protective layer that helps prevent coalescence and

separation of the phases. They have a wide range of applications in industries such as food, cosmetics, and pharmaceuticals, where stable emulsions are essential for product quality and performance. With this background, we focussed on fabricating Pickering particles from millet starch using physical modification method of micronization. These particles were then used for the preparation of stable O/W emulsions for food applications.

Chapter 1 provides a comprehensive introductory overview and literature review focused on the subject matter, encompassing millet starch and its inherent properties, the concept of Pickering emulsions, the technique of micronization as a means of physically modifying starch particles, the significance of and applications for coffee oil emulsions, and the relevance of plant-based dairy alternatives within the broader context.

Chapter 2 encompasses an extensive inquiry into the comprehensive nutritional, functional, phytochemical, and antioxidant profiles of nine distinct millet varieties, encompassing both their unaltered and germinated states. The process of germination was observed to yield significant enhancements in the nutritional, phytochemical, and antioxidant attributes of the millet grains. These initial findings laid the foundation for the subsequent exploration of potential applications for germinated millet grains in the realm of food production. Analysis of the proximate composition unveiled carbohydrates as the primary nutritional components in millets, followed by protein and fat. Notably, the carbohydrate content was predominantly constituted by starch, constituting 60% of the overall compositional makeup. Consequently, our study adopted a specific focus on the extraction and characterization of starch derived from millet grains.

Chapter 3 focusses on the isolation of starch fractions derived from millet grains, followed by their subsequent reduction in particle size. Extensive characterizations were conducted to assess the structural, functional, and thermal characteristics of these starch fractions.

The core objective of this chapter is to validate and demonstrate the feasibility of utilizing micronized starch particles derived from various millet varieties as Pickering particles for the purpose of stabilizing emulsions. Comparatively, among various millet types, micronized starch particles extracted from pearl millet and proso millet exhibited superior suitability for the formulation of oil-in-water (O/W) emulsions. This suitability was evaluated based on multiple parameters, including particle size, zeta potential, and hydrophilic properties. The emulsions were carefully prepared utilizing starch concentrations below its critical micelle concentration (CMC), specifically at 2.0%.

Chapter 4 involves the development of oil-in-water (O/W) Pickering emulsions containing coffee oil, employing varying concentrations of pearl millet starch as the stabilizing Pickering particle. Following multiple experimental iterations, three distinct emulsion formulations were chosen for comprehensive analysis, specifically a 40% coffee oil emulsion (designated as 40E), a 20% coffee oil emulsion (referred to as 20E), and a 20% ultrasonicated coffee oil emulsion (abbreviated as 20UE). After the formulation phase, the emulsions underwent a thorough characterization process to evaluate key parameters. These assessments encompassed droplet size, zeta potential, pH, density, color attributes, Fourier-transform infrared (FTIR) spectroscopy analysis, and rheological behavior. The microemulsions exhibited improved polydispersity index (PDI) and enhanced zeta potential values. Additionally, they demonstrated non-Newtonian shear-thinning behavior. Further validation through fluorescence microscopy images confirmed the effective encapsulation of coffee oil within micronized starch particles, thus underscoring the stability of the emulsions.

Chapter 5 is dedicated to the formulation of a plant-based dairy alternative beverage with coffee flavour. The foundational component of this beverage is sprouted finger millet milk, denoted as the Control (C). It incorporates Pickering emulsions at two varying

concentrations: 15% (v/v) designated as CB1 and 25% (v/v) denoted as CB2. This chapter meticulously scrutinizes the stability of the developed beverage over a 28-day period under refrigerated conditions. Routine evaluations were conducted at 7-day intervals to monitor key parameters, including brix, pH, color characteristics, and absorbance. The control sample displayed a brix value of 11.99%, closely aligning with that of traditional cow's milk. The prepared beverages exhibited brix values of 14.70% (CB1) and 14.41% (CB2), mirroring the standard. Notably, pH levels remained consistently at 6.00, with a minor reduction observed from the 21st to the 28th day of storage. A notable reduction in lightness was observed in the control samples compared to the test samples, where the incorporation of flavor emulsions notably elevated lightness values. Additionally, the introduction of coffee oil-flavored emulsions effectively maintained the color attributes of the test samples. Sensory analysis indicated that the 15.0% emulsions (CB1) received higher acceptance scores in terms of color, taste, aroma/flavor, mouthfeel, and overall acceptability, surpassing both the control sample (C) and the 25% emulsion sample (CB2) in these sensory aspects.

The current investigation illustrates the effective conversion of millet starch particles into high-quality Pickering particles through the physical process of size reduction. Size reduction, a straightforward method of physical modification, induces substantial alterations in starch particle properties. Reducing starch particle size results in a proportional decrease in Pickering emulsions' droplet size, concurrently enhancing their storage stability. This size reduction facilitates improved packing efficiency, creating a more cohesive barrier at the oil-water interface, thus enhancing Pickering particle characteristics.

Furthermore, the reduction in particle size leads to modified contact angles, rendering the particles superior Pickering agents. Additionally, the incorporation of the prepared

Pickering emulsion into sprouted finger millet milk demonstrates its potential as a flavor emulsion. It is noteworthy that millet milk stands out as a preferred dairy substitute due to its superior nutritional profile compared to other plant-based milk sources. This positions it as an excellent alternative to dairy, aligning with the prevailing dietary trends emphasizing high-nutrition and low-calorie choices. This study assumes heightened significance against the backdrop of the international recognition of 2023 as the International Year of Millets, further underscoring the relevance and timeliness of the research.

Chapter 1

Introduction and Review of Literature

1. Introduction

Starch, the second most abundant natural polysaccharide after cellulose, is widely present in the roots, seeds, and leaves of various plants, as well as in some algae. Starch is a highly sought-after biomaterial used in both food and non-food industries because of its abundance, cost-effectiveness, biodegradability, and nontoxic properties (Punia, 2020). Starch, known for its versatility, finds application in an extensive array of food products, including bakery goods, confectionery items, dairy products, soups, sauces, gravies, snacks, batters, coatings, and meat products. Additionally, starch has numerous non-food applications including pharmaceuticals, textiles, alcohol-based fuels, and adhesives (Kaur et al., 2012). Starch granules primarily consist of two main fractions, amylose (AMY) and amylopectin (AMP). AMY is a linear polymer of glucose units linked through α-(1→4) linkages, whereas AMP is a highly branched molecule with numerous short chains that are connected through α-(1→6) linkages to the linear segments of the macromolecule (Wu et al., 2013). Starch production worldwide amounts to approximately two billion tons annually, derived from crops such as rice, maize, wheat, barley, and others. With the projected increase in global population, demand for starch as a food source is expected to rise to 9 billion tons by 2050. However, due to urbanization, the availability of land for cultivating starchy crops is likely to be limited, potentially leading to reduced food supplies (Godfray et al., 2010). Therefore, there is a demand for newer sources of and millets offers a promising source material for the commercial production of starch.

Millet, a collection of small-seeded grains belonging to the Poaceae family, has served as a staple food for humans for over 10,000 years, predating the cultivation of wheat and rice. The term "millet" originated from the French word "Mile," meaning "thousand,"

symbolizing the abundance of grains in a small quantity of millet. These grains are commonly grown in regions characterized by limited rainfall and on marginal or depleted lands with low nutrient levels. Millets play a crucial role in sustaining the livelihoods of individuals living in famine-prone regions by providing a more reliable harvest than other crops in areas with low levels of precipitation (Tadele, 2016). Millets, with their gluten-free nature, low acidity, low glycemic index, and various other advantageous characteristics, possess significant potential for continued utilization due to their minimal maintenance requirements and drought tolerance. As a result, these grains are acknowledged as "nutri-cereals" and "cereals of the future" owing to their sustainable nature and compatibility with the environment (Kaur et al., 2023). The primary constituents of these underutilized grains are starch, proteins, and lipids, with relatively small quantities of non-starch polysaccharides and free sugars (Babu et al., 2019). Due to the decreasing accessibility of traditional starch sources to meet the escalating dietary demands of the expanding population, there exists a necessity to investigate novel starch sources exhibiting enhanced functional attributes. Starch, renowned for its diverse functionalities in the food industry, including surfactant, emulsifier, and thickening agent properties, has witnessed amplified market demand. Additionally, starch derived from underutilized sources like millet demonstrates compatibility with numerous food and non-food industrial applications.

1.1 “Millets” – The nutri-cereals

Millets are small, seeded grains that belong to several plant taxonomic groups and are produced in tropical and semi-arid regions of the world. They are considered as underutilized cereals whose potential is not fully exploited. Millets are designated as

‘superfoods’, owing to their high nutritional content (Tiwari et al., 2023). Referred to as nutri-cereals, millets are rich in minerals like calcium, iron, zinc, potassium, and magnesium as well as proteins, vital fatty acids, dietary fibre, and B vitamins. They assist in providing health benefits such as lowering blood sugar (diabetes), controlling blood pressure, and preventing thyroid, cardiovascular, and celiac disorders (Dayakar et al., 2017). Millets are members of the Poaceae family (formerly known as the Gramineae). Millets are taxonomically categorized into major millets and minor millets based on their grain size. The term "pseudo millets" is attributed to millets that do not belong to the Poaceae botanical family, which includes true grains; however, they exhibit similar nutritional characteristics and are utilized in comparable manners to true grains. As per the guidelines set forth by the Food Safety and Standards Authority of India (Guidance note no. 12/2019) in 2019, major millets encompass *Pennisetum glaucum* or pearl millet, finger millet (*Eleusine coracana*), and sorghum (*Sorghum bicolor*). On the other hand, minor millets comprise foxtail millet (*Setaria italica*), proso (or white) millet (*Panicum miliaceum*), Kodo millet (*Paspalum setaceum*), little millet (*Panicum sumatrense*), and barnyard millet (*Echinochloa utilis*). Additionally, buckwheat and amaranth are categorized as pseudo millets, despite their non-membership in the Poaceae family, due to their nutritional similarities and utilization patterns analogous to true millets (Liang and Liang, 2019; Sharma et al., 2022). *Pennisetum glaucum* or pearl millet, is a prominent variant that accounts for 40% of global output. Foxtail millet (*Setaria italica*), proso (or white) millet (*Panicum miliaceum*), and finger millet (*Eleusine coracana*) are the next most popular types. Kodo millet (*Paspalum setaceum*), small

millet (*Panicum sumatrense*), and barnyard millet (*Echinochlos utilis*) are other well-known millet variants (Liang, 2019).

According to FAOSTAT (2019), global production of millets was approximately 28.37 million tons, with India being the dominant producer, contributing to 40.62% of the total production. Currently, over 50% of the total millet production is being utilized for alternative purposes rather than just being consumed as a staple food. Millets are primarily used as food grains in India, Africa, Eastern Europe, and other Asian countries. Africa accounts for over 40% of the total global consumption of millets. Millets can be grown easily on shallow and low fertile soils with a pH range of 4.5 to 8.0, and they require very low amounts of water during their short growth period (Kumar et al., 2018). They are also drought-resistant and have a prolonged storage period if stored as whole grains. As a result, millets are considered to be important crops for food security due to their ability to sustainably grow in harsh agro-climatic conditions (Devi et al., 2014). Following the decision of United Nations General Assembly, 2023 is designated as the International Year of Millets to highlight the significance of these nutrient-dense crops and to place millets in the global forefront (Dayakar et al., 2021). The different kinds of millets are illustrated in fig. 1.1.

Fig. 1.1 Various types of millets

1.1.2 Nutritional profile of millets and its modification through germination

Millets, nutritionally rich grains, typically comprise 60-70% carbohydrates, 7-11% proteins, 1.5-5% fats, and 2-7% crude fiber. They are abundant in essential vitamins and minerals, particularly vitamin B, magnesium, and antioxidants. Additionally, millets serve as valuable sources of essential dietary minerals, including manganese, phosphorus, and iron. Millets are protein-rich, offering essential amino acids, albeit with lower levels of lysine and threonine. They are relatively high in sulfur-containing amino acids like methionine and cysteine (Singh et al., 2012). Millets also contain essential fatty acids such as linoleic, oleic, and palmitic acids, both in free form and bound within compounds like monogalactosyl diacylglycerols, digalactosyl diacylglycerols, phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine. Trace amounts

of fatty acids like arachidic acid, behenic acid, and erucic acid are present. Notably, millet oil is a valuable source of linoleic acid and tocopherols. Furthermore, millets are alkaline-forming grains and are gluten-free. They contain essential vitamins, including Niacin, folacin, riboflavin, and thiamine, as well as phosphorus, which play crucial roles in energy synthesis within the body. In summary, millets constitute a nutritionally dense grain with a diverse array of vital nutrients, making them a valuable component of a balanced diet (Saritha and Singh 2016).

In the realm of food research, an array of methodologies is explored to elevate the nutritional quality of food products. These methodologies encompass diverse techniques, including processing methodologies, biotechnological applications, and nutrient fortification strategies. Conventional methodologies such as steeping, germination, and fermentation are commonly employed to enhance the nutritional composition of food materials (Haritha et al., 2024). Of particular importance is the process of grain germination, which serves both practical and nutritional enhancement purposes. The nutritional significance of germinated millets lies in the augmentation of their nutrient profile facilitated by the germination process (Sharma, 2023). This process involves the activation of enzymes, which catalyze the hydrolysis of complex compounds such as starch, proteins, and fats into simpler forms, including sugars, amino acids, and fatty acids. Germination plays a crucial role in enhancing the physical characteristics and processability of grains, in addition to augmenting their palatability, flavor, and safety. Furthermore, germination is recognized as a significant technique for cost-effective and sustainable enhancement of the nutritional composition and health-promoting properties of grains (Xiang et al., 2023). The principal objective of germination is to activate

hydrolytic enzymes that remain inactive in raw seeds. Throughout the germination process, the biosynthetic potential of cereal grains is harnessed, leading to the development of various hydrolytic enzymes. These enzymatic reactions occurring in germinated grains induce structural alterations and the synthesis of novel compounds exhibiting bioactivity, thereby augmenting the nutritional profile and overall stability of the grains (Budhwar et al., 2020).

1.1.3 Antioxidant composition of millets

The growing interest in natural antioxidants can be attributed to the radical scavenging capacity (RSC) exhibited by phytochemicals, particularly phenolic compounds, flavonoids, and tannins. Millet, in particular, is rich in primary polyphenols such as phenolic acids and tannins, which are known for their antioxidative properties and their pivotal role in enhancing the body's immune system (Chandrasekhara and Shahidi, 2010). Devi et al. (2014) have reported a multitude of health benefits associated with the phenolic content in millets, including their capacity to act as antioxidants, mitigate inflammation, and exhibit antiviral properties. Furthermore, it is noteworthy that celiac disease is a genetically predisposed condition triggered by the ingestion of gluten. Since millets are devoid of gluten, their consumption can reduce the risk of celiac disease by mitigating the gastrointestinal irritation caused by conventional cereal grains containing gluten (Saleh et al., 2013).

1.2 Emulsions and emulsifiers – An overview

Oil-in-water (o/w) emulsions are widely utilized in the food and pharmaceutical industries as delivery systems for lipophilic flavorings, functional lipids, antioxidants, and various

other bioactive compounds (Jiang et al., 2014; Yang et al., 2012). To attain the desired stability, an emulsifier is required to decrease the interfacial tension between the oil and water phases. The various instability mechanisms in emulsions are shown in fig. 1.2 below. Emulsifiers are categorized based on their origin (natural/synthetic), hydrophilic-lipophilic balance (HLB), physical state (solid/liquid), and ionic character (ionic/non-ionic). Small molecular weight surfactants are conventionally utilized as emulsifiers, including amphiphilic biopolymers such as soluble proteins or polysaccharides, protein-polysaccharide complexes, and other ionic and non-ionic surfactants (Chen et al., 2020; Chen, et al., 2016 Rayner, et al., 2014). However, some commonly used synthetic emulsifiers, such as carboxymethylcellulose and polysorbate-80, can lead to low-grade inflammation, obesity/metabolic syndrome, and other chronic inflammatory diseases in wild-type mice due to their interference with the essential intestinal microbes that are critical to human health (Chassaing et al., 2015). Consequently, food colloids research groups worldwide are seeking alternative emulsifiers to address the limitations of conventional emulsifiers.

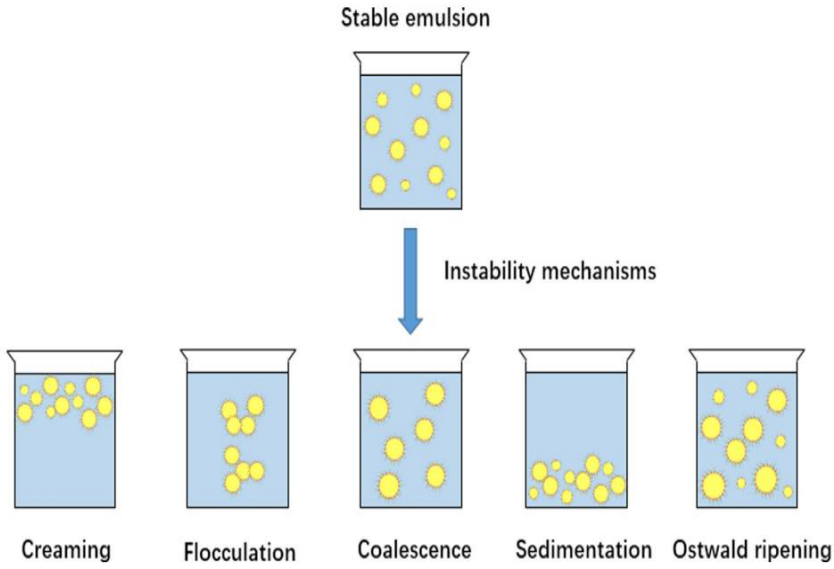

Fig. 1.2 Various instability mechanisms in emulsions (Reprinted with permission from Shao et al., 2020).

1.2.1 Pickering emulsions

In recent times, the use of solid colloidal particles to stabilize emulsions has gained traction. These particles, referred to as "Pickering particles," serve as interface stabilizers and result in the formation of "Pickering emulsions." Compared to conventional emulsifiers, Pickering particles offer numerous advantages. Due to their partial dual wettability, these solid particles can spontaneously and irreversibly adsorb onto the oil-water interface and surround the droplets, resulting in stable emulsions. The colloidal particles form a dense adsorption layer/film around the droplets to provide a physical barrier, preventing interactions between adjacent droplets and ultimately leading to emulsion stability (Chen et al., 2020; Xiao et al., 2016). Due to their strong adsorption, Pickering emulsions typically exhibit long-term stability and are more resistant to coalescence and Ostwald ripening than emulsions stabilized by conventional molecular

surfactants (Aveyard et al., 2003). Moreover, Pickering particles can stabilize emulsions at much lower concentrations than conventional emulsifiers, and they are mostly derived from natural sources, making them biocompatible and suitable for use as carriers for delivering bioactive substances (Yang et al., 2017). Two types of Pickering emulsions are shown in fig 1.3 based on the surface chemistry of the particle that conducts the wetting properties. If the contact angle of the particle is less than 90°, then its suitable for the preparation of o/w emulsions and if its more than 90° its suitable for the preparation of w/o emulsions.

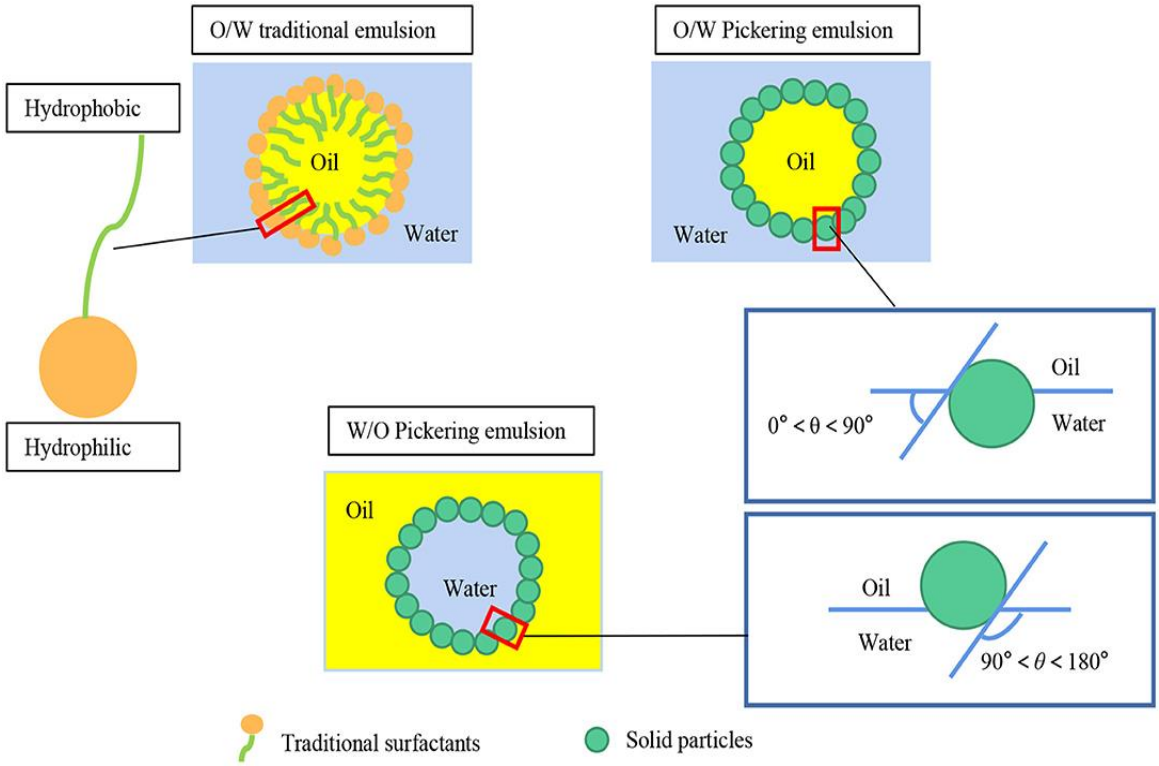

Fig 1.3 Description of two types of Pickering emulsions (Li et al., 2022)

1.2.2 Food-grade Pickering particles

Recently, a considerable number of food-grade Pickering particles have been identified and categorized into six major categories, including polysaccharides (such as starch and chitosan), proteins (including whey protein and zein), complex particles (including hydroxyapatite, silica, clay, and magnetic nanoparticles), flavonoids, food-grade wax, and fat crystals (Xia et al., 2021). Among these categories, starch, especially modified starch, has gained attention as an established Pickering particle with potential food applications. Starch is a Generally Recognized As Safe (GRAS), non-allergenic, abundant, and cost-effective food ingredient, which comprises linear AMY and branched AMP that are intrinsically assembled in their granular forms (Punia, 2020). The structure and properties of native starches from different plant sources are diverse, with well-known sources of plant starches being potatoes, corn, legumes, yams, and sweet potatoes. Millet varieties are recent additions to this list, where starch accounts for approximately 70% of the grain weight (Alcazar et al., 2015).

Due to their inherent properties, starch granules derived from various plants can act as food-grade Pickering particles, including millet starch. However, native starch granules have several limitations such as poor stability, inadequate hydrophobicity, and large size, which prevent their adsorption at the oil-water interface and hinder their ability to stabilize Pickering emulsions (Chen et al., 2020; Tavernier et al., 2016). To overcome these limitations, various physical and chemical methods have been utilized to modify native starch, including hydrothermal treatment, dry heat treatment, ultra-high-pressure treatment, acid hydrolysis, enzymatic modification, addition of cross-linkers, nanoprecipitation (Ge et al., 2017), and esterification (Kaur et al., 2012). Physical

modifications are preferred over chemical treatments due to their cost-effectiveness, non-toxicity, and natural properties (Chen et al., 2015). Furthermore, physically modified starch can be used as a clean-label ingredient in food products, making it more appealing to consumers in terms of food safety and preference (Park & Kim, 2021).

1.3 Millet starch as an emerging source of starch

The depletion of traditional reservoirs of starch to meet the escalating nutritional demands of the expanding global populace has catalyzed the investigation into novel starch reservoirs endowed with superior functional attributes. Starch, owing to its multifarious utility in the realm of nutrition, especially as a surfactant, emulsifying agent, and thickening agent, has witnessed a surge in market demand. Furthermore, the starch derived from under-explored sources, such as millets, exhibits compatibility with a wide spectrum of applications within both the food and non-food industrial sectors (Punia et al., 2021). The classification of millets is primarily predicated on their AMY content, delineating them into two principal categories: nonwaxy millets, characterized by high AMY content, and waxy millets, distinguished by their low AMY content (Kumar et al., 2023).

1.3.1 Isolation of millet starch and yield

To extract starch from millet grains, it is necessary to first solubilize the protein matrix, which then tightly binds the starch granules. This can be achieved by using various chemical reagents and methods. Wet milling, a prevalent technique employed for the extraction of millet starch, entails the immersion of millet kernels or flour in an aqueous medium, facilitating the isolation of starch from accompanying constituents. Typically,

this process utilizes either water exclusively or employs the acidic and alkaline steeping methods. Typically, starch extraction involves three consecutive stages: anatomical fragmentation, cellular breakdown, and final purification of the extracted starch (Mahajan et al., 2021). The selection of an appropriate extraction solution for millet starch extraction is contingent upon the specific composition and characteristics of the millet variety under investigation. It is essential to employ an extraction method that achieves a high starch concentration while minimizing protein residues. For instance, the acidic steeping method yields a higher protein residue (4.3%) within the granules compared to the alkaline method (0.7%) as reported by Yanez et al. (1986). The alkali isolation method, originally developed by Dimler et al. (1944), is widely acknowledged as the most efficacious technique for protein removal. Following this, the recovered starch is isolated using centrifugation and subsequently subjected to the drying process. To mitigate bacterial growth and amylase activity during starch extraction, a small quantity of sodium azide (0.01%) or mercury (II) chloride (0.01 M) is added. It is important to note that the choice of isolation solution can significantly influence the chemical composition and properties of the extracted starch. However, employing a neutral/near-neutral, alkaline, or acidic solution may lead to starch degradation or a low recovery of total starch from the millet flour. The yield percentage of millet starch typically ranges from 52% to 79%, with an AMY content ranging from 6% to 38.6% and an AMP content of approximately 70-80%. (Verma et al., 2021; Alcazar & Meireles, 2015). The method for isolation of millet starch is presented in fig.1.4.

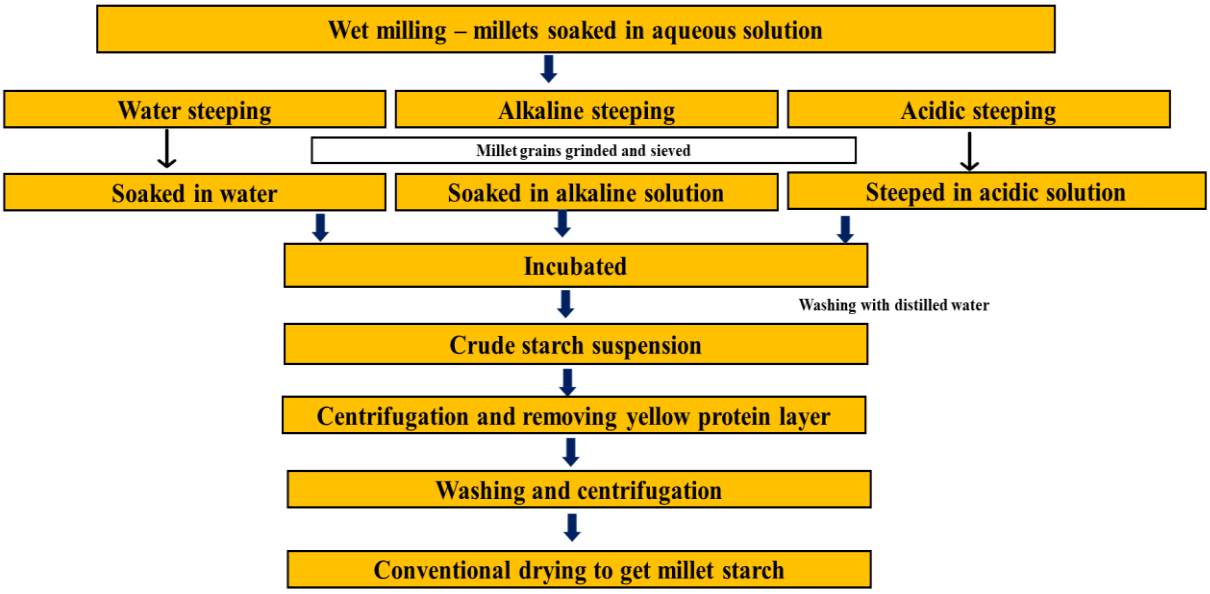

Fig 1.4 General method for millet starch isolation

The functionality of starch is significantly affected by the presence of AMY. Analysis of the AMY content in millet species indicated substantial variation (as shown in Table 1). Pearl and finger millet starches exhibit longer AMY chains and longer glucan chains between branch points compared to proso and foxtail starches, as demonstrated by Annor et al. (2014). The AMY AMP fractions of millet starch form complexes with iodine, resulting in the production of red and blue colors, respectively. The presence of branches within these fractions creates steric hindrance, influencing the ability of AMY and AMP to form helices with iodine molecules. This characteristic determines their iodine-binding affinity (%) and blue value, as described by Gaffa et al. (2004). Studies have reported that pearl millet AMY content is 21.3% with a blue value of 0.18 for AMP and a range of 1.3-1.41 for AMY. Foxtail millet starch is categorized into three types based on its AMY content: waxy, low AMY, and non-waxy starches, resulting in different colors upon

iodine staining ranging from reddish-brown to bluish-purple. Therefore, the AMY content of millets can serve as a useful indicator of its suitability for specific food product applications (Svihus et al., 2005).

Table 1.1 Comparison of starch fractions from various millet varieties

Grain	Starch (g/100 g dry weight)	AMY content (g/100 g starch)	AMP content (g/100 g starch)	Method of isolation	References
Foxtail millet	91.54	18.27±0.48	-	Alkali steeping	Dey & Sit, 2017
	56.2–73.1	1.38–12.35	87.65–98.62	Enzyme hydrolysis	Yin et al., 2019
		28.60			Annor et al., 2014
Kodo millet	94.18	-	-	Alkali steeping	Annor et al., 2013
	47.60–60.30	15.30–17.50	82.50–84.70	Enzymatic hydrolysis	Bora et al., 2019; Vali Pasha et al., 2018
Pearl millet	34.50-39.40	2.89-6.81	-	Alkali steeping	Suma & Urooj, 2105
	70.40	32.50			Annor et al., 2014
Barnyard millet	48.20–60.20	8.90–18.50	81.50–91.10	Enzymatic hydrolysis	Sharma & Gujral, 2020; Bora et al., 2019; Vali Pasha et al., 2018
Proso millet	58.0–77.87	2.24–38.67	25.44–69.00	Enzymatic hydrolysis	Bora et al, 2019; R. Shen et al., 2018; Yang et al., 2018a
	93.70			Alkali steeping	Annor et al., 2014
Little millet	52.00-57.00	11.90–17.90	82.10–88.10	Enzymatic hydrolysis	Sharma & Gujral, 2020; Bora et al., 2019; Vali Pasha et al., 2018
Finger millet	59.03–65.00	19.61–21.47	38.72–45.03	Enzymatic hydrolysis	Bora et al, 2019; Jayawardana et al., 2019
		32.40			

1.3.2 Starch modification

Native starches possess low shear and thermal stabilities, as well as high retrogradation rates, rendering them unsuitable for direct use in industrial applications. Consequently,

they undergo modifications via chemical, physical, or enzymatic means to overcome these limitations. Physical treatments are preferred over chemical methods due to their cost-effectiveness, innocuousness, and natural nature, as they do not require the treatment of effluents or produce chemical byproducts (Chen et al., 2015). Physical treatments can be divided into thermal and non-thermal methods. Heat-moisture treatment (HMT) and annealing are the most widely used thermal methods, both of which significantly improve starch's physicochemical properties without disrupting its granular structure. HMT is a low moisture content (10-30%) technique performed at high temperatures (90-120°C), whereas annealing is performed at intermediate or high moisture levels (50-60%) and low temperatures for a set time (Zavareze & Dias, 2011).

The most frequently used chemical modification methods are crosslinking, oxidation, and substitution, which involve linking functional groups to the starch backbone, resulting in modified starches with various applications. The various methods of starch modifications are presented in fig. 1.5.

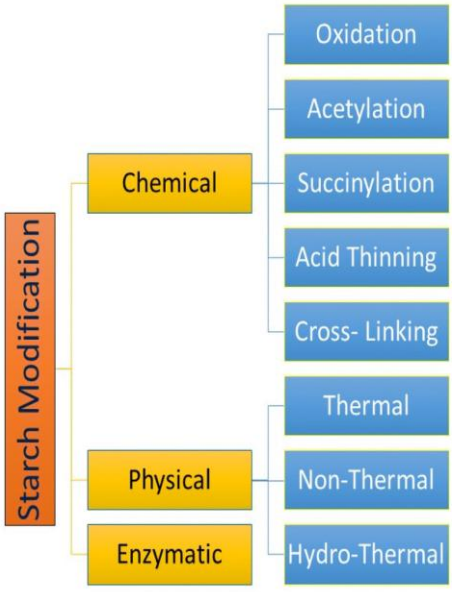

Fig. 1.5 Different modification techniques for starch modifications

1.3.2.1 Physical modification as a green approach for starch modification

In recent times, the food industry has exhibited a burgeoning interest in producing natural constituents for food products, thereby necessitating the enhancement of native starch properties without resorting to chemical alterations. Physical modifications of starch, which manipulate starch properties solely through physical treatments without introducing chemical modifications to the starch polysaccharide molecules, have gained considerable significance (Bemiller, 2018). These modifications are characterized by simplicity, cost-effectiveness, environmental friendliness, and safety, as they obviate the need for detrimental chemicals or biological agents. Physical treatments encompassing alterations in temperature/moisture conditions, shear pressure, irradiation, and mechanical attrition can influence the physical dimensions of native starch granules, consequently bringing about changes in the arrangement of starch polysaccharide molecules within the granules (Ashogbon & Akintayo, 2014). Physical modification is broadly categorized into thermal methods (pre-gelatinization and hydrothermal processing) and non-thermal methods (high pressure processing, micronization, ultrasonication, pulse electric field) (Bemiller, 2018). Pre-gelatinization entails depolymerization and fragmentation, which disrupt the molecular integrity of starch, while annealing and heat-moisture treatment sustain the granular structure of starch by subjecting it to temperatures below the gelatinization threshold but above the glass transition temperature. Non-thermal modification methods encompass high pressure processing, micronization, ultrasonication, and pulse electric field (Punia, 2020).

1.3.2.1.1 Heat moisture treatment (HMT)/hydrothermal treatment

Heat moisture treatment (HMT), also referred to as hydrothermal treatment, is a physical modification approach that employs precisely controlled levels of heat and moisture to modify the properties of starch. This technique is typically conducted at moisture levels below 35% and temperatures exceeding the gelatinization thresholds, spanning a duration ranging from 15 minutes to 16 hours (Arns et al., 2014). HMT induces partial disruption of the crystalline structure of starch, thereby influencing the interactions among polymer chains and causing dissociation of the double-helical arrangement. Eventually, the disrupted crystals undergo reorganization (Gunaratne and Hoover, 2002). The enzymatic digestibility of starch following HMT is primarily influenced by factors such as the starch source, moisture content, temperature, treatment duration, and interactions between starch fractions, including AMP-AMP, AMY-AMY, and AMY-AMP (Zeng et al., 2015).

Sandhu et al. (2020) reported lower pasting viscosities (683±11 cP), thermal stability, and retrogradation capacity in pearl millet starch after hydrothermal treatment. They additionally observed an increase in resistant starch (RS) and slow digestible starch (SDS) content post-modification. Thermal modification affected starch functionality and impacted compositional parameters, including a decrease in AMY content, as reported by Sandhu et al. (2020) for heat moisture-treated pearl millet starch.

1.3.2.1.2 Ultra-high pressure (UHP) treatment

There is a paucity of research on the utilization of Ultra-High Pressure (UHP) treatment for the modification of millet starch. Investigations focusing on the application of UHP at various pressures to modify proso millet starch have revealed an augmentation in hold viscosity (HV), final viscosity (FV) (2694 ± 64 cP in native form to 725 ± 1 cP @ 600

MPa), pasting temperature (PT) (57.40 ± 0.76 to 89.65 ± 0.00 °C), and peak time (P time) (4.33 ± 0.00 to 5.47 ± 0.00 min). This pattern was consistent across all treatments, except for the highest-pressure treatment, which resulted in a decrease in both peak viscosity (PV) and breakdown viscosity (BDV) (Li et al., 2018). Rice and maize starch granules exposed to pressures of 600 MPa and above experienced complete disintegration, leading to the formation of a gel-like structure (Li et al., 2012). These studies suggest that starch modified at higher pressures requires less heat for the disruption of intermolecular bonds. UHP treatment holds potential as an alternative technique for modifying millet starches intended for food applications. However, future studies should undertake comprehensive and extensive investigations into the mechanisms of gelatinization and retrogradation for all UHP-treated millet starches (Mahajan et al., 2021).

1.3.2.1.3 Cold plasma

Plasma, an extraordinary state of matter comprising a blend of electrons, photons, energized and non-energized molecules, free radicals, and positive and negative ions, has been acknowledged as the fourth phase of matter (Raghunathan et al., 2021). Plasma can be categorized into two forms based on thermodynamics: non-thermal/cold plasma and thermal plasma. In the realm of food processing, non-thermal plasma, also known as cold plasma, has garnered considerable interest owing to its capability to inactivate enzymes, degrade chemical residues, and extend the shelf life of food products (Gavahian et al., 2018).

A study conducted by Gao et al. (2019) documented an upsurge in gelatinization enthalpy, which quantifies the energy required to heat and gelatinize starch, in sorghum subjected to cold plasma treatment. The researchers also observed an augmentation in crystallinity

alongside a reduction in pasting properties, likely attributed to the rearrangement of depolymerized chains and the disruption of hydrogen bonding (Raghunathan et al., 2021). Collectively, plasma treatment induced alterations in the structural and functional characteristics of starch, thereby presenting potential applications in diverse food processing operations.

1.3.2.1.4 Ozonation

Ozone, a triatomic allotrope of oxygen present in the upper stratum of the atmosphere, can also be generated via corona discharge. Due to its elevated electrochemical potential, ozone manifests as an efficacious disinfectant and antimicrobial agent, thereby finding utility in prolonging the shelf life of diverse food commodities (Raghunathan et al., 2021). While conventional oxidizing agents can induce starch oxidation under specific pH and temperature conditions, there exists a potential risk of hazardous residuals in the food matrix. Consequently, ozone treatment represents a secure and environmentally friendly alternative, possessing Generally Recognized as Safe (GRAS) status (Sharma et al., 2021). Scientific investigations have evidenced that ozone treatment of corn starch granules yields a coarse and fibrous surface, resulting from starch depolymerization and the exposure of hydrophilic groups. This treatment elicits an augmentation in surface carbonyl and carboxyl content, engendering heightened gelatinization parameters, peak and final viscosities, and enhanced enzymatic susceptibility (Çatal, H., & İbanoğlu, 2012). Furthermore, ozone-treated foxtail millet flour exhibits improved swelling power, paste clarity, oil absorption, water absorption, and solubility (Sharma et al., 2022). Prominent consequences of ozonation commonly reported encompass heightened carbonyl and carboxyl group abundance, depolymerization of AMY and AMP molecules,

as well as a reduction in the apparent viscosity of starch pastes. Ozone treatment can also induce robust gel formation and ameliorate properties in the context of biodegradable films (La Fuente et al., 2019).

1.3.2.1.5 Micronization approach for the physical modification of starch

The physical modification of starch through different stimuli, such as milling, moisture, temperature, pressure, pH, radiation, pulse-electric field, and ultrasonic waves, leads to changes in its morphology, including size and shape, and its three-dimensional structure (Zia et al., 2017). Recently, research in the food industry has focused on reducing the particle size of food ingredients, also known as "micronization," as it has been found to improve functional properties, alter structures, and increase surface areas properties (Ogawa et al., 2003; Raghavendra et al., 2004; Sangnark et al., 2003). Certain food particles incorporate surface-active compounds, owing to their amphiphilic molecular structure, which tend to migrate preferably to oil/water or air/water interfaces. Micronization facilitates the enhanced release of soluble compounds into the surrounding medium. Insoluble surfactants positioned on the particle surface promote the alignment of the entire particle towards the interface, contingent upon adequate mobility. Such entities are referred to as Pickering stabilizers or particle-stabilized emulsions and foams (Flach et al., 2019).

To be effective as Pickering emulsifiers, solid particles must be at least one order of magnitude smaller than the emulsion droplets they aim to stabilize. This is particularly important for native starches and cellulose, whose original dimensions are at the micron level. Li et al. (2013) showed that rice starch with the smallest particle size outperformed waxy maize, wheat, and potato starch in stabilizing Pickering emulsions at a minimal

concentration, demonstrating that particle size, rather than morphology or surface chemistry, governs their ability to stabilize emulsions.

Micronized apple pomace was shown to be a superior emulsifier compared to larger native particles, as smaller particles improved physicochemical and antioxidant properties by altering initial structures (Lu et al., 2020). Similarly, micronized apple pomace, oat bran, and sugar beet produced more stable emulsions than their larger counterparts, indicating the potential for micronization to create Pickering starch particles (Huc et al., 2021). Micronization can be achieved using various techniques, such as grinding, which breaks down internal links in materials through compression, impact, attrition, and shearing forces (Joshi, 2011; Berk, 2018; Sahin et al., 2009).

In the realm of solid particulate materials, predominant methods such as jet milling, ball milling, and colloid milling are extensively employed. Conversely, for liquid materials, high-pressure homogenization, ultrasonic homogenization, and microfluidization technologies are predominantly utilized. Micronization, facilitated by these techniques, substantially augments the physico-chemical and functional attributes of food materials, thereby leading to an amelioration in food quality (Chen et al., 2017).

In contrast to samples pulverized utilizing traditional mechanical methodologies, superfine powders using methods such as micronization offer enhanced physicochemical properties. These improvements encompass superior flowability and hydration, heightened bioavailability, reduced interfacial tension, as well as enhanced flavor release and mouthfeel (Gao et al., 2020). However, using micronization to design Pickering starch particles is a novel concept in the food industry.

1.3.3 Potential applications of millet starch

Millet starch has demonstrated potential in several applications, such as stabilizers and thickening agents in sauces, gravies, and ketchup, as well as film-forming agents and packaging materials in food items (Shaikh et al., 2017a, Sharma et al., 2021). Fig. 1.6 illustrates the wide array of applications associated with millet starch.

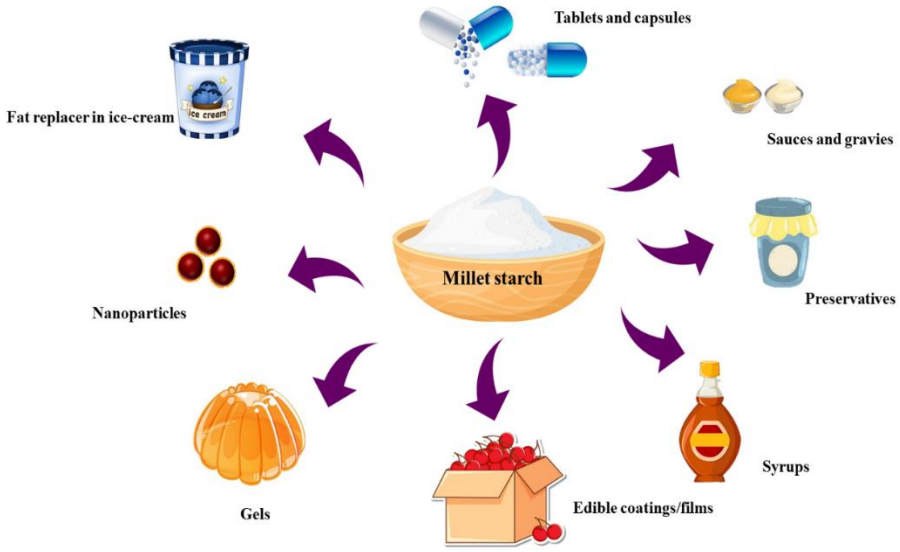

Fig 1.6 Range of applications of millet starch

The utilization of millet starch in various applications is hindered by its limited availability compared to sources like maize, cassava, and wheat. Additionally, challenges such as low AMY content, insufficient functionality, and processing difficulties arising from the small grain size further restrict its use. To overcome these limitations, starch modification techniques can be employed to achieve desired characteristics. Despite the potential of millet starch, its application in the food industry is still constrained and requires further exploration. The various applications of millet starch in detail have been presented in table 1.2.

Table 1.2 Applications of millet starch in food industry

Starch source	Native/modified starch	Application	Characteristics	Reference
Finger millet	Oxidized and acetylated	Tablet and capsule	• Decrease in disintegration time and increased resistance to friability. • Increase in tensile strength	Afolabi et al; 2012
Foxtail millet	• Ultrasonication and succinylation	Functional food	• Increased resistant starch content. • Significant cholesterol and bile/salt binding ability	Babu and Mohan; 2019
Pearl millet	Octenyl succinyl anhydride (OSA)	Fat replacer in ice-cream	• Heightened viscosity and increased gel strength • Low melting and overrun value • Acceptable sensorial and rheological characteristics	Sharma et al., 2017
Pearl millet	• Hydroxypropylated • Succinylated • Oxidized • Acetylated	Custard	• Reduced syneresis • Improved low temperature stability in storage • Improved sensorial and rheological properties	Shaikh et al., 2019
Pearl millet	• Hydroxypropylated • Succinylated	Sauces and gravies	• High swelling power and solubility • Improved paste viscosity and clarity • Low temperature requirement for gelatinization	Shaikh et al., 2017b
Sorghum	Pregelatinized	Tablet	• Higher tensile strength and brittle fracture index • Increased densification • Faster plastic deformation	Alebiowu and Itiola, 2002
Pearl millet	• Hydroxypropylated • Succinylated	White sauce	• Reduced syneresis • Better thermal and shear stability • Enhanced sensory characteristics	Shaikh et al., 2020
Proso millet	Starch nanoparticles	Nutraceutical	• Good stability and visco-elastic nature • Decrease in thermal transition temperature	Jhan et al., 2020
Barnyard millet	Cross linking by STMP	Films	• Lower water vapor permeability (WVP) • Higher tensile strength and solubility	Sharma et al., 2021

Pearl millet	Crosslinking	Edible films as preservatives	• Lower moisture, solubility, water vapor permeability (WVP), and elongation at break (EB) values	Dhull et al., 2021
Finger millet	Native starch	Flexible and edible thin films	• Excellent functional properties, including solubility, swelling index, and water vapor permeability	Gautam et al., 2021
Barnyard millet	Native starch	Antioxidant packaging material	• Good antioxidant and light barrier properties	Cao et al., 2017
Foxtail millet	Native starch	Packaging of *Queso Blanco* Cheese	• Antioxidant and UV light barrier properties • Enhanced shelf life for cheese	Yang et al., 2018b
Millet and sorghum	Enzymatically hydrolyzed starch	Glucose syrup	• Good substrate for glucose syrup production with good viscosity	Zainab et al., 2011
Foxtail millet	Native starch	Starch gels	• Well set gels with 3-D cellular structure with organized hexagonal pores • Good opacity	Nagaprabha et al., 2018

1.4 Coffee oil emulsions – Relevance and applications

Within the vast range of bioactive compounds, the usage of substances derived from food co-products has been growing in popularity. One such example of a bioactive-rich co-product is coffee oil, which is obtained from the roasted coffee beans (Speer, 2006). Despite being underutilized, roasted coffee oil (RCO) has demonstrated significant potential in the food industry as it contains substantial quantities of essential fatty acids and phenolic compounds. In addition, the fat-soluble aromatic compounds present in roasted coffee beans can be utilized as flavoring agents in a broad range of food preparations (Anese et al., 2000). Coffee is found to contain approximately 10–15% lipids, with the majority of these lipids constituting coffee oil. These lipids are primarily located within the endosperm of the coffee bean. Coffee oil predominantly consists of triacylglycerols, making up about 75% of its composition, along with fatty acids accounting for approximately 18% (Deotale et al. 2019). The interfacial and surface-active properties of coffee oil have been extensively documented in the scientific literature, with relevant studies conducted by Ferrari et al., in 2010, as well as research by Illy and Navarini in 2011.

Owing to its constituent components, coffee oil demonstrates potential as an ingredient in various food formulations, including ice cream, chocolates, instant beverages, confectionery, and nutraceutical foods. Coffee oil is particularly vulnerable to lipid oxidation, resulting in the development of undesirable off-flavors. Moreover, the aromatic compounds it contains are delicate and prone to loss during processing. The encapsulation of coffee oil with an appropriate wall material has proven effective in enhancing its resistance to oxidation and enabling controlled release of these hydrophobic aroma

compounds (Prasad et al., 2019). Nonetheless, the challenge of insolubility in water could impede its application. Therefore, emulsion-based encapsulation has emerged as a promising approach to mitigate this issue by safeguarding the oil against oxidative degradation and enhancing its compatibility with food matrices (Ribeiro et al., 2020). One of the most common techniques involves the use of solid particles with partial wettability, instead of surfactants, to emulsify the coffee oil. This process is known as the Pickering mechanism, which involves the adsorption of solid particles at the interface of the oil droplets. The solid particles prevent droplet coalescence by providing a high energy barrier for detachment, while also protecting the oil from degradation by external agents (Atarian et al., 2019). This can be carried out with the help of millet starch-based Pickering particles.

1.5 Plant-based dairy alternatives – A sustainable food for the future

In the past 20 years, the demand for plant-based dairy alternatives, derived from legumes such as soybeans, cereals like rice and oats, or nuts such as almonds and hazelnuts, has risen significantly. This growth is driven by health and environmental concerns, lactose intolerance, and the preference for a flexitarian diet, despite the beverages' taste (Silva et al., 2020). The global market for plant-based dairy alternatives was worth $US12.1 billion in 2018 and is projected to reach $US25.1 billion by 2026 (Craig et al., 2021). Moreover, non-dairy plant-based beverages are expected to account for around 5% of the milk and dairy products market in the next six years (Research and Markets Global Dairy Products Market Report 2019; Research and Markets $35+ Billion Dairy Alternatives Market & Consumption Report, 2020–2026). In addition to their use as "milk," plant-based drinks are also widely used as ingredients in recipes. The various steps involved in the production of plant-based milks are shown in fig 1.7.

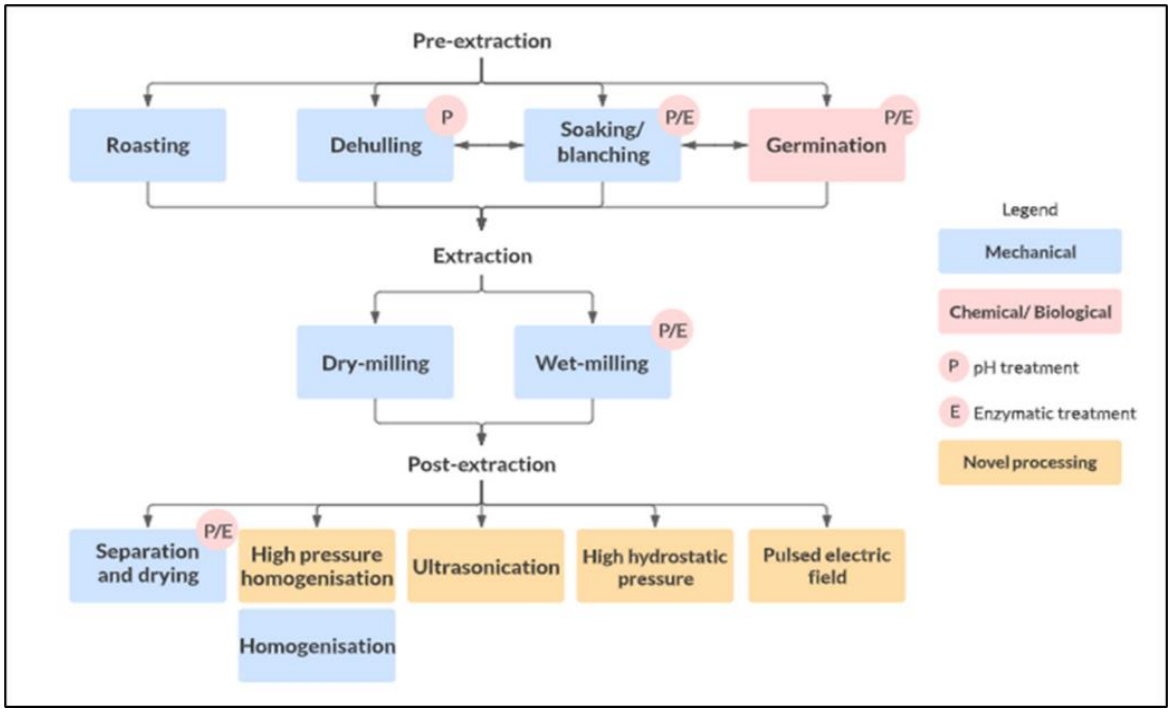

Fig 1.7 Overview of possible processing techniques for plant-based ingredient extraction for dairy analogue production (Pua et al., 2022)

1.5.1 Nutritional aspects of plant-based milk substitutes

Milk offers a superior nutritional profile compared to plant-based beverages, and this distinction is a crucial consideration when exploring alternative products to replace milk. Table 1.3 provides an overview of the nutritional characteristics and limitations of various plant sources in comparison to cow's milk. Plant-based beverages typically contain lower and less variable levels of micronutrients and amino acids. However, these alternatives offer functional benefits by supplying dietary fibres, isoflavonoids, and antioxidants from their plant sources (Chalupa-Krebzdak et al., 2018).

In contrast, milk is a high-energy source, providing approximately 64 kcal per 100 g of the product. This energy primarily derives from the carbohydrates, fats, and proteins naturally present in milk (Vanga and Raghavan, 2018). Consequently, for plant-based

beverages to serve as viable substitutes for animal milk, they must exhibit a highly energetic composition while maintaining an appropriate nutritional balance.

Table 1.3 Nutritional aspects and limitations of some plant matrices compared with cow's milk (Reproduced with permission from (Bocker & Silva, 2022))

Plant matrix	Nutritional aspects	Limitations	References
Soy *(Glycine max)*	High PDCAAS and DIAAS. High availability of magnesium, iron and copper ions. Presence of bioactive constituents (especially isoflavones such as glycitein, genistein, and daidzein). Moderate emulsifying properties due to amphipathic nature of its proteins. Presence of phytochemicals such as phytic acid, saponins, and sterols. Considerable amount of polyunsaturated fatty acids (especially linoleic (18:2) and linolenic (18:3) acids).	High content of antinutrients (trypsin inhibitors). Presence of proteins with allergenic potential. Off-flavors (beany flavors and astringency). Methionine and cysteine are limiting amino acids.	Astolfi et al. (2020); Chalupa-Krebzdak et al. (2018); Lai et al. (2013); Rizzo and Baroni (2018)
Rice (*Oryza sativa*)	High carbohydrate content. No significant allergenic potential. Gluten free. Presence of phytosterols (β-sitosterol and γ-oryzanol). Considerable amount of phosphorus, magnesium	Low content of monounsaturated and polyunsaturated fatty acids. Lysine is a limiting amino acid. Low protein content and poor digestibility. Presence of anti-nutritive compounds (phytates and trypsin inhibitors). Difficult emulsification due to high	Biswas et al. (2011); Boye et al. (2012); Sethi et al. (2016); Chalupa-Krebzdak et al. (2018)

	and potassium. Good source of vitamin E and B-complex vitamins. High starch content.	presence of starch. High sugar content.	
Almond (*Prunus dulcis*)	High protein content. High content of monounsaturated fatty acids. Presence of bioactive compounds such as alpha-tocopherol and arabinose. Good source of vitamin E, vitamin A and manganese. Low-calorie content (provides approximately 50 calories and 290 kJ per 200 g).	Presence of proteins with allergenic potential. Low PDCAAS. Methionine and cysteine are limiting amino acids. Susceptibility to rancidification due to its high concentration of polyunsaturated fatty acids.	Vanga and Raghavan (2018); Grundy et al. (2016); Sathe et al. (2002)
Oat (*Avena sativa*)	High carbohydrate content. High lipids content (higher than the other cereals). No significant allergenic potential. Good source of fibers (especially beta-glucan). Gluten-free.	Difficult emulsification due to high presence of starch. Presence of antinutrients (trypsin inhibitors and phytates). Lysine is a limiting amino acid. Low calcium content. High amount of lipases that can promote its rancidification.	Basinskiene and Cizeikiene (2020); Vanga and Raghavan (2018); Deswal et al. (2014)
Coconut (*Cocos nucifera*)	High saturated fat content. Presence of lauric acid. Good source of vitamin E. High availability of magnesium, iron, and copper ions. No significant allergenic potential.	Low content of monounsaturated and polyunsaturated fatty acids.	Abdullah et al. (2018); Vanga and Raghavan (2018); Sethi et al. (2016)

Quinoa (*Chenopodium quinoa*)	Quinoa presents good amount of cysteine, methionine, and lysine. Gluten-free. High quality of the protein profile (approximately 80% of digestibility). Presents chemical composition comparable to cereal grains. High starch content. Good source of iron, potassium, magnesium, calcium, copper, and manganese. High amount of tocopherol.	Bitter taste due to the presence of saponins	Vilcacundo and Hernández-Ledesma (2017); Nowak et al. (2016); Dakhili et al. (2019)
Chickpea (*Cicer arietinum*)	Presents low content of antinutrients. Good availability of iron (higher than the other legumes). High bioavailability of its protein content. Considerable amount of polyunsaturated fatty acids (especially linoleic (18:2) and oleic (18:1) acids). Good source of vitamins (especially thiamine, riboflavin, niacin, and folate).	Lysine and methionine are limiting amino acids.	Jukanti et al. (2012); Ferreira et al. (2006); Brazaca and Silva (2003)
Sesame seed (*Sesamum indicum*)	Presence of lignans (sesaminol, sesamin, and sesamolin). No significant allergenic potential. Low content of saturated fatty acids. High amount of amino acids that contains sulfur. Good lipid profile (the main fatty acids are	Presence of antinutrients (oxalates and phytates). Lysine is a limiting amino acid. Protein content with more solubility in salt than it´s in water. Thermosensitive protein content. Off-flavors (chalkiness and bitterness).	Vanga and Raghavan (2018); Sethi et al. (2016); Silva et al. (2020

	oleic (18:1), palmitic (16:0), linoleic (18:2), and stearic (18:0)).		
Sunflower seed (*Helianthus annuus*)	Low-calorie and good source of lipids.	Presence of antinutrients (phytates). Poor gel-formation properties of its proteins.	Silva et al. (2020)
Tiger nut (*Cyperus esculentus*)	High percentage of carbohydrates (approximately 12–17%). Gluten-free. Moderate fat content (considerable presence of oleic (18:1) and linoleic (18:2) acids). Moderate amount of nutritional minerals, such as phosphorus and calcium.	Percentage of protein is usually lower than 1%.	Codina-Torrella et al. (2017); Corrales et al. (2012)

*PDCAAS: Protein digestibility corrected amino acid score.

DIAAS: Digestible indispensable amino acid score.

1.5.2 Millet milk as a dairy substitute

Millets, when evaluated for their high protein content, low starch levels, moderate flavor, and low-calorie content, have the potential to be a suitable source of dairy substitutes. Millets present an edge for production of dairy alternatives due to their widespread cultivation and ease of maintenance. Millet milk is a preferred dairy substitute option as it is a more nutritious plant milk source compared to others. This makes it an excellent substitute for dairy, especially in the current trend of high-nutrition and low-calorie diets (Raajeswari & Nithya, 2018). Studies indicate that millet milk retains its nutritional value under both high and low processing temperatures (Nair et al., 2020). Some of the companies that are already using millets for production of dairy beverages include Alt Foods, Nourish You Millet Mlk, Oatey etc.

1.6 Future prospects and conclusion

Despite having many benefits, the use of millets is limited due to their small grain size, which makes them difficult to process and handle. The major component of millet grain is starch, but it is not often used in its native form because of its inadequate physicochemical and functional properties such as solubility, shear stability, retrogradation, and pasting properties. However, modifying the structure and functionality of starch can enhance its utilization for specific food products. Modified starch can be produced through physical, chemical, enzymatic, and dual methods. Its relatively high gelatinization temperature compared to other cereal starches can be advantageous in food processing techniques that require thermal stability. The pasting properties, viscosity, and gel strength of millet starch contribute to its functionality in food formulations, providing texture enhancement, moisture retention, and stability to the

end products. The available reports on isolation, modification, and utilization of starch from millets are limited, however, millets are a cost-effective and promising alternative source of starch that can compete with other major starch sources (corn, potato, wheat, cassava) in the food industry as a food additive, thickening, gelling, and binding agent.

In the context of Pickering stabilization, it is imperative that the particle size is substantially smaller, ideally by a factor of at least ten, in comparison to the size of the oil droplets. Consequently, various methods for size reduction are widely employed to modify starch, and researchers have explored an array of techniques for this purpose, including environmentally-friendly or "green" technologies. The effectiveness of these modification methods may be intricately linked to the inherent characteristics of native starch granules, encompassing attributes such as granule size, crystalline structure, the ratio of amylose to amylopectin, and protein content.

Therefore, the selection of appropriate modification methods must also be guided by the specific type and source of native starch, as these factors play a pivotal role in determining both the quality of the modified starch and the efficiency of the modification process. Furthermore, it is noteworthy that the combination of multiple methods represents an intriguing approach, as it enables a more precise tailoring of the properties of the modified starch. These tailored properties are paramount for optimizing its suitability for highly specialized applications.

In the above background information and the gaps identified, the present work focusses on the physical modification of native millet starch granules by micronization and validates its ability to function as a stable colloidal particle to stabilize Pickering emulsions encapsulating coffee oil.

Objectives

The detailed objectives of the study are as follows:

1. Determination of the nutritional, functional and phytochemical composition of nine millet varieties in their raw and germinated form.
2. Isolation of starch from millet grains, size reduction and its characterisation based on structural, functional and thermal properties.
3. Optimisation and characterisation of coffee oil encapsulated Pickering emulsions stabilised by physically modified pearl millet starch granules.
4. Development of new product formulation (plant-based beverages) from sprouted finger millet and, its sensory and shelf-life studies.

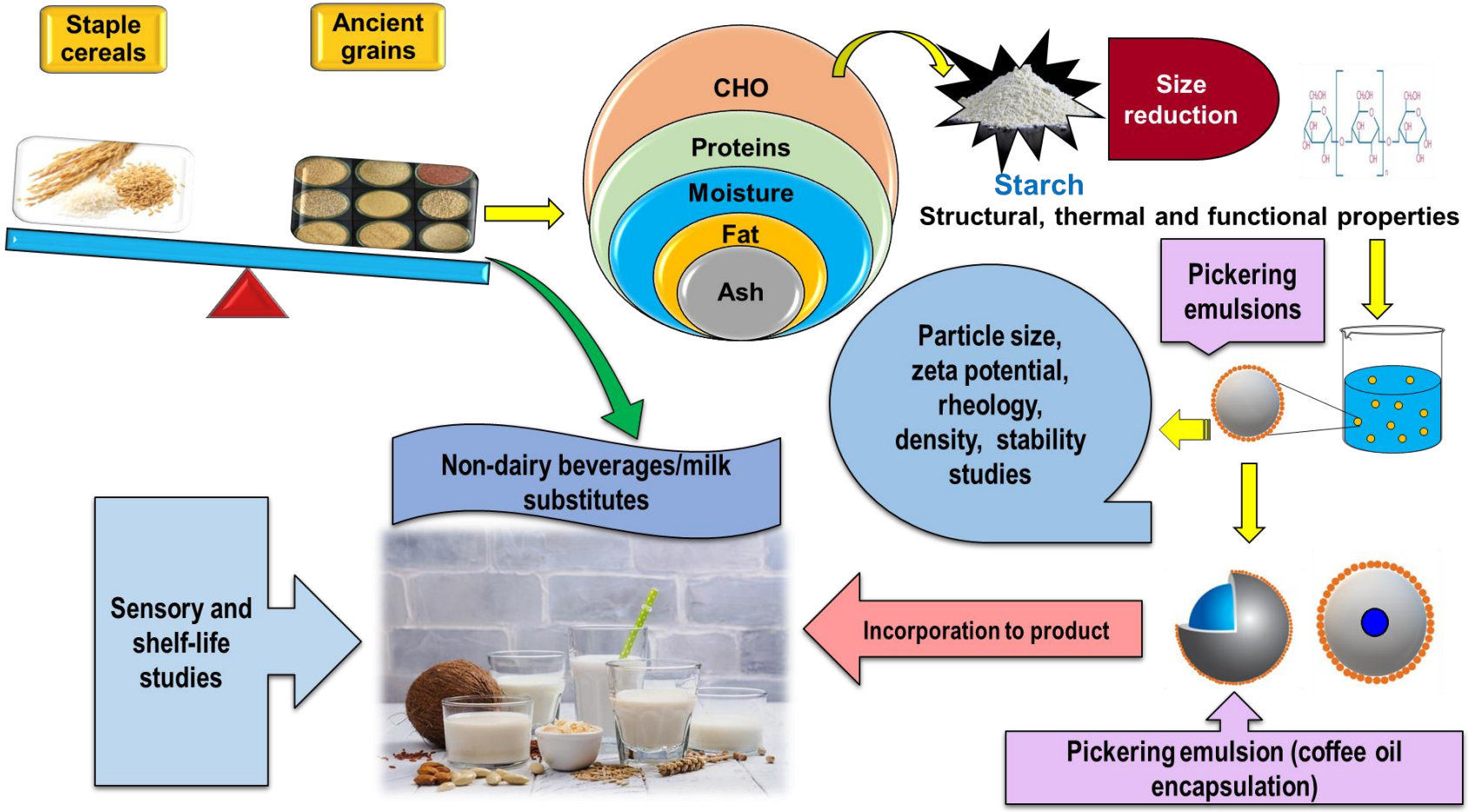

Fig 1.8 Overall workflow of the study

1.7 References

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Chapter 2

Determination of the nutritional, functional and phytochemical composition of nine millet varieties in their raw and germinated form

2.1 Introduction

Ancient grains can be defined as species or particular varieties of true cereals (sorghum, millets, teff, and wild rice), pseudocereals (amaranth, buckwheat, and quinoa) and pulses (cowpea, Marama bean). These are traditional staple foods cultivated hundreds of years ago and consumed by communities outside the mainstream of technological development. Consequently, these grains have undergone relatively limited genetic improvement. Further, the ancient grains are gluten-free and can be consumed by celiac – patients, which is a major criterion that adds to their nutritive value (Taylor and Awika, 2017). Among ancient grains, millets and pseudocereals are important crops in the semi-arid and tropical regions of Asia and Africa due to their resistance to diseases, pests, short growing season, and ability to thrive in less productive soils under heat and drought conditions (FAO, 1995). Asian countries are the second most important block of millet producers that accounts for 38% of the global area, as well as 42% of worldwide production. As per the Food and Agriculture Organization Corporate Statistical Database (FAOSTAT) 2018, global millet production for the year 2016 was found to be 30.35 million tonnes. In India, millet production is ~10 million tons, out of which the production of small millet is about 467 thousand tons (Himanshu et al. 2018). Following the decision of United Nations General Assembly, 2023 is designated as International Year of Millets highlighting the significance of these nutrient-dense crops which is expected to place millets in the global forefront (Rao et al., 2021). Millets are nutri-cereals, enriched with minerals like calcium, iron, zinc, potassium, and magnesium as well as proteins, vital fatty acids, dietary fibre, and B vitamins. They assist in providing health benefits such as lowering blood sugar

(diabetes), controlling blood pressure, and preventing thyroid, cardiovascular, and celiac disorders (Dayakar et al., 2017).

Millets are small, seeded cereals that belong to the family Poaceae, which are grown mainly in the semi-arid regions of Asia and Africa. Sorghum and pearl millet are the major millet varieties. Other millet varieties comprise finger millet (*Eleusine coracana*), foxtail millet (*Setaria italica*), proso millet (*Panicum miliaceum*), kodo millet (*Paspalum scrobiculatum*), barnyard millet (*Echinochloa spp*.), and little millet (*Panicum sumatrense*) (Himanshu et al., 2018). A pseudocereal is a plant grown to produce starchy grain suitable for human food (excluding cereals, legumes, oilseeds, and nuts). The term "pseudocereal" combines "cereal," referring to grains of grass species, with the prefix "pseudo-" meaning "false" or "resembling." The major pseudocereals are grain amaranth (*Amaranthus caudatus*; A. *cruentus*; *A. hypochondriacus*; family: Amaranthaceae), quinoa, and buckwheat (Fletcher, 2015). The nutrient density of the staple grains can be increased by natural methods like soaking, sprouting and fermentation. Germination or sprouting of cereal grains followed by hydrothermal treatment under ambient environmental conditions leads to the synthesis of new compounds and elevates the nutritional quality of grains (Uppal & Bains, 2012). The core purpose of germination is the increment of certain hydrolytic enzymes that are usually dormant in raw seeds (Kaukovirta-Norja et al., 2004). The phytic acid and polyphenols that cohere to the enzymes in our digestive tract hinders the absorption of carbohydrates and proteins. These detrimental effects can be curbed by the process of germination (Onyango et al., 2004). Sprouting is known to improve protein digestibility (Annor et al., 2017). During germination, a dynamic and complex flow of nutrients takes place that are essential for

the growth of plants (Theodoulou et al., 2012). During germination, nutrients and bioactive compounds are reported to increase (Koehler et al., 2007, Donkor et al., 2012) and then decrease, while being utilized by the plant in the process of growth (Yang et al., 2001). Sprouting is known to improve the antioxidant properties which further reduces the oxidative stress (Pasko et al., 2009).

As the food industry is looking for alternative sources for developing non-glutinous products, there is an excellent scope for ancient grain-based health foods. Even though there are studies that suggest the nutritional superiority of ancient grains, there are no comprehensive reports on the influence of germination on the nutritive value, phytochemical and antioxidant activities of these grains. As there is an increase in demand for gluten-free products in the market, documentation of the nutritional profile of these ancient grains will add value to their food applications. Moreover, germination leads to a decrease in the anti-nutritional factors present in these grains. Therefore, in our present study, we aim to elucidate the complete nutritional profile of these ancient grains in their raw and germinated forms.

2.1.1 Objective

Determination of the nutritional, functional and phytochemical composition of nine millet varieties in their raw and germinated form.

2.2 Materials and methods

2.2.1 Experimental design

The experiment protocol for the study is given in Fig 2.1 below:

Fig 2.1 Outline of the chapter

2.2.2 Chemicals and consumables

Grain samples (pearl millet, sorghum, kodo millet, finger millet, little millet, barnyard millet, and foxtail millet) used in the study was received as gift samples from Indian Council of Agriculture Research-Indian Institute of Millet Research, Hyderabad, India. Proso millet and amaranth were purchased from authenticated dealers in Trivandrum, Kerala, India.

All the chemicals used in the study were obtained from Sigma Aldrich (St. Louis MO, USA) unless otherwise stated. L-Amino acids standard reference kit was purchased from Sisco Research Laboratories, Maharashtra, India. Ammonium formate, formic acid (MS grade) and acetonitrile (MS grade) were purchased from Biosolve Ultra International, Bangalore, India. HPLC grade water was used throughout the study obtained from a water purification system (Millipore Milli-Q, Bangalore, India). All the solvents used were of

LCMS grade. Minigen Nylon syringe filters (0.2µm diameter) were obtained from Genetix Biotech Asia Pvt. Ltd, New Delhi, India.

The freeze-dried cultures of *Lactobacillus casei* (NCDC17) were supplied by National Dairy Research Institute, Karnal, Haryana, India.

2.2.3 Germination of millet grains

The germination of the millet samples was carried out according to the procedure of Badau et al., 2005. The grain (50 g; moisture content ranging between 6 – 12%) was immersed in 1.25% (w/w) sodium hypochlorite solution (seed: water ratio of 1:5, w/v) at room temperature (32 ± 2°C) for 30 min to disinfect them. Then it was washed thoroughly under tap water for 15-20 min for rinsing off the excess sodium hypochlorite. The grains were then covered with moist cotton cloth and left to germinate at room temperature (32 ± 2°C) for 0, 24, 48 and 72 hours respectively. After germination, the grains were oven dried at 40 °C for 48 h (moisture content of 5 – 12%). Each raw grain sample (50 g) and germinated sample was ground to powder using a grinder (Prestige Dry masala grinder PDMG 02, India) and passed through a 50 mm mesh. The samples were then stored in airtight containers at 4 °C for further analysis.

2.2.4 Sample preparation for LC-MS/MS analysis

The extracts were prepared using the procedure reported by Siroha et al., (2016) with some modifications. A defatted homogenous sample (1.0 g) was weighed and added with methanol and water in the ratio of 3:1 at room temperature (30 ± 2 °C). The mixture was vortexed and centrifuged at 4 °C at 10,000 rpm for 15 min followed by filtration through 0.2 µm nylon membrane filter.

2.2.5 Proximate composition

The standard procedures of AOAC (1990 (method 930.15, 923.03, 920.39)) were used for the determination of moisture, ash, crude fat, and protein contents of all the grain flours. Triplicate samples of all the samples were oven-dried at 100 °C transferred to a desiccator and allowed to cool at room temperature for moisture content. The sample weights were recorded before and after heat treatment in a muffle furnace (550°C for 12 h) for the ash content determination. Micro Kjeldahl method was used for the protein estimation with nitrogen to a protein conversion factor of 6.25, and fat content was determined using Soxhlet extraction. The total carbohydrate content was calculated using the difference method (100 - sum of protein, ash, fat and moisture).

2.2.6 Functional properties of grain flours

The water retention capacity (WRC), water holding capacity (WHC), swelling capacity (SwC), and oil holding capacity (OHC) of the raw samples were determined according to the procedure by Ruperez and Saura Calixto (2001). Specifically, 500 mg of millet flour was placed in a 50 mL centrifuge tube to which 30 mL of distilled water was added. The mixture was stirred and left at room temperature for 1 hour and centrifugation was performed at 3000 x g for 20 minutes, followed by removal of the supernatant. The residue was then weighed, and WRC was determined as grams of water per gram of dry sample. For WHC determination, one gram of sample was mixed with 10 mL of distilled water and allowed to stand at ambient temperature (32± 2 °C) for 30 minutes before centrifugation at 3000 rpm or 2000 × g for 15 minutes.

Similarly, for OHC determination, one gram of sample was mixed with 10 mL of sunflower oil and allowed to stand at ambient temperature (30 ± 2 °C) for 30 minutes before centrifugation at 3000 rpm or 2000 × g for 15 minutes. The water and oil holding capacity were calculated based on the difference in weight of the material before and after centrifugation. The swelling capacity (SC) was evaluated by weighing 500 mg of the sample in a 10 mL measuring cylinder and adding 10 mL of distilled water. The mixture was gently stirred to eliminate trapped air bubbles and left undisturbed at room temperature overnight to facilitate settling of the sample. The volume (in mL) occupied by the settled sample was measured, and SC was expressed as mL per gram of dry sample.

2.2.7 Total dietary fiber content

The determination of total dietary fiber content in the millet varieties was conducted in accordance with the Bureau of Indian Standard Method (1984). Initially, 3 grams of fat and moisture-free samples were subjected to autoclaving with water, followed by cooling. Subsequently, the pH of the mixture was adjusted to 1.3 using 5M HCl. Pepsin and chloroform were then introduced into the solution, which was then incubated for 20 hours at 37°C. Following this, the pH was readjusted to 6 using 3N NaOH, and 25 mL of phosphate buffer was added before treating the solution with pancreatin and glucoamylase. The resultant mixture was further incubated at 37°C for 18 hours. Subsequent to the enzymatic treatment, centrifugation was performed at 3000 rpm for 30 minutes to separate the insoluble dietary fiber (IDF). The soluble dietary fiber (SDF) present in the supernatant was precipitated by the addition of ethanol. Both SDF and IDF fractions were subjected to washing with alcohol, acetone, and diethyl ether, followed by lyophilization until a constant weight was achieved.

2.2.7.1 Prebiotic efficacy of dietary fiber

Lactobacillus casei (NCDC17) were cultured in MRS (de Man, Rogosa and Sharpe) broth and were preserved in MRS broth containing 50% glycerol at -80°C. To assess the prebiotic effectiveness, the MRS broth was supplemented with 1% (w/v) of autoclaved SDF that was heated at 121°C for 15 minutes. Inulin (1%) was used as a positive control. The culture was then inoculated with 100 µL of the suspension containing 5x10^3 CFU/mL cells and incubated at 37°C for up to 72 hours. Samples were collected at intervals of 0, 24, 48, and 72 hours to monitor the growth of bacteria and the utilization of SDF by measuring the pH and absorbance of the culture broth. Turbidity was measured using a UV-Visible spectrophotometer at 600 nm (Arun et al., 2017). The short chain fatty acid (SCFA) synthesis in supernatant collected at different time intervals was analyzed and quantified by HPLC following the protocol of Guerrant et al., 1982 with some modifications.

2.2.8 Mineral analysis

The mineral analysis was carried out using ICP-MS (Thermo Scientific iCAP RQ, single quadrupole). The samples were digested using the microwave digestion procedure. The microwave digester used was MARS 5 194A07. The samples were taken in a digestion vessel and added with 5 ml of 65% HNO_3 and 2 ml H_2O_2. The microwave-assisted digestion was done by 15 minutes ramp till 130 °C from ambient temperature and then held for 2 minutes at 800 W applied power. Further, the temperature was increased to 185 ^{0}C in a 10 min ramp time and then keeping the samples at 185 ^{0}C for 30 min at 800 W applied power. After digestion, the samples were diluted again to 50 ml.

2.2.9 Amino acid analysis using LC-MS/MS

Free amino acids (essential amino acids (E) – phenylalanine, leucine, methionine, threonine, valine, histidine, tryptophan, lysine, isoleucine and non-essential amino acids (NE) – glutamic acid, glycine, proline, aspartic acid, tyrosine, hydroxyproline, alanine, serine, asparagine, cysteine, arginine, cysteine, glutamine) were standardized using the LC-MS/MS system (Nexera with LCMS-8045, Shimadzu Corporation, Kyoto, Japan) - HPLC (Nexera LC-30AD) equipped with an autosampler (SIL-30AC), temperature-controlled column oven (CTO-20AC) and prominence diode array detector (SPD-M20A) coupled to triple quadrupole mass spectrometer (Nexera with LCMS-8045, Shimadzu Corporation, Kyoto, Japan). Working standards were prepared from the stock solution by dilution with milli-Q water with a concentration ranging between 0.01- 1 µg/ml. The quantification of all the amino acids was carried out using Shimadzu Shim-pack GISS C18 column (150 X 2.1 mm i.d, 1.9 µm) using a mobile phase of water / formic acid (100/0.1) for solvent A and 100% methanol for solvent B. Amino acids were eluted with a linear gradient system as follows: 0.5 – 4.9 min 5% of solvent B, 5.0 – 13 min 85% of solvent B, and 13.1 – 15 min 5% of solvent B, a flow rate of 0.3 ml/min and oven temperature of 40 °C. The LC-MS/MS with electrospray ionization (ESI) was operated in multiple reaction-monitoring (MRM) mode, both positive and negative. The injection volume was 10 µL, and ion spray voltage was 4 kV. The collision-induced dissociation (CID) gas was 230 kPa. Each calibration solution was analyzed in triplicate, and the average value of the results was used as the representative for each point. The results obtained are represented as amino acids µg/g with standard deviation (SD) (n = 3).

2.2.10 Analysis of water-soluble vitamins

Vitamins (thiamine, riboflavin, niacin, pyridoxine, folic acid and ascorbic acid) were analyzed using the LC-MS/MS system (Nexera with LCMS-8045, Shimadzu Corporation, Kyoto, Japan). The quantification was carried out using a Shimadzu Shim-pack GISS C18 column (150 X 2.1 mm i.d., 1.9 µm). The mobile phase used was 10 mM ammonium formate in water and 0.1% formic acid for solvent A and 10 mM ammonium formate in methanol and 0.1% formic acid for solvent B. A linear gradient system was used as follows: 0.01 – 2 min 5% of solvent B, 2.0 – 5.0 min 30% of solvent B, 5.0-8.0 min 45% of solvent B, 8.0-12 min 90% of solvent B and 12 – 16 min 5% of solvent B, flow rate of 0.3 ml/min and oven temperature of 33 °C. The ESI was operated in both positive and negative MRM. The negative mode was used only for ascorbic acid. The injection volume was 10 µl. The ion spray voltage was 4 kV. The nebulizing gas flow was 3.0 L/min, and drying and heating gas flow was 10 L/min. Each calibration solution was analyzed in triplicate, and the average value of the results was used as the representative for each point. The results obtained are represented as vitamins ng/g with SD (n = 3).

2.2.11 Phytochemical profiling of raw and germinated grains

2.2.11.1 Determination of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

The millet extracts were evaluated for their total phenolic content using the Folin Ciocalteau reagent. Gallic acid was used as a standard at concentrations ranging from 1-100 µg/mL (Singleton and Rossi, 1965). The reaction mixtures consisted of 10-100 µL of

millet extracts, Folin–Ciocalteu reagent (0.5 mL), and 20% sodium carbonate, and were incubated for 90 min. The absorbance of the reaction mixture was measured at 760 nm using a Shimadzu ultraviolet-visible 2600 (UV) spectrophotometer from Kyoto, Japan. The flavonoid content in the millet extracts was determined using the aluminum chloride method (Chang et al., 2002), and the absorbance was read at 510 nm using a multimode reader (Biotek Synergy 4, Winooski, VT, USA). The total phenolic and flavonoid contents were expressed as milligrams of gallic acid equivalents (mg GAE)/g sample (dry basis; db) and milligrams of quercetin equivalents (mg QE)/g sample (db), respectively.

2.2.11.2 Chemical profiling using LC-MC/MS

The quantitative analysis of polyphenols (28 compounds – catechol, catechin, quinine, naringenin, tocopherol, gallic acid, chlorogenic acid, epicatechin, syringic acid, vanillic acid, caffeic acid, epigallocatechin, ferulic acid, myricetin, quercetin, p-Coumaric acid, luteolin, apigenin, kaempferol, rutin, daidzein, hesperetin, shikimic acid, ellagic acid, morin, genistein, cinnamic acid and chrysin) was carried out using LC-MS/ MS system (Nexera with LCMS-8045, Shimadzu Corporation, Kyoto, Japan) using the method developed by our group (Abraham et al., 2020). Working standards were created using concentrations ranging from 0.01-1 µg/ml, and the quantification was conducted on a Shimadzu Shim-pack GISS C18 column using a linear gradient system with water/formic acid (100/0.1%) mobile phase for solvent A and 100% methanol for solvent B. Polyphenols were eluted with a linear gradient system as follows: 0.5 – 1.9 min 5% of solvent B, 2.0 – 10.0 min 98% of solvent B, 10.1 – 15 min 98% of solvent B and 15.1 – 17 min 5% of solvent B. The flow rate was 0.3 mL/min, the injection volume was 10 µl, and the oven temperature was maintained at 40 °C. LC-MS/MS data was collected and

processed by Lab Solutions software (Shimadzu, Kyoto, Japan. The samples were analyzed in triplicate, and the results were represented as polyphenols in μg/g with SD (n = 3). The average value of the results was used as the representative for each point. Lab Solutions software (Shimadzu, Kyoto, Japan) was used to collect and process the LC-MS/MS data.

2.2.11.3 Antioxidant analysis by 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay

DPPH scavenging activity of millet extracts was estimated using the procedure proposed by Brand-Williams et al., 1995. The absorbance of the assay mixture was read at 517 nm using multimode reader (Biotek Synergy 4, Winooski, VT, USA). The % inhibition was calculated from the IC_{50} (μg/mL) values obtained.

The ABTS assay was carried out according to the method by Lee et al., (2015) with slight modifications. After incubation, the absorbance was measured at 734 nm using multimode reader (Biotek Synergy 4, Winooski, VT, USA). The IC_{50} ABTS values (the concentration of sample required for inhibition of 50% of ABTS radicals) were obtained using the following formula:

$$\text{Inhibition (\%)} = 100 \times (A_0 - A_1)/A_0$$

Where absorbance of the control and sample/standard are A_0 and A_1, respectively. The IC_{50} values were expressed as μg/mL of ascorbic acid equivalent (AAE).

2.2.12 Statistical analysis

The results obtained from the experiments were presented as mean ± SD of triplicate measurements, unless otherwise mentioned. The data was analyzed using one-way analysis of variance (ANOVA) and Duncan's multiple range tests were used to calculate the significance of differences between means. The statistical analysis was performed using SPSS Statistics 20 Software (IBM, USA) and significance was considered at $p \leq 0.05$.

2.3 Results and discussion

2.3.1 Proximate composition

The proximate composition of the various millet grains is presented in Table 2.1. Results showed that carbohydrates are the primary nutrient present in millets followed by protein and fat. Amaranth was found to be the richest source of protein (14.52 ± 0.25%; dry basis (d.b)) followed by proso (13.04 ± 0.14%; d.b) and foxtail millets (12.78 ± 0.10%; d.b). It is reported to have the highest protein content (15.5–16.10%) among the pseudocereals and wheat (Alvarez et al., 2010). The protein content of ancient grains from the present study was within the ranges of the various reported studies (Saleh et al. 2013; Verma et al. 2015). The protein content of millet grains was found to be much higher (14.00 %) when compared to the staple cereals, e.g., maize (9.00 %), wheat (12.00 %) and rice (6.80 %) (Meherunnahar et al., 2018). Thilagavathi et al., (2015) reported that proso millet contained the highest amount of protein among all the millets followed by pearl millet. Kodo and finger millet had significantly lower protein content when compared to the other small millets. Compared to the raw grains, the protein content decreased

significantly ($p < 0.05$) for almost all the millet varieties upon germination as observed by Singh et al., (2019). This may be due to the fact that the protease enzymes are activated during soaking and sprouting process, leading to degradation of protein content in the grains (Singh et al., 2019).

The crude fat content indicated the following trend: amaranth (4.8 ± 0.14 % d.b) > pearl millet (4.65 ± 0.07 % d.b) > foxtail (4.30 ± 0.14 % d.b), followed by little millet (3.53 ± 0.04 % d.b) and barnyard millet (1.99 ± 0.01 % d.b). The fat content of foxtail, proso, little and kodo millets was reported to range between 2.3 - 5.9, 2.1 - 5.2, 3.10 - 4.1 and 1.1 - 3.3%, respectively (Kumar and Parameshwaran, 1998). The values obtained in the present study fall within these reported ranges, except for proso. The lipid content of amaranth is reported to vary between 1.9 – 9.7% depending on the species and genotype (Caselato and Amaya, 2012). The fat content of the germinated grains decreased significantly when compared to the raw samples, which are in accordance with the findings of Traore et al. (2004). This decrease could be due to the fact that lipids are used to produce the necessary energy for the biochemical and physiological modifications that occur in the seed during germination (Elmaki et al., 1999).

Amaranth, barnyard and proso millets were found to contain the highest ash content. There was not much significant change in the ash content of the grains upon germination. Total carbohydrate content was found to be higher in raw proso millet (67.04 ± 0.36 % db) followed by sorghum (66.55 ± 0.06 % db), finger millet (65.92 ± 0.55 % db) and which are close to the reported values (Longvah et al., 2017). The carbohydrate content of germinated grains was seen to be gradually decreasing. This reduction in starch content can be attributed to the hydrolytic activity of the amylase enzyme degrading starch

polymer chains. Another reason could be due to the use of starch as an energy source in the sprouting process (You et al., 2016). Thus, the results suggest that sprouting modifies the proximate composition of these grains by enhancing the hydrolysis of complex insoluble organic compounds present in the seeds to form simpler water-soluble organic compounds (Singh et al., 2019).

Table 2.1 Proximate composition of raw and germinated millet grains (% dry weight basis)

Parameters (% db)	Germination time (GT)	Pearl millet	Sorghum	Barnyard millet	Foxtail millet	Finger millet	Kodo millet	Little millet	Proso millet	Amaranth
Moisture	0^{th}	8.92 ± 0.09^{f}	12.35 ± 0.07^{h}	6.55 ± 0.14^{b}	6.24 ± 0.06^{a}	10.77 ± 0.02^{g}	8.92 ± 0.09^{f}	6.61 ± 0.06^{c}	7.21 ± 0.13^{e}	7.08 ± 0.01^{d}
	24^{th}	48.27 ± 0.06^{d}	46.15 ± 0.03^{c}	42.00 ± 0.03^{b}	38.39 ± 0.37^{a}	51.42 ± 0.05^{e}	42.24 ± 0.06^{b}	51.30 ± 0.04^{e}	38.05 ± 0.25^{a}	52.63 ± 0.08^{f}
	48^{th}	45.04 ± 0.17^{g}	42.57 ± 0.04^{e}	39.63 ± 0.08^{d}	35.45 ± 0.05^{a}	44.55 ± 0.07^{f}	36.70 ± 0.03^{b}	36.45 ± 0.07^{b}	38.32 ± 0.45^{c}	61.47 ± 0.01^{h}
	72^{nd}	48.69 ± 0.26^{h}	39.42 ± 0.06^{d}	37.20 ± 1.20^{c}	41.28 ± 0.06^{e}	46.29 ± 0.26^{g}	34.86 ± 0.06^{a}	42.59 ± 0.06^{f}	35.64 ± 0.56^{b}	53.33 ± 0.02^{i}
Total ash	0^{th}	1.78 ± 0.04^{a}	1.43 ± 0.14^{a}	2.57 ± 0.09^{d}	2.36 ± 0.10^{c}	2.03 ± 0.01^{b}	1.99 ± 0.07^{b}	1.70 ± 0.09^{a}	2.60 ± 0.01^{c}	3.03 ± 0.12^{d}
	24^{th}	2.19 ± 0.08^{a}	2.17 ± 0.25^{a}	2.17 ± 0.80^{a}	2.33 ± 0.56^{a}	2.07 ± 0.35^{a}	2.30 ± 0.03^{a}	2.54 ± 0.48^{a}	2.03 ± 0.25^{a}	2.04 ± 0.02^{a}
	48^{th}	2.14 ± 0.35^{a}	2.29 ± 0.06^{a}	2.06 ± 2.46^{a}	2.13 ± 0.25^{a}	2.10 ± 0.05^{a}	2.24 ± 0.56^{a}	2.05 ± 0.65^{a}	2.06 ± 0.40^{a}	2.21 ± 0.06^{a}
	72^{nd}	2.23 ± 0.50^{a}	2.13 ± 0.03^{a}	2.40 ± 0.04^{a}	2.19 ± 0.58^{a}	2.32 ± 0.09^{a}	2.03 ± 0.08^{a}	2.02 ± 0.08^{a}	2.50 ± 0.34^{a}	2.23 ± 0.45^{a}
Crude fat	0^{th}	4.65 ± 0.07^{h}	1.30 ± 0.14^{b}	1.99 ± 0.01^{c}	4.30 ± 0.14^{g}	2.20 ± 0.07^{e}	2.09 ± 0.07^{d}	3.53 ± 0.04^{f}	1.13 ± 0.09^{a}	4.80 ± 0.14^{i}
	24^{th}	2.76 ± 0.50^{b}	1.10 ± 0.72^{a}	3.50 ± 0.23^{c}	2.95 ± 0.04^{b}	2.30 ± 0.03^{b}	3.03 ± 0.05^{c}	2.69 ± 0.06^{b}	2.93 ± 0.26^{b}	2.40 ± 0.67^{b}
	48^{th}	3.14 ± 0.04^{b}	2.87 ± 0.03^{a}	3.00 ± 0.78^{b}	3.68 ± 0.06^{c}	2.46 ± 0.60^{a}	3.55 ± 0.58^{c}	3.60 ± 0.07^{c}	2.94 ± 0.50^{a}	2.77 ± 0.57^{a}
	72^{nd}	2.65 ± 0.06^{a}	2.75 ± 0.50^{a}	2.92 ± 0.79^{a}	3.23 ± 0.25^{b}	2.70 ± 0.47^{a}	2.89 ± 0.03^{a}	3.00 ± 0.97^{b}	2.77 ± 0.62^{a}	2.70 ± 0.36^{a}
Crude protein	0^{th}	12.46 ± 0.25^{f}	10.89 ± 0.78^{e}	9.60 ± 0.14^{b}	12.78 ± 0.10^{g}	8.04 ± 0.28^{a}	9.55 ± 0.14^{b}	10.18 ± 0.06^{d}	13.04 ± 0.14^{h}	14.52 ± 0.25^{i}
	24^{th}	5.08 ± 0.04^{a}	8.50 ± 0.06^{d}	8.00 ± 0.16^{d}	10.92 ± 0.45^{e}	5.50 ± 0.35^{a}	6.26 ± 0.05^{b}	6.96 ± 0.45^{b}	11.76 ± 0.40^{f}	7.19 ± 0.36^{c}
	48^{th}	5.27 ± 0.08^{a}	7.77 ± 0.07^{c}	11.73 ± 0.02^{f}	9.55 ± 0.05^{e}	7.38 ± 0.05^{c}	6.48 ± 0.06^{b}	7.10 ± 0.26^{c}	12.65 ± 0.07^{g}	8.41 ± 0.25^{d}
	72^{nd}	7.42 ± 0.15^{d}	6.87 ± 0.58^{c}	5.70 ± 0.04^{b}	7.58 ± 0.45^{d}	4.25 ± 0.34^{a}	9.70 ± 0.45^{e}	6.16 ± 0.59^{c}	12.70 ± 0.04^{f}	7.29 ± 0.58^{d}
Carbohydrate	0^{th}	59.25 ± 0.06^{c}	66.55 ± 0.06^{h}	58.80 ± 0.11^{b}	60.68 ± 0.39^{d}	65.92 ± 0.55^{g}	63.72 ± 0.39^{e}	64.64 ± 0.39^{f}	67.04 ± 0.36^{i}	57.60 ± 0.21^{a}

24th	41.7±0.34d	42.08±0.04e	44.33±0.60f	45.41±0.06g	38.71±0.05c	46.17±0.65h	36.51±0.56b	45.23±0.25g	35.74±0.25a
48th	44.41±0.40c	44.5±0.02c	43.58±0.57b	49.19±0.67d	43.51±0.36b	51.03±0.08f	50.72±0.56e	44.03±0.45c	25.14±0.05a
72nd	39.01±0.46b	48.83±0.06	51.78±0.32g	45.72±0.26d	44.44±0.24c	50.52±0.06f	46.23±0.68e	46.39±0.02e	34.45±0.09a

*Values are means ± SD (n = 3), followed by different superscript letter are significantly different ($p \leq 0.05$). The letter "a" represents the least value.

2.3.2 Functional properties of grain flours

The results of the functional properties of the grain flours are depicted in figure 2.1a. WHC was found to be the highest in pearl millet (3.41 g/g) followed by barnyard (3.39 g/g), proso millet (3.26 g/g), and kodo millet (3.23 g/g). WRC and OHC were found to be highest in kodo and pearl millet, followed by barnyard and finger millet and lowest in proso millet. The swelling capacity was found to be the highest in amaranth (9.43 mL/g) and lowest in pearl millet (6.20 mL/g).

Functional properties are important with regard to their application in various food products. Water/Oil holding capacity denotes the ability of flour to hold the water/oil. The water holding capacity increases with carbohydrates (starch), soluble sugars and proteins (polar amino acids), and other hydrophilic components. The OHC depends on the surface polarity, hydrophobic components, and presence of lipophilic compounds. An increase in water and oil-holding capacity favours enhanced mouthfeel and flavour retention in food products. The observed variation in different flours may be due to the different protein, fibre, and carbohydrate concentration, their degree of interaction with water as well as oil and conformational characteristics, as reported by Butt and Batool (2010). The swelling capacity indicates the increase in the volume of the sample on imbibing water when soaked in water with respect to its initial volume. The SC of flours depends on various factors like particle size, variety, and types, processing methods or unit operations employed, the difference in the composition, etc. (Suresh and Samsher, 2013).

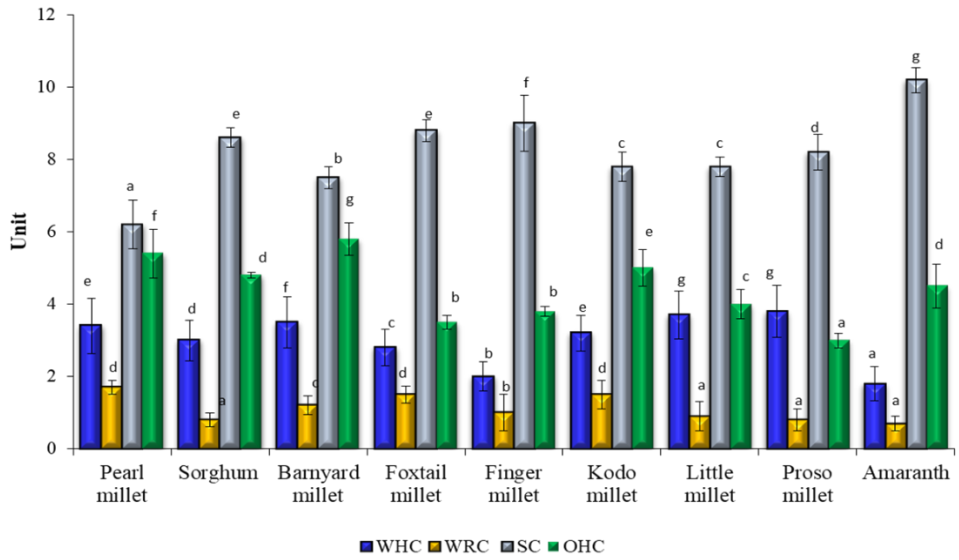


Fig 2.1a Bar graph showing the functional properties of grain flours. WHC – water holding capacity (g of water/g of flour), WRC – water retention capacity (g of water/g of flour), SwC – swelling capacity (mL of water/g of flour), OHC – oil holding capacity (g of oil/g of flour). Each value represents mean ± SD (standard deviation) from triplicate measurements. Columns in the graph bearing different superscripts differ significantly ($p<0.05$). The letter "a" represents the least value.

2.3.3 Total dietary fibre content of millet grains

The total dietary fibre content of millet grains is presented in table 2.2. The major constituents of millet dietary fibre are hemicellulose, lignin, cellulose, cutin and silica (Devi et al., 2014). Bran of the millets are a rich source of dietary fibre (complex polysaccharide). Due to higher viscosity, glycaemic index and water holding capacity dietary fibres plays a major role in reduction of blood glucose level, insulin response, cholesterol level and the risk of bowel disorders (Dayakar Rao et al., 2017). The total dietary fibre (TDF) content in millets ranged between 11.61 – 19.38 % on dry weight

basis. Pearl millet contain total fibre of 20.4% whereas the same for finger millet is 18.6%, which is higher than sorghum (14.2%). The total dietary fibre content of wheat and rice is reported to be 17.2 and of 8.3 % respectively (ElSheikh *et al.*, 2000). The soluble dietary fibre was found to be highest in barnyard millet (7.00 ± 0.36%) and insoluble fibre in pearl millet (16.54 ± 0.27%). Verma et. al, (2015) has stated that barnyard millet is a rich source of dietary fibre with good amounts of both soluble and insoluble fractions. Saleh et al., 2013 has reported that kodo millet and little millet had about 37% to 38% of dietary fibre, which was found to be the highest among the cereals.

Table 2.2 Dietary fibre content of millets (% dry weight basis)

Parameters (% db)	Bajra	Jowar	Barnyard millet	Foxtail millet	Finger millet	Kodo millet	Little millet	Proso millet	Amaranth
Total dietary fibre	19.38 ± 0.05^{e}	11.61 ± 44^{a}	17.51 ± 0.49^{d}	11.50 ± 0.70^{a}	13.78 ± 0.58^{b}	18.23 ± 1.03^{d}	15.31 ± 0.56^{c}	15.22 ± 0.41^{c}	17.01 ± 0.78^{d}
Soluble fibre	3.56 ± 0.57^{b}	2.02 ± 0.24^{a}	7.00 ± 0.36^{f}	3.92 ± 1.02^{c}	2.27 ± 0.20^{a}	5.39 ± 0.44^{e}	4.94 ± 0.26^{d}	4.44 ± 0.18^{c}	5.27 ± 0.33^{e}
Insoluble fibre	16.54 ± 0.27^{g}	9.60 ± 0.34^{c}	10.52 ± 0.26^{d}	7.96 ± 0.23^{a}	11.35 ± 0.27^{e}	8.77 ± 0.67^{b}	12.89 ± 0.63^{f}	11.10 ± 0.61^{a}	11.41 ± 0.20^{e}

Values are means ± SE (n = 3), followed by different superscript letter are significantly different ($p \leq 0.05$). The letter “a” represents the least value.

2.3.3.1 Prebiotic efficacy of millet samples and SCFA production

The prebiotic efficacy of all the grain samples based on pH and OD are represented in figures 2.2 and 2.3. This was assessed in terms of decreasing pH, increasing OD, colony count and SCFA production. From the following figures it was evident that there was a decrease in the pH and increase in the OD of the growth medium. When DF is incorporated in the growth medium for the probiotics, they feed on the DF producing SCFA as secondary metabolites which decreases the pH of the growth media. SCFAs produced because of the fermentation of non-digestible oligosaccharides result in the reduction of the pH of culture broth. Such decrease in pH can be used as an indication of the prebiotic effect of the oligosaccharides incorporated in the culture broth (Berggren, 1993; Azmi et al., 2012). The increase in turbidity of the growth medium can be related to the growth of microorganisms indirectly. Carbohydrates reaching the colon undergo different degree of fermentation and results in the production of short chain fatty acids (SCFA) such as acetate, propionate, and butyrate, which provide metabolic energy for the host and help in the acidification of the bowel (Swennen K et al. 2006). Millet's whole grain also shows prebiotic activity, which helps to increase the population of friendly bacteria that plays a key role to promote digestion. The present study demonstrated that the dietary fibre in traditional millets have good prebiotic efficiency that helps in maintaining healthy gut with sufficient normal bacterial flora. Maintenance of gut homeostasis is reported to have a significant role in prevention and management of lifestyle diseases. The SCFAs present have been known to promote the integrity of the intestinal epithelium. The SCFA - acetate, butyrate and propionate- produced as secondary metabolites by the probiotics.

Colonic bacteria prefer butyrate as their sole source of energy (Arun et al., 2019). There is an increase in the SCFA content of grains indicating good prebiotic activity.

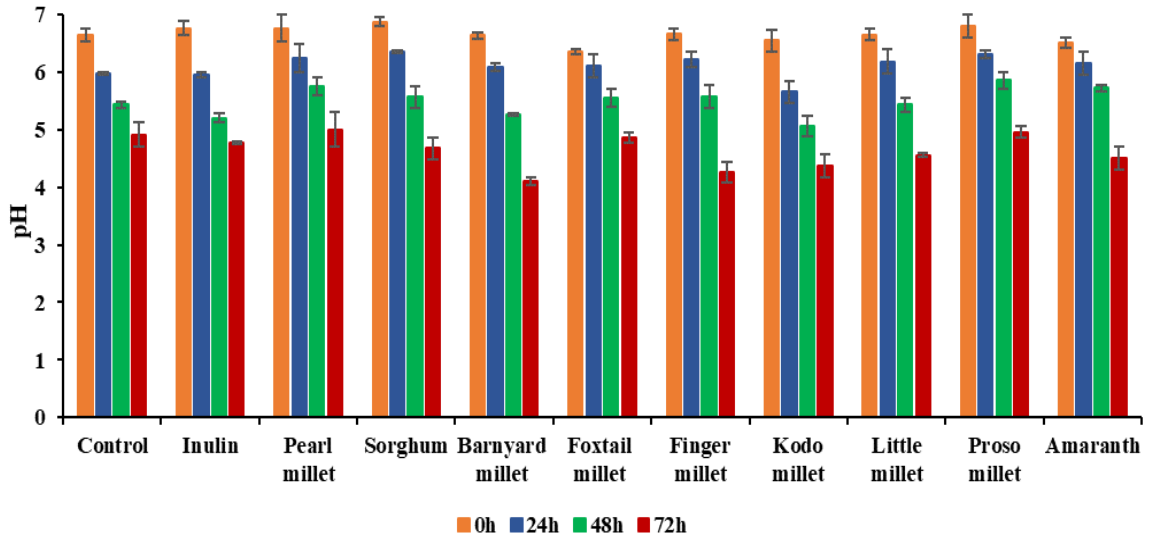


Fig 2.2 Prebiotic efficacy of SDF in millets based on pH

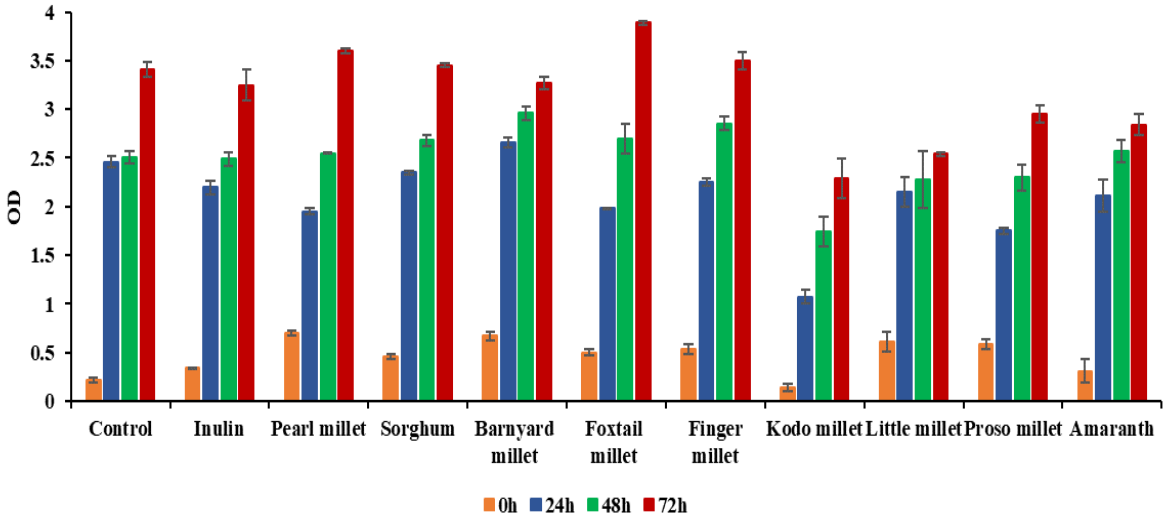
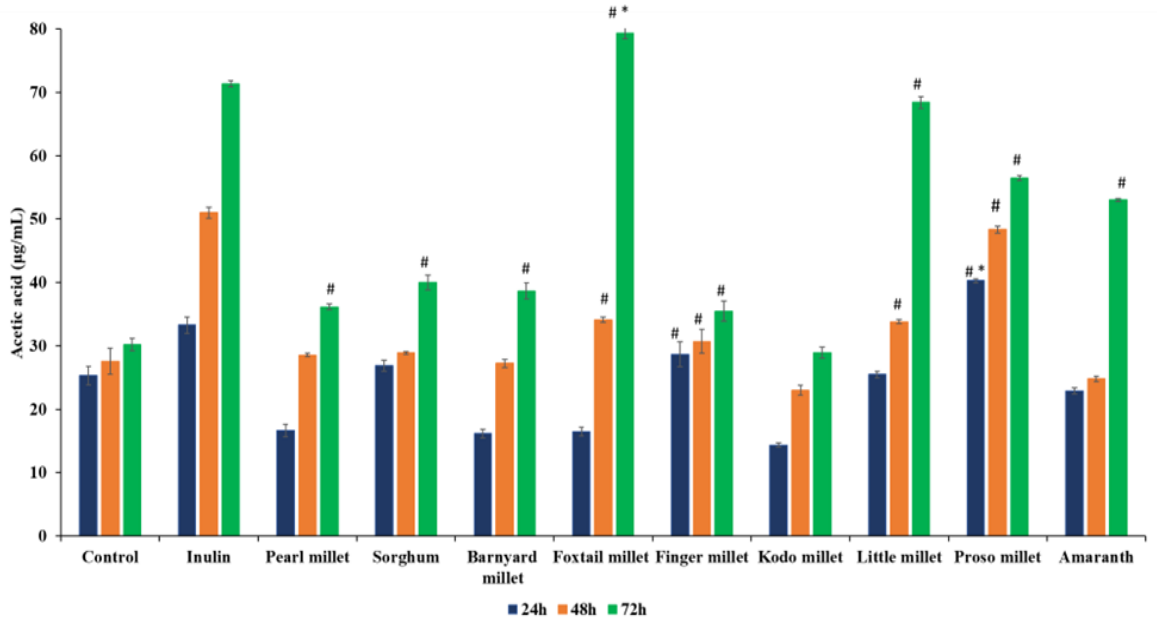
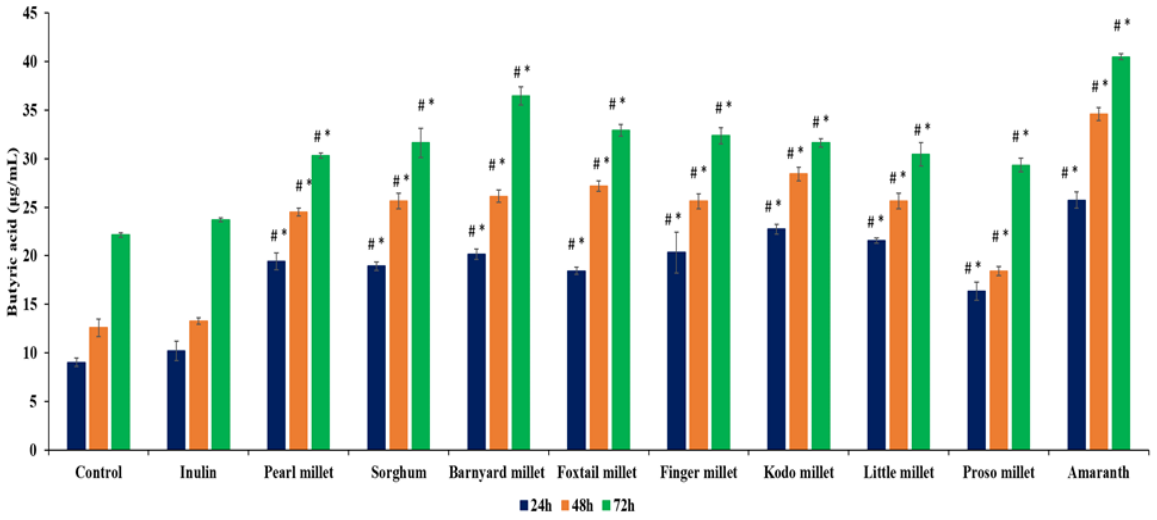


Fig 2.3 Prebiotic efficacy of SDF in millets based on optical density (OD)

a


Acetic acid (µg/mL)
0
10
20
30
40
50
60
70
80
Control
Inulin
Pearl millet
Sorghum
Barnyard millet
Foxtail millet
Finger millet
Kodo millet
Little millet
Proso millet
Amaranth
24h
48h
72h

b


Butyric acid (µg/mL)
0
5
10
15
20
25
30
35
40
45
Control
Inulin
Pearl millet
Sorghum
Barnyard millet
Foxtail millet
Finger millet
Kodo millet
Little millet
Proso millet
Amaranth
24h
48h
72h

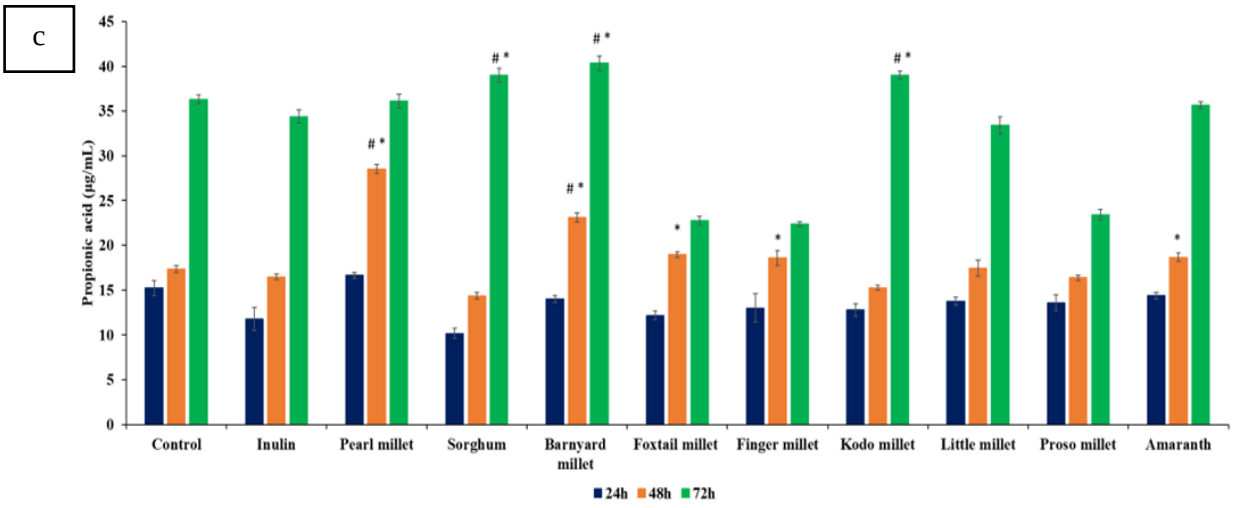


Fig 2.4 Prebiotic efficacy of SDF in millets based on SCFA production: a – Acetic acid (µg/mL); b – Butyric acid (µg/mL); c – Propionic acid (µg/mL) (#$p \leq 0.05$ versus Control, * $p \leq 0.05$ versus Inulin.

Table 2.3 Prebiotic efficacy of SDF in millets based on viable cell count (CFU/mL)

Sample	0^{th} h	24^{th} h	48^{th} h	72^{nd} h
Control	$5.00x10^{6}$±0.28	$2.09x10^{11}$±0.44	$5.15x10^{15}$±0.35	$1.65x10^{18}$±0.21
Inulin	$8.65x10^{6}$±0.21	$4.23x10^{11}$±0.94	$8.15x10^{15}$±0.68	$9.55x10^{17}$±0.12
Foxtail millet	$7.40\ x10^{6}$±0.14	$2.13x10^{11}$±0.42	$5.70x10^{15}$±0.42	$1.80x10^{18}$±0.28
Barnyard millet	$9.55\ x10^{6}$±0.07	$6.17x10^{11}$±0.74	$7.92x10^{15}$±0.40	$1.55x10^{18}$±0.32
Little millet	$5.25\ x10^{6}$±0.21	$1.71x10^{11}$±0.83	$4.58x10^{15}$±0.36	$9.08x10^{17}$±0.45
Proso millet	$1.61\ x10^{7}$±0.38	$2.72x10^{11}$±0.12	$5.51x10^{15}$±0.38	$8.86x10^{17}$±0.61
Kodo millet	$1.45\ x10^{7}$±0.07	$3.99x10^{11}$±0.35	$6.40x10^{15}$±0.19	$1.45x10^{18}$±0.54
Finger millet	$1.50\ x10^{7}$±0.42	$4.59x10^{11}$±0.53	$9.55x10^{15}$±0.35	$2.00x10^{18}$±0.42
Amaranth	$6.50\ x10^{6}$±0.14	$2.69x10^{11}$±0.34	$3.99x10^{15}$±0.30	$7.44x10^{17}$±0.62
Sorghum	$7.35\ x10^{6}$±0.35	$3.15x10^{11}$±0.36	$5.88x10^{15}$±0.16	$8.41x10^{17}$±0.04
Pearl millet	$9.50\ x10^{6}$±0.42	$4.55x10^{11}$±0.57	$7.40x10^{15}$±0.11	$9.59x10^{17}$±0.20

2.3.4 Mineral analysis of millet grains

Proximate composition indicated presence of comparatively higher amount of minerals in amaranth and proso millet. From the mineral analysis, it is noted that amaranth was a rich source of dietary minerals such as Mg, K, Fe, Ca and Zn. Foxtail was found to be the most abundant source of iron (143.26 ± 0.57 mg/kg) and zinc (22.17 ± 0.56 mg/kg) among the millets, which were in accordance with that observed by Girish et al. (2014). Finger millet was found to be the richest source of calcium (3037.65 ± 0.48 mg/kg). It is an excellent source of natural calcium for growing children and aging people. These nutrients (zinc and iron), play an essential role in enhancing immunity. Millets are found to be excellent sources of magnesium and phosphorus. The mineral content in millets ranges from 1.7 to 4.3 g/100 g, which is many folds higher than the other staple cereals like wheat and rice (Aggarwal et al., 2012). Calcium content of finger millet is about eight times higher than wheat, and being the richest source of calcium (348 mg/100 g), it can prevent osteoporosis (Dayakar et al. 2017). Kruger et al. (2014) has also confirmed the high calcium content of finger millet and also proved that calcium was distributed throughout the endosperm, where phytate levels were low. Barnyard millet and pearl millet are good sources of iron, and their consumption can meet the iron requirements of pregnant women suffering from anaemia. The incorporation of millets in the diet can help to eradicate nutritional deficiencies in developing and underdeveloped countries. The unique combination of high levels of Mg, Ca, Fe, and folate in amaranth can be used to supplement food products for celiacs, children, or pregnant women for compensating nutritional deficiencies that result from

special diets or high requirements (Taylor and Awika, 2017). The mineral composition of the grains is presented in table 2.4.

Table 2.4 Mineral composition of millets (mg/kg)

Samples	Bajra	Jowar	Barnyard millet	Foxtail millet	Finger millet	Kodo millet	Little millet	Proso millet	Amaranth
Sodium	39.46 ± 0.64^{f}	22.02 ± 0.29^{a}	33.01 ± 0.39^{d}	27.32 ± 0.85^{c}	805.98 ± 0.32^{g}	25.80 ± 0.60^{b}	26.81 ± 0.30^{c}	35.68 ± 0.42^{e}	24.95 ± 0.74^{e}
Magnesium	512.18 ± 0.20^{a}	590.89 ± 0.36^{c}	52.77 ± 0.57^{b}	623.28 ± 0.76^{d}	752.12 ± 0.35^{h}	701.01 ± 0.02^{e}	703.28 ± 0.99^{f}	730.77 ± 0.38^{g}	1232.71 ± 0.49^{i}
Potassium	5809.19 ± 0.03^{g}	4233.67 ± 0.59^{e}	3261.53 ± 0.27^{c}	4590.66 ± 0.48^{f}	6468.00 ± 0.83^{h}	3181.28 ± 0.22^{b}	3050.92 ± 0.38^{a}	4003.12 ± 0.72^{d}	6494.78 ± 0.72^{i}
Calcium	336.18 ± 0.26^{f}	164.15 ± 0.92^{d}	145.77 ± 0.44^{c}	534.84 ± 0.69^{g}	3037.65 ± 0.48^{i}	165.78 ± 0.57^{e}	142.78 ± 0.47^{b}	89.95 ± 0.23^{a}	1763.20 ± 0.81^{h}
Manganese	22.11 ± 0.75^{g}	20.90 ± 0.46^{f}	17.86 ± 0.35^{d}	15.96 ± 0.16^{c}	195.75 ± 0.73^{i}	15.07 ± 0.26^{b}	195.75 ± 0.73^{a}	15.07 ± 0.26^{e}	13.84 ± 0.53^{h}
Iron	84.98 ± 0.23^{e}	58.91 ± 0.38^{d}	22.40 ± 0.34^{a}	143.26 ± 0.57^{h}	109.95 ± 0.47^{f}	22.24 ± 0.28^{a}	36.77 ± 0.42^{b}	55.21 ± 0.32^{c}	129.07 ± 0.33^{g}
Zinc	18.49 ± 0.61^{e}	11.91 ± 0.30^{a}	16.88 ± 0.41^{d}	22.17 ± 0.56^{g}	11.36 ± 0.31^{a}	14.93 ± 0.29^{c}	13.75 ± 0.42^{b}	18.97 ± 0.24^{e}	19.46 ± 0.55^{f}
Copper	4.75 ± 0.40^{d}	2.89 ± 0.28^{b}	2.20 ± 0.10^{a}	5.85 ± 0.37^{e}	4.41 ± 0.45^{d}	7.61 ± 0.43^{f}	3.55 ± 0.31^{c}	5.86 ± 0.30^{e}	5.01 ± 0.16^{d}
Selenium	0.81 ± 0.05^{f}	ND	0.53 ± 0.05^{e}	0.04 ± 0.05^{a}	ND	0.19 ± 0.01^{c}	ND	0.41 ± 0.03^{d}	0.08 ± 0.01^{b}

Values are means ± SE (n = 3), followed by different superscript letter are significantly different ($p \leq 0.05$). The letter “a” represents the least value.

2.3.5 Amino acid composition of millets

In the present study, the quantification of free amino acids in grains was carried out using LC-MS/MS (Table 2.6). The details of method validation of the experiment are presented in table 2.6. The samples were analyzed, and the peaks were compared with those of reference compounds analyzed under the same conditions. Millets were found to be fair sources of both essential and non-essential amino acids. Among the grains studied, raw amaranth was found to be the best source of amino acids (both essential and non-essential) as it contained the highest amount of almost all the amino acids, followed by barnyard, pearl, and proso millet. Amaranth was rich in phenylalanine (2.743 ± 0.08 µg/g), methionine (6.413 ± 0.02 µg/g), valine (5.583 ± 0.41 µg/g), and arginine (1.427 ± 0.09 µg/g). Amaranth grains are reported to be superior over other conventional cereals as they possess a more balanced composition of essential amino acids (Fletcher, 2015). The grain proteins of common wheat and durum wheat are rich in glutamic acid and proline, but low in essential amino acids, especially lysine and threonine, as well as tryptophan, methionine, and isoleucine (Wrigley & Bietz, 1988). Gorinstein et al., (2002), has reported that amaranth grains contained higher amounts of lysine, methionine, and arginine. Nimbalkar et al. (2012) has reported that amaranth was a good source of amino acids especially phenylalanine (4.17 µg/g), methionine (4.09 µg/g), valine (3.78 µg/g), tryptophan (7.79 µg/g), lysine (3.33 µg/g) and isoleucine (03.20 µg/g), which are comparable to the values found in the present study. In this study, cysteine was found to be present in meager quantities in millet grains, similar to that reported by Kamara et al. (2009) which means that S-S bonds were absent in them. The breakdown of protease resistant prolamins and the increase of essential amino acids upon germination have been

reported by Traore et al. (2004). An increase in the amount of phenylalanine and valine has been reported in sorghum varieties upon germination by Afify et al. (2012), which are in line with our results as the content of phenylalanine increased from 0.011 µg/g in the raw form to 2.600 µg/g in germinated grain. The lysine content of sorghum increased four times upon germination from 0.069 µg/g to 4.194 µg/g. Besides, the non-essential amino acid content was found to decrease in the grains and glutamic acid increased in sorghum as observed by Afify et al. (2012). Kalinova and Moundry (2006) have reported that the protein content of proso millet (11.6% of dry matter) is comparable to that of wheat, and its grain was also significantly more abundant in essential amino acids like leucine, isoleucine, and methionine than wheat protein as wheat is deficient in lysine and methionine (Wrigley and Bietz, 1988). The present study revealed a higher concentration of free amino acids in millets. Thus, conventional cereals, which are usually poor sources of lysine and methionine can be replaced with traditional grains such as millets.

Table 2.5 MRM (Multiple Reaction Monitoring) transitions for amino acid standards using LC-MS/MS

Sl. no	Standards	Retention time (min.)	Ion monitoring (m/z)		Calibration equation	R^2	Collision energy (CE) (V)
			Precursor ion	Product ion			
1	Phenylalanine[E]	4.11	166.00	120.15	Y =700156*x+3.49769e+006	0.993	14
2	Leucine[E]	2.74	132.20	86.10	Y= 925582*x+3.13800e+006	0.996	12
3	Methionine[E]	2.19	150.10	104.05	Y = 35990.3*x+55624.5	0.995	10
4	Threonine[E]	1.84	120.10	74.10	Y = 21694.6*x+113466	0.996	12
5	Valine[E]	2.07	118.00	72.00	Y = 116187*x+409357	0.996	14
6	Glutamic acid[NE]	1.84	148.00	84.15	Y = 49143.9*x+105552	0.993	15
7	Glycine[NE]	1.81	76.10	29.95	Y = 4535.71*x+28101.1	0.998	12
8	Proline[NE]	1.89	116.10	70.10	Y = 85808.8*x+143127	0.993	16
9	Aspartic acid[NE]	1.85	133.80	73.85	Y = 8417.32*x+56124.9	0.995	14
10	Tyrosine[NE]	2.69	182.00	136.00	Y = 68825.9*x+396713	0.993	12
11	Hydroxyproline[NE]	2.69	132.00	86.15	Y = 793538*x+813857	0.996	15
12	Alanine[NE]	1.84	90.00	44.15	Y = 49891.0*x+300448	0.998	13

13	Tryptophan^{E}	7.00	204.8	188.10	Y=178790*x+339027	0.995	12
14	Serine^{NE}	1.83	106.10	60.25	Y=11585.1*x+161971	0.994	15
15	Histidine^{E}	1.82	155.80	110.20	Y = 17259.5*x+52977.3	0.996	16
16	Aspargine^{NE}	1.83	133.10	74.05	Y = =1539.33*x+24738.6	0.999	17
17	Cystine^{NE}	1.82	241.10	152.00	Y =16758.4*x+120222	0.998	13
18	Lysine^{E}	1.82	146.90	84.10	Y = 36698.0*x+31338.3	0.996	16
19	Arginine^{NE}	1.78	174.50	70.15	Y = 1058.91*x-1534.15	0.996	21
20	Cysteine^{NE}	1.90	122.00	76.05	Y = 817.330*x-4957.09	0.997	17
21	Glutamine^{NE}	1.81	146.80	84.05	Y = 27689.3*x+234182	0.991	18
22	Isoleucine^{E}	2.69	132.00	86.30	Y = 871511*x+2.59353e+006	0.997	11

†Abbrevations : E-Essential amino acids, NE-Non essential amino acids

Table 2.6 Amino acid composition of raw and germinated millet grains (µg/g)

Sl. no	Amino acid Standards	GT	Pearl millet	Sorghum	Barnyard millet	Foxtail millet	Finger millet	Kodo millet	Little millet	Proso millet	Amaranth
1	Phenylalanine^{E}	0^{th}	1.311±0.23^{g}	0.011±0.05^{b}	1.541 ± 0.24^{h}	0.008±0.38^{a}	0.182±0.07^{d}	0.168±0.09^{c}	0.324±0.10^{e}	1.048 ± 0.20^{f}	2.743± 0.08^{i}
		24^{th}	2.467± 0.21^{e}	2.299±0.18^{d}	<0.001	1.362±0.07^{c}	0.185±0.01^{a}	0.696±0.03^{b}	0.654±0.02^{b}	2.802±0.16^{f}	1.346±0.21^{c}
		48^{th}	1.697±0.21^{f}	2.529±0.40^{g}	0.509±0.04^{b}	0.854±0.07^{d}	0.378±0.04^{a}	0.613±0.05^{c}	0.580±0.16^{b}	1.581±0.11^{e}	<0.001
		72^{nd}	2.641±0.31^{g}	2.603±0.29^{g}	0.406±0.07^{b}	0.610±0.04^{c}	0.273±0.01^{a}	1.036±0.14^{e}	0.935±0.04^{d}	1.276±0.14^{e}	1.556±0.07^{f}
2	Leucine^{E}	0^{th}	0.435±0.12^{h}	0.422±0.05^{g}	0.021±0.41^{d}	0.097±0.18^{f}	<0.001	0.030±0.13^{e}	0.006±0.07^{b}	0.002±0.06^{a}	0.013±1.27^{c}
		24^{th}	1.953±0.01^{f}	2.087±0.03^{g}	<0.001	1.579±0.03^{e}	0.755±0.03^{b}	0.570±0.08^{a}	1.295±0.03^{d}	2.546±0.19	0.970±0.03^{c}
		48^{th}	1.760±0.10^{c}	1.978±0.01^{e}	0.847±0.01^{a}	1.168±0.15^{b}	1.810±0.02^{d}	0.874±0.07^{a}	1.062±0.02^{b}	1.819±0.04^{d}	<0.001
		72^{nd}	2.353±0.08^{f}	1.955±0.03^{e}	0.778±0.08^{a}	1.341±0.07^{b}	1.654±0.10^{c}	0.758±0.14^{a}	1.305±0.06^{b}	1.772±0.11^{d}	1.235±0.05^{b}
3	Methionine^{E}	0^{th}	0.453±0.05^{f}	0.049±0.01	3.902±0.11^{h}	0.200±0.41^{e}	0.145±0.07^{c}	0.069±0.26^{b}	0.178±0.02^{d}	1.48±0.04^{g}	6.413±0.02^{i}
		24^{th}	2.622±0.10^{d}	2.357±0.36^{d}	<0.001	1.831±0.05^{c}	0.478±0.04^{a}	0.387±0.05^{a}	0.881±0.08^{b}	4.861±0.06^{e}	0.849±0.05^{b}
		48^{th}	2.489±0.31^{g}	2.271±0.17^{f}	0.700±0.04^{b}	1.082±0.03^{d}	1.933±0.05^{e}	0.577±0.02^{a}	0.967±0.02^{c}	2.578±0.13^{g}	<0.001
		72^{nd}	3.412±0.13^{f}	1.271±0.17^{c}	0.578±0.06^{a}	1.092±0.03^{b}	1.515±0.04^{d}	0.566±0.04^{a}	1.784±0.11^{e}	1.661±0.06^{d}	1.320±0.25^{c}
4	Threonine^{E}	0^{th}	0.097±0.04^{f}	0.094±0.49^{e}	0.188±0.10^{g}	0.019±0.11^{a}	0.092±0.01^{d}	0.061±0.07	0.068±0.06^{c}	0.499±0.08^{h}	1.583± 0.01^{i}
		24^{th}	2.594±0.20^{f}	1.821±0.10^{e}	<0.001	1.757±0.06^{d}	0.616±0.08^{c}	0.241±0.02^{b}	0.076±0.01^{a}	1.739±0.13^{d}	0.382±0.06^{b}
		48^{th}	2.701±0.23^{f}	2.557±0.30^{e}	0.804±0.05^{b}	0.523±0.05^{a}	1.629±0.08^{d}	0.778±0.16^{b}	0.651±0.06^{a}	1.565±0.10^{c}	<0.001
		72^{nd}	3.592±0.19^{e}	2.576±0.28^{e}	0.614±0.07^{a}	1.241±0.10^{c}	1.707±0.10^{d}	0.677±0.05^{a}	0.679±0.03^{a}	1.705±0.07^{d}	0.894±0.06^{b}
5	Valine^{E}	0^{th}	2.801±0.11^{f}	0.145±0.08^{b}	5.739±0.07^{i}	0.065±0.06^{a}	0.524±0.07^{d}	0.277±0.11^{c}	0.633±0.10^{e}	3.390±0.06^{g}	5.583±0.41^{h}
		24^{th}	4.415±0.18^{e}	4.639±0.20^{e}	2.547±0.14^{c}	3.401±0.26^{d}	1.350±0.07^{a}	1.310±0.14^{a}	1.682±0.17^{b}	5.739±0.07^{f}	1.391±0.26^{a}

		48th	3.74 ±0.19c	4.257±0.08d	1.207±0.09a	1.330±0.11a	2.711±0.14b	1.476±0.16a	1.359±0.09a	2.638±0.18b	<0.001
		72nd	5.705±0.22e	4.210±0.15d	1.294±0.12a	2.691±0.04c	2.800±0.13c	1.299±0.18a	1.522±0.15b	2.501±0.23c	1.585±0.15b
6	LysineE	0th	0.492±0.07f	0.069±0.15b	6.177±0.12i	0.012±0.16a	0.142±0.07d	0.447±0.06e	0.761±0.03g	0.131±0.11c	3.082±0.17h
		24th	0.963±0.04c	2.618±0.19e	1.188±0.10d	1.290±0.21d	0.727±0.10b	0.265±0.03a	0.304±0.03a	0.911±0.07c	0.398±0.04a
		48th	0.816±0.08b	1.287±0.14c	1.647±0.10e	1.344±0.11c	0.430±0.06a	1.436±0.10d	1.640±0.10e	0.894±0.08b	<0.001
		72nd	0.210±0.08a	4.194±0.17e	0.466±0.06b	0.573±0.05b	<0.001	1.578±0.17c	2.912±0.08d	0.567±0.03b	1.639±0.10c
7	IsoleucineE	0th	1.004±0.01h	0.087±0.02b	0.844±0.06f	0.036±0.05a	0.304±0.11e	0.093±0.11c	0.186±0.12d	0.944±0.06g	2.102±0.02i
		24th	1.855±0.08e	2.196±0.21f	<0.001	1.463±0.12d	0.569±0.04a	0.558±0.10a	1.230±0.09c	2.459±0.08g	0.921±0.05b
		48th	1.791±0.06c	1.872±0.03d	0.814±0.07b	0.840±0.14b	1.779±0.06c	0.633±0.05a	0.882±0.05b	1.872±0.05d	<0.001
		72nd	2.391±0.15e	1.660±0.26c	0.609±0.02a	1.361±0.15b	1.551±0.10c	0.667±0.45a	1.337±0.09b	1.746±0.10d	1.321±0.11b
8	TryptophanE	0th	8.220±0.05i	0.041±0.20b	6.050±0.02g	0.021±0.20a	0.150±0.11c	0.280±0.05e	0.170±0.06d	3.660±0.04f	8.160±0.17h
		24th	2.410±0.26d	3.173±0.13e	<0.001	1.636±0.21c	0.380±0.02a	0.215±0.06a	0.851±0.06b	3.489±0.20	2.816±0.18d
		48th	1.340±0.26d	2.690±0.22f	<0.001	0.628±0.06c	1.458±0.08d	0.286±0.03a	0.464±0.06b	1.398±0.12d	1.818±0.07e
		72nd	0.310±0.01b	1.471±0.12e	<0.001	0.603±0.03c	0.843±0.05d	0.193±0.38a	0.507±0.36c	0.876±0.09d	<0.001
9	HistidineE	0th	1.069±0.10f	0.037±0.04a	0.160±0.20d	0.039±0.11a	0.084±0.14b	0.125±0.02c	0.273±0.76e	1.268±0.05g	5.587±0.01h
		24th	4.472±0.27d	<0.001	4.733±0.09d	3.736±0.17c	<0.001	1.557±0.28b	3.464±0.29c	8.214±0.14e	0.331±0.10a
		48th	7.606±0.26g	<0.001	10.222±0.11h	1.279±0.06b	1.645±0.13c	2.288±0.30d	3.273±0.31e	4.675±0.28f	0.041±0.02a
		72nd	12.671±0.11e	1.283±0.26a	11.363±0.30	1.673±0.10b	2.491±0.27c	1.464±0.20a	4.429±0.28d	1.454±0.11a	2.586±0.18c
10	Glutamic acidNE	0th	0.543±0.30c	1.277±0.06f	4.394±0.08i	0.078±0.06a	0.468±0.02b	0.606±0.13d	1.905±0.05g	0.836 ± 0.10e	3.027±0.09h
		24th	3.779±0.11f	2.674±0.21d	2.440±0.25c	2.842±0.16e	0.938±0.05b	0.546±0.14a	2.417±0.19c	5.703±0.21g	<0.001

		48th	4.666±0.31e	5.256±0.17f	1.646±0.19b	2.639±0.33c	3.378±0.29d	1.370±0.10b	2.553±0.06c	3.532±0.11d	0.551±0.11a
		72nd	5.845±0.10e	6.624±0.17f	1.498±0.10b	3.863±0.10d	3.721±0.18d	1.355±0.08a	2.390±0.15	1.939±0.05c	1.404±0.21b
11	GlycineNE	0th	0.034±0.08c	0.072±0.06e	0.177±0.07g	0.005±0.07b	0.048±0.04d	0.001±0.11a	0.129±0.11f	1.346±0.06h	1.485±0.31i
		24th	2.314±0.16g	1.913±0.03f	1.190±0.07d	1.427±0.12e	0.768±0.11c	0.575±0.06b	0.782±0.07c	2.455±0.17g	0.268±0.06a
		48th	2.420±0.23f	1.757±0.17e	0.613±0.07c	0.514±0.07b	1.308±0.14d	0.446±0.07a	0.523±0.02b	1.440±0.27d	<0.001
		72nd	2.542±0.28e	1.292±0.23c	0.628±0.06a	1.602±0.06d	1.675±0.11d	0.585±0.05a	1.209±0.18c	1.392±0.07c	0.887±0.08b
12	ProlineNE	0th	0.064±0.05d	0.038±0.10a	0.058±0.02c	0.041±0.05b	0.062±0.07d	0.105±0.02e	1.071±0.12g	0.146±0.06f	4.368±0.09h
		24th	2.776±0.22d	3.337±0.11e	<0.001	1.408±0.06c	0.535±0.03a	0.540±0.11a	1.199±0.15b	3.803±0.16f	0.595±0.03a
		48th	3.433±0.17d	4.278±0.09d	0.517±0.07a	0.544±0.08a	0.876±0.37b	0.580±0.06a	1.441±0.09c	1.405±0.15c	<0.001
		72nd	5.389±0.45f	4.786±0.16e	0.423±0.06a	1.620±0.13d	1.440±0.12c	0.405±0.17a	1.316±0.15c	0.760±0.10b	1.313±0.17c
13	Aspartic acidNE	0th	0.299±0.17h	0.275±0.02g	0.166±0.07d	0.002±0.02a	0.038±0.06b	0.059±0.07c	0.839±0.02i	0.171±0.02e	0.199± 0.05f
		24th	2.723±0.10d	3.328±0.18e	<0.001	1.549±0.12c	1.473±0.11c	0.062±0.72a	0.499±0.06b	0.375±0.69b	0.388±0.04b
		48th	3.664±0.14e	3.551±0.12e	1.690±0.17d	0.264±0.14a	1.590±0.05d	0.793±0.21c	0.409±0.13b	0.431±0.11b	0.565±0.14b
		72nd	4.784±0.22f	6.298±0.11g	1.575±0.08e	0.417±0.08b	1.278±0.14d	0.225±0.10a	0.831±0.14c	1.166±0.12d	0.511±0.07b
14	TyrosineNE	0th	0.824±0.10f	0.040±0.04a	1.588±0.11g	0.058±0.20b	0.444±0.03e	0.233±0.05c	0.436±0.11d	1.672±0.09h	8.877± 0.27i
		24th	4.429±0.10f	3.537±0.11f	1.299±0.05d	2.373±0.18e	0.232±0.02a	0.619±0.08b	0.616±0.05b	2.733±0.08e	0.925±0.05c
		48th	3.420±0.14f	0.473±0.06a	0.813±0.09b	1.333±0.17c	2.660±0.11e	0.743±0.04b	0.289±0.45a	1.619±0.24d	<0.001
		72nd	5.606±0.19c	3.743±0.13b	0.477±0.05a	0.398±0.03a	1.720±0.15	0.585±0.01a	0.497±0.05a	8.641±0.20d	0.496±0.05a
15	HydroxyprolineNE	0th	0.951±0.15h	0.077±0.03b	0.783±0.10f	0.034±0.09a	0.271±0.03e	0.083±0.06c	0.171±0.02d	0.896±0.05g	2.148±0.02i
		24th	1.849±0.10e	2.170±0.14f	<0.001	1.651±0.10c	0.590±0.07a	0.483±0.04a	1.232±0.19d	2.578±0.13f	0.918±0.02b
		48th	1.781±0.07b	1.848±0.08c	<0.001	0.861±0.10a	1.758±0.10b	0.678±0.02a	0.896±0.03a	1.838±0.11c	<0.001

		72nd	2.350±0.08e	1.924±0.08d	0.425±0.06a	1.433±0.10b	1.501±0.06c	0.629±0.05a	1.350±0.07b	1.666±0.08c	1.286±0.05b
16	Alanine[NE]	0th	0.110±0.10f	0.017±0.10b	0.023±0.04c	0.010±0.15a	0.058±0.10e	0.032±0.10d	0.372±0.06g	1.066±0.17h	1.708 ± 0.11i
		24th	1.228±0.09c	2.260±0.14e	1.228±0.16c	1.450±0.09d	0.925±0.06b	0.748±0.04a	0.891±0.05b	3.855±0.07f	0.624±0.10a
		48th	2.859±0.05f	2.171±0.10e	0.703±0.04a	0.939±0.01b	1.733±0.26d	0.680±0.15a	1.200±0.14c	2.433±0.10f	<0.001
		72nd	3.169±0.12e	2.311±0.20c	0.620±0.03a	2.359±0.07c	2.517±0.06d	0.580±0.07a	1.532±0.15b	2.145±0.09c	1.266±0.15b
17	Serine[NE]	0th	0.058±0.16f	0.018±0.14b	0.023±0.08c	<0.001	0.003±0.06a	0.047±0.18d	0.047±0.11e	0.071±0.07g	0.14±0.20h
		24th	1.251±0.11c	1.702±0.14e	0.942±0.04b	1.489±0.08d	0.482±0.12a	<0.001	0.577±0.11a	0.584±0.14a	0.542±0.12a
		48th	2.384±0.25d	2.415±0.23d	0.921±0.03b	0.474±0.03a	1.320±0.08c	0.922±0.05b	0.862±0.25b	1.385±0.25c	<0.001
		72nd	2.702±0.14f	3.330±0.14g	0.775±0.07b	0.378±0.45a	1.438±0.06d	0.764±0.02b	1.267±0.08d	1.844±0.08e	0.927±0.06c
18	Aspargine[NE]	0th	0.254±0.08e	0.010±0.06c	0.001±0.05a	<0.001	<0.001	0.004±0.07b	0.374±0.06f	0.068±0.10d	0.814±0.26g
		24th	0.917±0.03c	1.845±0.14c	<0.001	0.458±0.16b	0.064±0.18a	0.032±0.58a	0.317±0.09b	1.872±0.07c	0.066±0.01a
		48th	0.881±0.12c	0.826±0.06c	1.203±0.12d	0.243±0.05b	0.845±0.15c	0.083±0.02a	0.432±0.07b	0.858±0.09c	<0.001
		72nd	1.442±0.12e	1.703±0.16f	0.728±0.09c	0.341±0.05b	0.084±0.34a	0.214±0.05b	0.640±0.08c	0.848±0.06d	0.383±0.14b
19	Cystine[NE]	0th	0.051±0.11a	<0.001	0.279±0.12b	0.008±0.05a	<0.001	0.041±0.12b	<0.001	0.078±0.04a	0.435±0.02c
		24th	0.055±0.03a	0.047±0.60a	<0.001	0.079±0.14a	<0.001	<0.001	<0.001	0.247±0.09b	<0.001
		48th	0.379±0.05b	0.074±0.47a	0.022±0.08a	<0.001	0.041±0.02a	<0.001	0.031±0.14a	0.266±0.10b	<0.001
		72nd	0.716±0.11c	0.026±0.01a	0.042±0.01b	0.015±0.02a	0.064±0.62b	<0.001	0.072±0.02b	0.066±0.02b	0.045±0.06b
20	Arginine[NE]	0th	0.679±0.25f	0.581±0.17e	0.837±0.22g	0.006±0.10a	0.237±0.13c	0.255±0.09d	0.146±0.06b	1.218±0.02h	1.427±0.09i
		24th	1.901±0.02f	0.255±0.06b	<0.001	0.816±0.55d	0.352±0.18c	0.055±0.15a	0.315±0.04c	1.213±0.16e	0.033±0.01a
		48th	0.441±0.80a	0.882±0.06c	<0.001	0.531±0.09a	0.909±0.05d	1.201±0.16e	0.660±0.09b	0.881±0.06c	<0.001
		72nd	0.231±0.08b	1.383±0.13f	0.157±0.06a	0.785±0.01d	0.255±0.03b	0.639±0.05c	0.918±0.02e	0.682±0.05c	0.147±0.68a

21	Cysteine^{NE}	0th	0.039± 0.01^{a}	0.091±0.11^{d}	0.642 ± 0.20^{g}	0.056±0.11^{b}	0.142±0.01^{e}	0.078±0.02^{c}	0.168±0.03^{f}	0.167±0.02^{f}	0.696±0.11^{h}
		24th	0.766±0.07^{d}	0.087±0.01^{a}	0.254±0.01^{b}	0.237±0.06^{b}	0.047±0.45^{a}	0.070±0.04^{a}	0.085±0.26^{a}	0.637±0.09^{c}	0.042±0.01^{a}
		48th	0.298±0.06^{c}	0.139±0.03^{b}	0.489±0.04^{d}	0.088±0.08^{a}	0.181±0.08^{b}	0.076±0.14^{a}	0.084±0.34^{a}	2.886±0.06^{c}	0.007±0.04^{a}
		72nd	0.487±0.06^{d}	0.138±0.25^{b}	0.406±0.02^{d}	0.265±0.05^{c}	0.186±0.04^{b}	0.780±0.09^{e}	0.836±0.04^{f}	0.287±0.03^{c}	0.085±0.02^{a}
22	Glutamine^{NE}	0th	0.509±0.14^{f}	0.063±0.06^{b}	6.873±0.12^{i}	0.003±0.06^{a}	0.136±0.07^{d}	0.439±0.12^{e}	0.992±0.02^{g}	0.113±0.07^{c}	3.112±0.01^{h}
		24th	1.456±0.07^{e}	2.876±0.06^{g}	<0.001	1.355±0.18^{f}	0.735±0.09^{c}	0.293±0.05^{a}	0.332±0.10^{b}	1.580±0.07^{f}	0.918±0.09^{d}
		48th	1.184±0.09^{c}	1.765±0.16^{d}	2.389±0.22^{e}	1.138±0.08^{c}	0.667±0.08^{a}	1.202±0.13^{c}	1.350±0.07^{c}	0.826±0.08^{b}	<0.001
		72nd	0.230±0.10^{a}	6.298±0.16^{e}	0.598±0.04^{b}	0.637±0.12^{b}	<0.001	1.343±0.10^{c}	2.401±0.20	0.543±0.11^{b}	1.868±0.07^{d}

*Values are means ± SD (n = 3), followed by different superscript letter are significantly different ($p \leq 0.05$). The letter "a" represents the least value.

†Abbrevations : E-Essential amino acids, NE-Non essential amino acids, GT – Germination time

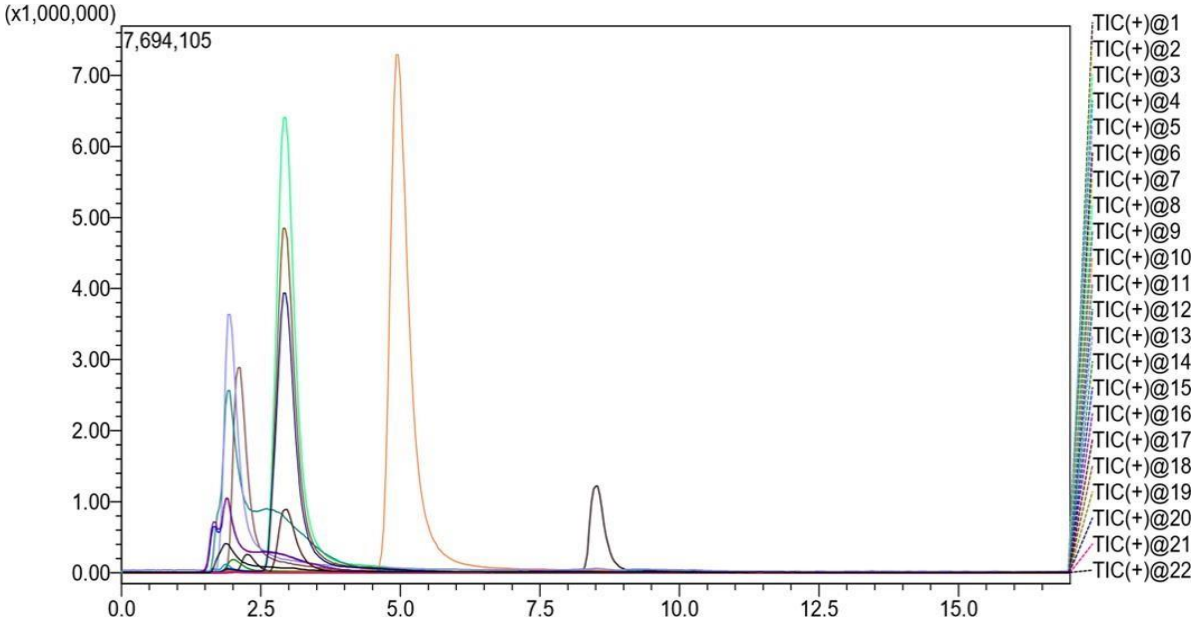


Fig 2.5 Representative LC-MS/MS chromatogram showing standard amino acids (10 ppb mix)

1–tryptophan, 2–serine, 3–leucine, 4–histidine, 5–aspargine, 6–methionine, 7–cystine, 8-threonine, 9-valine, 10-phenylalanine, 11-glutamic acid, 12-glycine, 13-proline, 14-aspartic acid, 15-glutamine, 16-lysine, 17-tyrosine, 18-isoleucine, 19-arginine, 20-hydroxyproline, 21-cysteine, 22-alanine

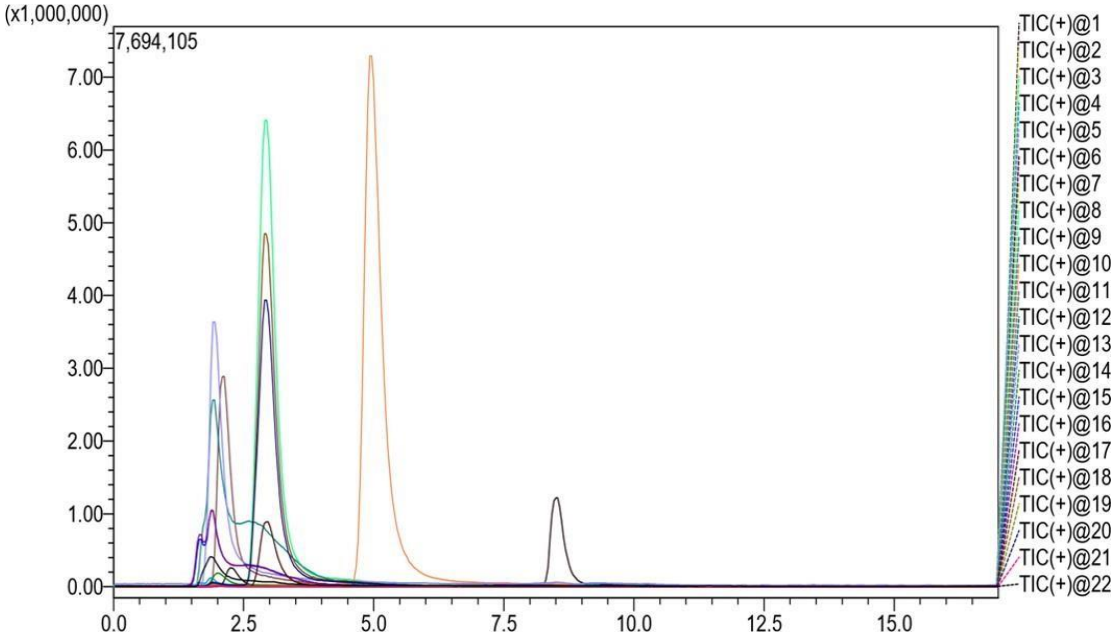


Fig 2.6 Representative LC-MS/MS chromatogram showing amino acid concentration in sample (little millet)

1–tryptophan, 2–serine, 3–leucine, 4–histidine, 5–aspargine, 6–methionine, 7–cystine, 8-threonine, 9-valine, 10-phenylalanine, 11-glutamic acid, 12-glycine, 13-proline, 14-aspartic acid, 15-glutamine, 16-lysine, 17-tyrosine, 18-isoleucine, 19-arginine, 20-hydroxyproline, 21-cysteine, 22-alanine

2.3.6 Vitamin composition of raw and germinated millets

Ancient grains were found to contain adequate amounts of B vitamins and ascorbic acid (Table 2.8). The method validation for vitamin composition using LC-MS/MS is represented in table 2.7. Proso followed by amaranth, foxtail, and pearl millet were found to be the richest source of most of the vitamins studied. Thiamine content was found to be highest in foxtail millet (11.57±0.10 ng/g) followed by proso and pearl millet. Proso millet was found to be a good source of riboflavin (21.24±0.07ng/g). According to Kumar et al. (2018), foxtail millet (1.65 mg/100 g) and pearl millet (1.48 mg/100 g) contained reasonable amounts of riboflavin. The riboflavin content of the millets is found to be several folds higher than the staple cereals. Saleh et al. (2013) has observed that the riboflavin content was highest in proso millet (0.28 mg/100 g) and thiamine in foxtail millet (0.59 mg/100g), which was in accordance with the results of the present study. Niacin content was found in good amounts in almost all the grains. Saleh et al. (2013) have stated that the niacin content of sorghum (jowar) is 4.3 mg/100 g, common millet (proso) is 4.5 mg/100 g and barnyard millet is 4.2 mg/100g. Pyridoxine was found to be the highest in finger millet (11.28±0.08ng/g). Foxtail millet (189.79 ± 0.31 ng/g) followed by amaranth (137.86 ± 0.11 ng/g) and proso millets (136.52 ± 0.20 ng/g) contained good amounts of folic acid. The vitamin B content in cereal grains generally increases due to sprouting and it also supports the seedling development and growth (Lemmens et al.,2019). Riboflavin content of the grains diminished under germination. The reduction of some B vitamins during germination can be attributed to the fact that the *de novo* synthesis of vitamins is only initiated in later sprouting stages and water-soluble vitamins can leach into the steeping water (Moongngarm and Saetung, 2010). Variations in the

vitamin contents of sprouted grains can be attributed to the type of grain and conditions of steeping and sprouting (Lemmens et al. 2019).

Barnyard millet was found to be the most abundant source of ascorbic acid (98.39 ± 0.11ng/g). There was a significant increase in the vitamin C content of the ancient grains upon germination. The increase in vitamin C during malting/germination is steered by the enzymatic hydrolysis of starch by amylases and diastases that increase the availability of glucose for the biosynthesis of vitamin C (Nkhata et al., 2018). This escalated content of glucose acts as a predecessor to the formation of vitamin C (Desai et al., 2010).

Table 2.7 MRM (Multiple Reaction Monitoring) transitions for vitamin standards using LC-MS/MS

Sl. no	Standards	Retention time (min.)	Ion monitoring (m/z)		Calibration equation	R^2	Collision energy (CE) (V)
			Precursor ion	Product ion			
1	Thiamine	1.84	265.20	122.15	Y = 609825*x-1.58242e+006	0.999	14
2	Riboflavin	8.33	442.20	295.05	Y= 4883.69*x+4919.37	0.995	15
3	Niacin	2.19	124.10	78.15	Y = 17332.0*x+50658.5	0.999	24
4	Pyridoxine	2.20	170.20	152.05	Y = 220729*x+432966	0.998	16
5	Folic acid	9.18	377.20	243.10	Y = 15205.7*x+124427	0.999	23
6	Ascorbic acid	1.95	175.20	115.10	Y = 594.230*x-4989.32	0.999	13

Table 2.8 Vitamin content of raw and germinated millets (ng/g)

Vitamins	GT	Pearl millet	Sorghum	Barnyard millet	Foxtail millet	Finger millet	Kodo millet	Little millet	Proso millet	Amaranth
Thiamine	0th	2.80±0.16c	1.55±0.26b	1.40±0.04b	11.57±0.10g	3.53±0.13d	3.50±0.13d	4.46±0.07e	5.17±0.03f	0.18±1.04a
	24th	3.40±0.19a	6.31±0.30b	6.29±0.17b	22.75±0.09e	<0.001	22.33±0.26e	10.54±0.19d	7.91±0.06c	<0.001
	48th	3.81±0.18c	4.63±0.38d	0.50±0.07a	26.66±0.22g	1.43±0.12b	16.46±0.20f	6.81±0.07e	4.80±0.13d	<0.001
	72nd	1.68±0.27b	6.73±0.27d	0.85±0.07a	12.42±0.11e	<0.001	5.69±0.10c	2.47±0.15b	<0.001	<0.001
Riboflavin	0th	4.24±0.15d	2.25±0.09b	3.71±0.07c	2.57±0.40b	1.63±0.24a	2.20±0.10b	2.15±0.09b	21.24±0.07f	9.60±0.27e
	24th	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	48th	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	72nd	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Niacin	0th	219.34±0.19f	390.67 ± 0.09i	194.69±0.16e	277.34±0.32g	177.62±0.15c	136.81±0.15a	182.44±0.06d	283.94±0.09h	166.47±0.24b
	24th	165.13±0.09f	244.06±1.86g	127.70±0.76e	95.13±0.80d	64.97±0.79a	83.30±0.54c	167.24±1.35f	294.35±1.47h	70.92±1.30b
	48th	146.62±1.26g	296.10±0.68i	63.99±0.79d	51.90±0.47b	96.99±0.60e	29.30±0.70a	107.34±0.93f	205.49±1.20h	57.41±0.53c
	72nd	348.81±1.16h	181.70±1.02g	67.83±0.62c	57.36±0.94b	22.32±2.11a	51.67±1.08b	107.37±1.30d	167.69±0.60f	119.01±0.47e
Pyridoxine	0th	0.85±0.06c	2.12±0.09d	0.25±0.05a	0.74±0.05c	11.28±0.08h	2.75±0.04e	4.89 ± 0.06f	6.50±0.07g	0.48±0.05b
	24th	4.20±0.09c	4.78±0.10c	5.71±0.15d	3.73±0.17b	6.36±0.15e	5.74±0.12d	14.17±0.14f	3.41±0.06b	2.14±0.05a
	48th	3.85±0.09b	5.20±0.10c	3.63±0.06b	2.79±0.05a	5.62±0.15c	6.80±0.07d	5.64±0.08c	2.22±0.12a	2.57±0.07a
	72nd	5.82±0.06c	1.24±0.09a	1.47±0.19a	<0.001	1.62±0.14a	7.50±0.29d	2.13±0.08b	2.51±0.08b	2.85±0.03b
Folic acid	0th	125.46±0.12f	84.50±0.04b	111.74±0.13e	189.79±0.31i	104.58±0.60d	91.80±0.09c	67.65±0.06a	136.52±0.20g	137.86±0.11h
	24th	134.14±0.07f	98.66±0.57c	6.58±0.15a	148.63±0.62g	25.20±0.19b	152.95±0.23h	106.68±0.57d	737.37±3.91i	123.20±0.86e

	48th	44.12±0.07c	234.61±0.12h	<0.001	83.27±0.25f	61.69±0.64e	1.33±0.09a	2.33±0.12b	171.70±0.59g	55.83±0.09d
	72nd	168.71±0.52g	118.71±0.57d	22.49±0.05a	126.91±0.33e	<0.001	29.47±0.34b	61.89±0.12c	162.88±0.64f	115.45±0.76d
Ascorbic acid	0th	55.34±0.34b	86.88±0.38g	98.39±0.11i	78.03±0.36e	46.60±0.17a	68.84±0.30c	73.21±0.12d	99.67±0.16h	83.24±0.21f
	24th	557.11±0.66e	901.85±0.71g	246.70±1.05b	244.66±1.08b	357.73±0.77c	505.46±1.20d	384.30±0.62c	807.51±0.90f	42.96±0.68a
	48th	670.72±0.70c	1812.84±0.49i	137.86±0.58a	1694.90 ±0.72g	374.78±0.81b	723.96±0.74d	1143.64±1.08f	1737.19±1.61h	1005.58±0.99e
	72nd	623.36±0.80b	827.47±1.01d	709.16±1.32c	30452.90±2.02h	703.46±1.21c	4556.93±3.25g	283.31±1.11a	3214.49±2.39f	1248.62±1.19e

GT – germination time

*Values are means ± SD (n = 3), followed by different superscript letter are significantly different ($p \leq 0.05$). The letter "a" represents the least value.

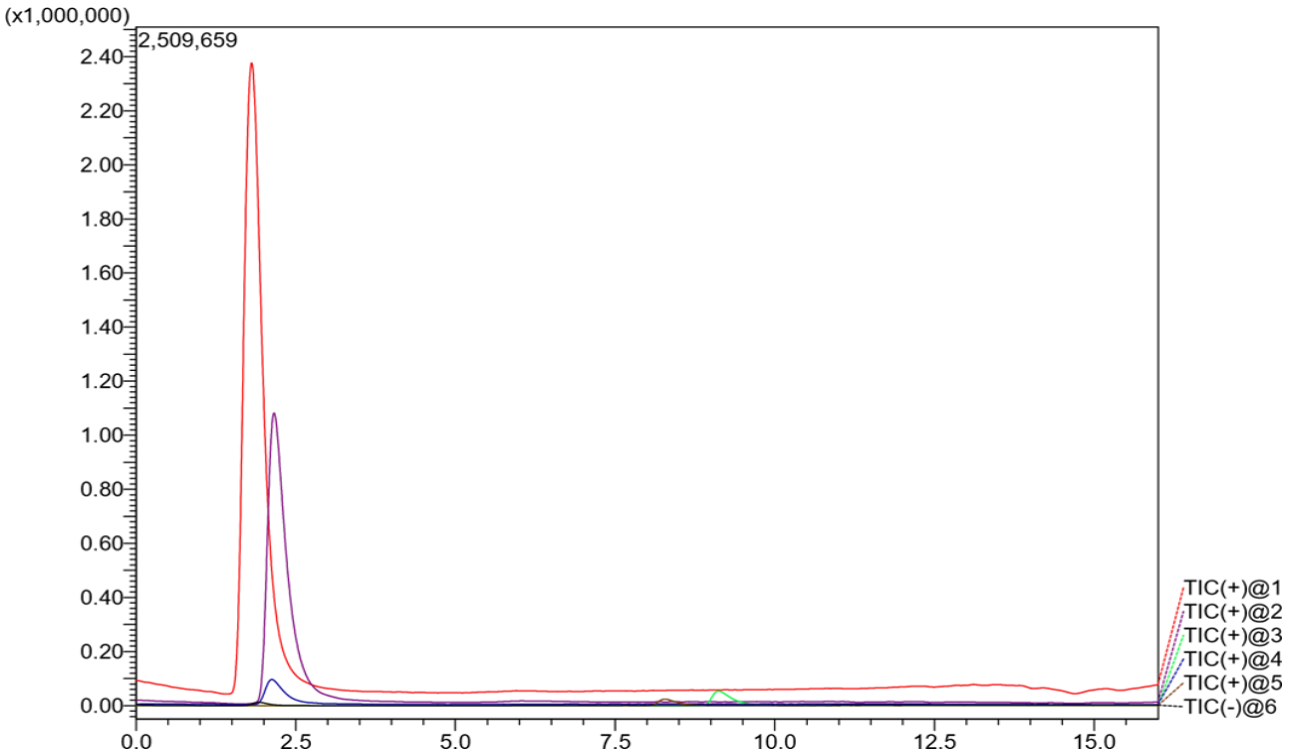


Fig 2.7 Representative LC-MS/MS chromatogram showing standard vitamins (100ppb mix+350ppbascorbic acid)

1-thiamine, 2-pyridoxine, 3-folic acid, 4-nicotinic acid (niacin), 5-riboflavin, 6-ascorbic acid

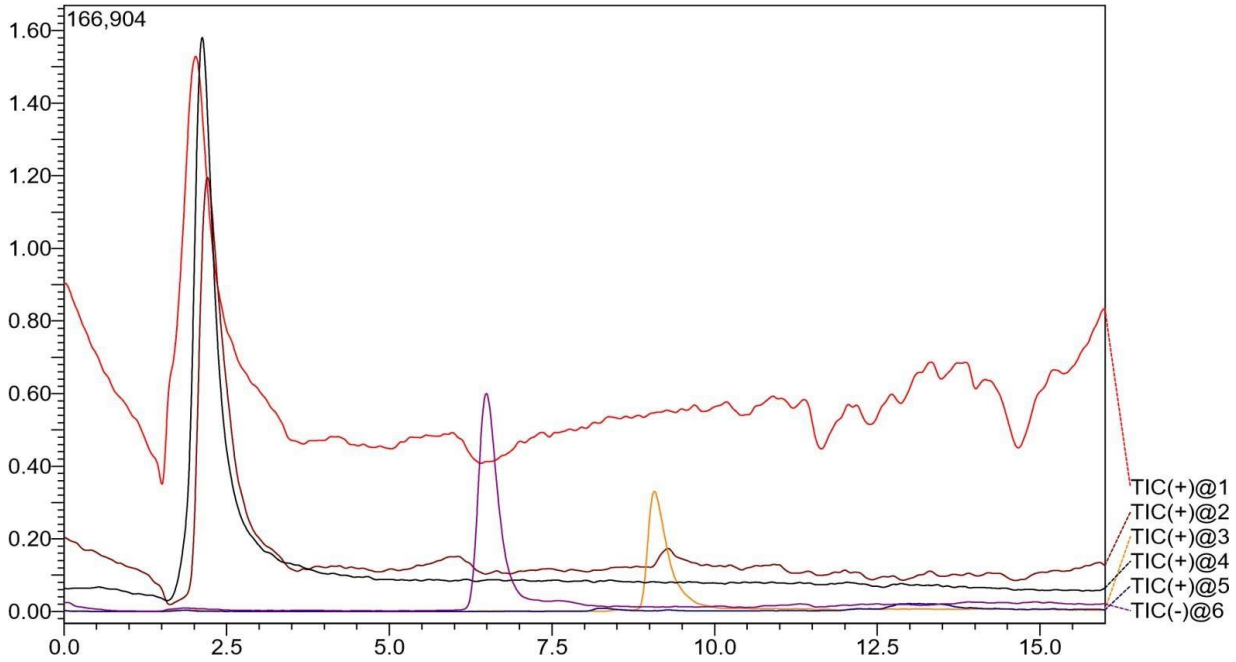


Fig 2.8 Representative LC-MS/MS chromatogram showing vitamin content in sample (little millet)

1-thiamine, 2-pyridoxine, 3-folic acid, 4-nicotinic acid (niacin), 5-riboflavin, 6-ascorbic acid

2.3.7 Phytochemical profiling of raw and germinated millet grains

2.3.7.1 *Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)*

Polyphenolic compounds are reported to be involved in potentiating the redox defense of the body, prevention, and counteracting oxidative stress and reducing free radical-related cellular damage (Adebo and Gabriela, 2020). They have generated interest because of their broad human health promoting effects, most of which are related to their antioxidant properties (Mira et al., 2002) and to the synergistic effects with other antioxidants (Felipe et al., 2001). Both phenolics and flavonoids are powerful antioxidants giving protection against oxidative and free radical damage and exert greater antioxidant effects than vitamin C, vitamin E, selenium, and zinc (Nambiar et al., 2012). Therefore, the presence of these compounds signifies the nutraceutical potential of the commodity. In the present study, we have evaluated and compared the phenolic and flavonoid contents of nine millets in their raw and germinated form. Also, the change in composition of these bioactive during germination was investigated to understand the evolution of nutraceutical property during germination.

Figure 2.9 shows the TPC of the raw and germinated grains. The TPC of all the millet types increased significantly ($p \leq 0.05$) upon germination. The TPC followed the order amaranth>foxtail millet>little millet>barnyard millet >kodo millet> sorghum> pearl millet> finger millet> proso millet in the raw form. As noted, among the raw grains, amaranth had the highest phenolic content (10.58±0.95 mg GAE/g dry weight (d.w)) followed by foxtail millet (9.42±0.80 mg GAE/g d.w), little millet (7.91±0.65 mg GAE/g d.w), and barnyard millet (7.26±0.95 mg GAE/g d.w). Proso millet exhibited the least phenolic content (2.80±0.08 mg GAE/g d.w). With progress in germination time, there

was a gradual increase in the phenolic content of all grains. The TPC after 72 h of germination was found to follow the order amaranth>barnyard millet>foxtail millet>kodo millet> little millet> sorghum> finger millet> proso millet. The TPC of amaranth grain increased from 11.63±0.50 mg GAE/g d.w after 24 h to 31.50±1.40 mg GAE/g d.w after 72 h of germination. On the other hand, foxtail millet showed a 1.9-fold increase in the TPC from 24th (11.90±0.58 mg GAE/g d.w) to 72nd h (23.57±0.85 mg GAE/g d.w) of germination.

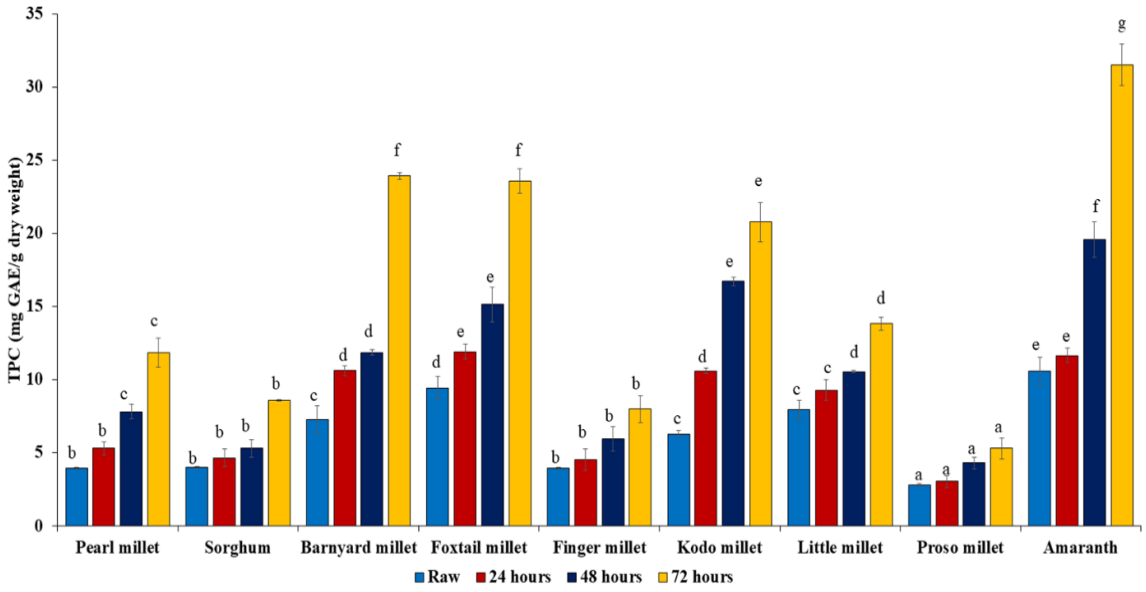


Fig. 2.9 Total phenolic content (TPC) of raw and germinated grains (mg GAE/g dry weight). (*Values are means ± SD (n = 3), followed by different superscript letter are significantly different ($p \leq 0.05$). The letter "a" represents the least value).

Similar to TPC, TFC of the grains showed a gradational increment upon germination as shown in Figure 2.10. TFC in raw millets followed the order of amaranth>foxtail millet>barnyard millet>proso millet> pearl millet> little millet> sorghum> kodo millet>

finger millet. As in the case of TPC, amaranth grains showed the highest flavonoid content in the raw form (479±2.10 mg QE/g d.w). This was followed by raw foxtail and barnyard millet with 142.33±1.60 mg QE/g d.w and 105.66±1.50 mg QE/g d.w., respectively. Whereas in germinated form after 72 h pearl millet had the highest TFC (1762.33 ±1.90 mg QE/g). Upon germination, the flavonoid content of amaranth and sorghum increased to 1272.33±1.80 mg QE/g d.w d.w and1399.00±1.20 mg QE/g d.w respectively. This was followed by barnyard millet> kodo millet> foxtail millet> finger millet> little millet> proso millet.

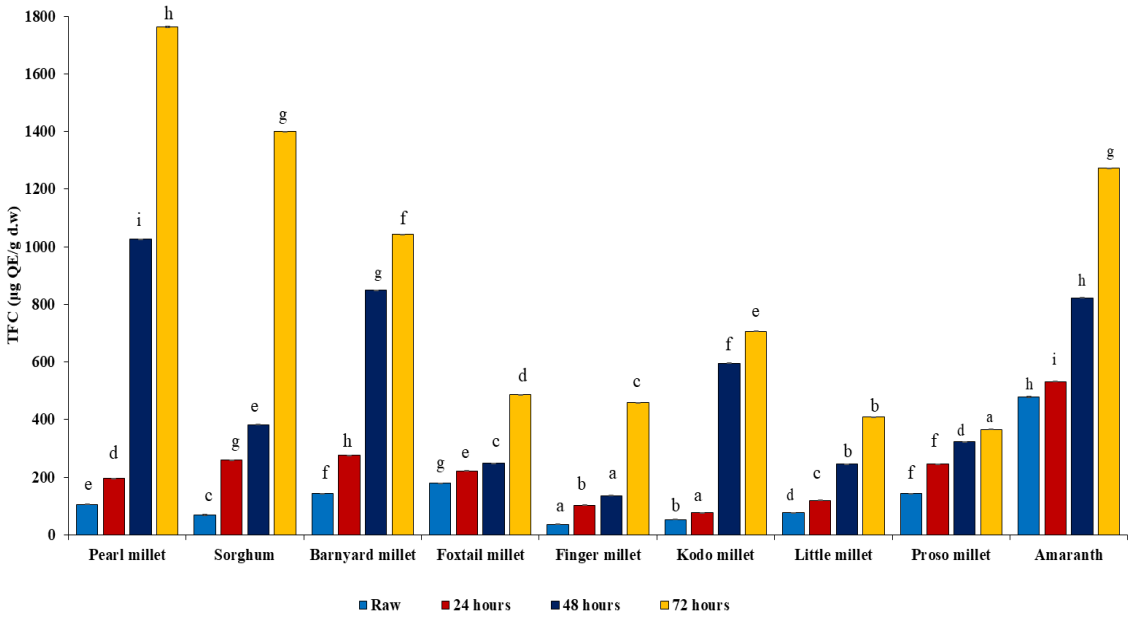


Fig 2.10 Total flavonoid content (TFC) of raw and germinated grains (mg QE/g dry weight). (*Values are means ± SD (n = 3), followed by different superscript letter are significantly different ($p \leq 0.05$). The letter "a" represents the least value).

Similar observations were reported by Guardianelli et al. (2019). Sharma, Saxena and Riar (2018) reported germination-mediated increase in phenolic content of amaranth after

24 h of germination and foxtail millet after 16 h germination, respectively. It is reported that germination is an adequate and effective bioprocess for increasing the concentration of phenolic compounds in amaranth seeds, and hence their nutraceutical quality (Alvarez-Jubete, 2010). The influence of germination on total phenolic contents has been investigated in many edible seeds, such as edible beans and cereal grains. Most studies found that germination can gradually accumulate soluble phenolics in germinated edible seeds and sprouts compared with raw seeds (Gan et al., 2017). This could be due to the release and biosynthesis of phenolic compounds. Cell wall-degrading enzymes are active during germination, and they contribute to modification of the cell wall structure of the grain (Mishra et al; 2022; Gowda et al., 2022). The significance of this relies on the fact that phenolic compounds such as hydroxycinnamates (*e.g*., ferulic and *p*-coumaric acids) are bound to non-starch polysaccharides in grain cell walls through associations such as ester and ether bonds. The action of cell wall-degrading enzymes (mainly esterases) on these bonds contributes to the release of bound phenolic compounds. Increase in the seed polyphenolic contents during germination can influence the functional properties of millets, since polyphenolic compounds having antioxidant properties can protect against degenerative disease (heart and cancer) in which reactive oxygen species are involved (Lopez-Amoros, Hernandez, and Estrella, 2006). The flavonoid content in millet grains is reported to be higher than most of the cereals (Yang et al; 2022; Hoskeri et al., 2022). Perales-Sánchez et al., 2014 reported that there was a significant increase in the TFC of germinated amaranth seed flours relative to its raw form. Sharma, Saxena and Riar (2016) reported similar observations on the increase in TFC of barnyard millet after germination. There was a major increase in the flavonoid content of kodo millet upon germination

(52.33±1.83 mg QE/g d.w to 705.66±1.30 mg QE/g d.w), as observed by Sharma, Saxena and Riar (2017) and Sharma et al. (2015). The increase in the TFC values of the grains is due to the synthesis of new flavonoid compounds during germination (Saleh et al., 2019). The variation in the contents of individual flavonoids during germination may be attributed to the release of aglycons from glucosides through the catalysis of activated glucosidases or conversion and biosynthesis of flavonoids in germinated millet grains (Rani et al., 2018).

2.3.7.2 Chemical profiling using LC-MS/MS

The TPC and TFC content indicated that the millets are good sources of phenolic compounds, which increased during germination. The major polyphenol and flavonoid compounds of these grain samples were characterized using LC-MS/MS analysis. Presence of about 28 polyphenol compounds (as listed in table 2.10) was analyzed using this method, among which, gallic acid, epicatechin, syringic acid, vanillic acid, ferulic acid, p-coumaric acid, shikimic acid and ellagic acid were found to be the major components and the presence of these compounds were confirmed using authentic standards. The analytical parameters of the LC-MS/MS method is provided in Table 2.9. The individual polyphenol content also increased in almost all the samples upon germination.

Amaranth

Amaranth contains considerable amounts of shikimic acid> syringic acid> vanillic acid> cinnamic acid>ferulic acid> p-coumaric acid> ellagic acid> epigallocatechin. Amaranth had the highest vanillic acid content in the raw form (215.01±0.25 µg/g). Epicatechin

content (90.00±0.80 μg/g) was highest in the 72 h germinated amaranth seed grains. The syringic acid content improved from 232.18±1.10 μg/g in raw amaranth to 620.50±2.18 μg/g in 72 h-germinated seed. There was an increase in the caffeic acid of amaranth from 3.52±0.30 to 31.22±0.95 μg/g when germinated for 72 h. The ferulic acid content of amaranth escalated from 107.93±1.25 to 926.10±2.07 μg/g after 72 h. The ellagic and cinnamic acid contents of amaranth grains too increased gradually following 72 h of germination. From the phenolic acids determined, vanillic acid was the most abundant in amaranth followed by chlorogenic acid, p-coumaric acid and caffeic acid as reported by Procopet and Oroian (2022). Polyphenols including rutin (10.1 mg/g) and isoquercetin (0.5 mg/g), were found in amaranth seed, and three phenolic acids, including 4-hydroxybenzoic acid (2.2 mg/g), syringic acid (0.8 mg/g), and vanillic acid (1.8 mg/g), were also measured by Barba de la Rosa et al 2009. The phenolic acids in the methanol extract of A. spinosus (amaranthus) seeds are characterized by the predominance of caffeic acid followed by cinnamic, gallic, vanillic, protocatechuic, ferulic, and coumaric acids (Rjeibi et al., 2016). The polyphenols in amaranth seed present high antioxidative activity and anti-inflammatory capacity as well as miscellaneous effects like anti-fibrotic, anti-bacterial, anti-hypertensive, anti-atherogenic and anti-proliferative effects (Antoniewska et al., 2018). From the present study, we confirmed the presence of considerable amounts of hydroxycinnamic acids of raw and germinated amaranth grains which is found to increase considerably upon germination. The study also reports the presence of a range of phenolic compounds which are not reported earlier.

Pearl millet

Characterization of phenolic compounds revealed the presence of catechin, quinine, naringenin, tocopherol, gallic acid, chlorogenic acid, epicatechin, syringic acid, vanillic acid, caffeic acid, epigallocatechin, ferulic acid, myricetin, p-coumaric acid, luteolin, apigenin, kaempferol, hesperetin, shikimic acid, ellagic acid, genistein, cinnamic acid and chrysin. The major polyphenols found were shikimic acid> syringic acid> cinnamic acid> vanillic acid> ferulic acid> p-coumaric acid> epicatechin. Pearl millet raw form contained the highest catechin content (4.66±1.25 µg/g) among all the millets studied which increased upon germination. Pearl millet contained good amounts of epicatechin upon germination (157.29±0.55 µg/g). The vanillic acid content of pearl millet increased from 155.56±0.95 µg/g in the raw form to 335.08±1.20 µg/g after 48 h of germination. Pearl millet had substantial amounts of cinnamic acid. The amount varied from 236.94± 1.20 µg/g (raw form), 249.39±0.07 µg/g (after 24 h), 309.45±1.20 µg/g (after 48 h) and 482.30±1.35 µg/g (after 72 h). Trans-cinnamic, protocatechuic, and hydroxybenzoic acids were the main phenolic compounds of pearl millet extracts as observed by Radhouane (2013) and Bouajila (2020). Methanolic extracts of pearl millet contain syringic acid, caffeic acid, p-coumaric acid and ferulic acid according to Triki et al., 2022 as observed in our present study. Apart from this the present study also reports the presence of polyphenols like luteolin, naringenin and hesperitin in raw and germinated pearl millet samples for the first time.

Sorghum

Raw sorghum showed good amounts of shikimic acid> ferulic acid> cinnamic acid> syringic acid> epicatechin> ellagic acid> p-coumaric acid> vanillic acid. In sorghum, quinine content was found to be highest in the 72 h germinated sample (5.42±0.60 µg/g)

which was the highest among all the millet samples. Chlorogenic acid was found to be the highest in 72 h-germinated sorghum (11.79±0.90 µg/g). The syringic acid content in sorghum showed an increase from 67.87±1.09 µg/g in the raw form to 497.24±1.00 µg/g after 72h germination. The vanillic acid content of sorghum increased from 14.49±0.07 µg/g in raw form to 772.16±0.50 µg/g after 3-day germination. Hithamani and Srinivasan (2014) identified gallic acid, protocatechuic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, sinapic acid, salicylic acid, and cinnamic acid in native sorghum. Ghinea et al., 2021 has reported that the chlorogenic acid in sorghum has doubled after 48 h of germination as observed in our study. The authors also report the increase of naringenin and p-coumaric acid after germination.

Barnyard millet

Barnyard millet possessed considerable amounts of shikimic acid> syringic acid> ferulic acid > p-coumaric acid> epicatechin> ellagic acid > cinnamic acid. Barnyard millet had the highest content of syringic acid (403.10±0.80 µg/g) among the raw grains. Naringenin content was also found to be the highest in germinated barnyard millet and there was nine-fold increment in its value (46.11±0.80 µg/g). After 72 h of germination, the catechin content was found to be the highest in barnyard millet (69.73±0.04 µg/g). The vanillic acid content in the grains had significant increase from 13.25±0.50 to 283.89±1.10 µg/g upon 72 h germination. Sharma et al., (2016) reported that free, bound, and total polyphenolic contents of barnyard millet increase upon germination.

Foxtail millet

Raw foxtail millet had ample amount of shikimic acid> syringic acid> p-coumaric acid> cinnamic acid> vanillic acid> ellagic acid> ferulic acid> epicatechin. It also showed the highest tocopherol content (9.64±0.25 µg/g) upon germination for 72 h. Syringic acid content escalated from 336.49±0.25 µg/g in the raw form to 562.43±0.90 µg/g after germination for 72h. There was a remarkable increase in the ferulic acid content of foxtail millet from 20.18±0.05 µg/g in raw form to 565.61±1.10 µg/g following 72 h of germination. Vanillic acid content increased to 534.21±1.25 µg/g in the germinated foxtail millet from 28.93±0.09. p-Coumaric acid content increased from 31.95±0.15 to 474.81±1.10 µg/g following germination. Chandrasekara & Shahidi, (2011) also reported the presence of apigenin, vanillic acid and gallic acid at various levels in the soluble and bound fractions of different millets including foxtail millet. Ferulic acid, caffeic acids and p-coumaric acids were detected in foxtail millets by Pradeep and Sreerama (2018) and Chandrasekara and Shahidi (2011). Presence of flavonoids such as kaempferol and lutein were confirmed in the present study. They also contained ferulic and p-coumaric acids in good amounts. All these contributed to the high TPC content of foxtail millet.

Finger millet

Finger millet in its raw form had considerable amounts of ferulic acid> shikimic acid> syringic acid> p-coumaric acid> cinnamic acid> epicatechin> vanillic acid. In the case of finger millet, there was a twenty-fold increase in the catechin content after germination of 72 h (2.01±0.10 to 21.30±0.20 µg/g). p-Coumaric acid of germinated finger millet grain (72 h) increased ~ 7 times to 489.98 ± 1.20 µg/g. Ferulic acid content of raw samples were 387.88±2.80 µg/g which then decreased to 208.12±0.90 µg/g after 24 h. But it increased to 973.20±1.20 µg/g after 72 h. Vanillic acid content of the samples also

increased significantly upon germination to 72 h. Chethan et al. (2008) identified nine phenolic acids which include gallic acid, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, ferulic acid, syringic acid, trans-cinnamic acid and p-coumaric acid in finger millets varieties. Caffeic (CA) and p-coumaric (p-CoA), the two common hydroxycinnamic acid derivatives in grains (Van Hung, 2016), which are mostly bound forms are present ranging from 0.83 to 2.43 and 1.88–3.94 mg/100 g, respectively. Ferulic acid was the dominant polyphenol in the bound fraction in finger millet, ranging from 59.58 to 67.36 and from 35.19 to 40.12 mg/100 g DW for central and northern Malawi millets, respectively (Xiang et al., 2019), which were in accordance with our results. Chethan and Malleshi (2007) found a relatively high content of free phenolic acids mainly composed of benzoic acid derivatives and cinnamic acid derivatives in finger millet from India. This may be a result of different extraction methods and growing environments (Van Hung, 2016). Ferulic acid which is predominant in finger millet has therapeutic potential including antioxidant, anti-inflammatory and antimicrobial properties. Hydroxycinnamic acids compose of majority of the polyphenol fraction in finger millet. Finger millet seed coat is abundant in many polyphenols when compared to major cereals like wheat or rice (Onipe et al., 2022). The dark colored finger millet that we used in our study possesses higher amounts of antioxidants than light colored ones as reported by Kumar et al., (2021).

Kodo millet

Kodo millet contained all the major polyphenols in subtle quantities including shikimic acid> cinnamic acid> syringic acid> ferulic acid> p-coumaric acid> ellagic acid> vanillic acid. In kodo millet, the gallic acid increased from 5.55±0.60 µg/g to 16.21±0.90 µg/g

after 72h of germination. Also, the epicatechin, syringic acid and shikimic acid levels escalated gradually upon germination. The vanillic acid content in kodo millet increased in the order 17.10±0.09 µg/g (raw) < 22.87±0.20 µg/g (24 h) < 54.52±0.20 µg/g (48 h) < 305.22±0.95 µg/g (72h). The ferulic acid content increased from 77.24 ± 0.85 µg/g in raw form to 231.21±1.20 µg/g after 72 h germination. Sharma et al. (2017) reported higher amounts of phenolic content and antioxidant activity in methanolic extracts of kodo millet grains especially cinnamic and ferulic acids. Chandrasekara and Shahidi (2011); have reported the presence of vanillic acid, cinnamic acid, apigenin and gallic acid in kodo millet as seen in our study. In recent years, phenolic acids mainly ferulic and p-coumaric acids are of major interest to food researchers and manufacturers due to their bioactive properties and prevention of chronic diseases.

Little millet

The major polyphenols found in raw little millet includes shikimic acid> syringic acid> ferulic acid> cinnamic acid> ellagic acid > epicatechin >apigenin. Upon germination, the highest syringic acid content was seen in little millet (1773.47±1.28 µg/g) followed by pearl millet (1501.38±1.80 µg/g). Though the presence of other phytochemicals investigated were not significant compared to the above listed compounds, there was a remarkable increase in some of them during germination. E.g., vanillic acid content increased from 26.58±0.45 to 270.70±0.95 µg/g and p-coumaric acid content increased from 7.95±0.06 to 402.63±1.50 µg/g upon g72 h of germination. Tocopherol content of little millet post 3-day germination was 5.99±0.02 µg/g. Bioactive compounds such as gallic acid, vanillic, *p*-hydroxybenzoic acid, sinapic acid, chlorogenic acid, caffeic acid, ferulic acid, and *p*-coumaric acid are all rich in little millets (Kaur et al., 2019). Pradeep

and Guha (2011) have reported that gallic acid contents of little millet increased during germination. Presence of flavonoids e.g., like naringenin, myricetin in millet grains have increased many folds during germination etc. Reports on the individual flavonoids is limited.

Proso millet

Major polyphenols in raw proso millet were shikimic acid> ferulic acid> syringic acid> cinnamic acid> vanillic acid> epicatechin. Syringic acid and vanillic acid content of proso millet shot up from 241.07±0.95 µg/g, 26.58±0.45 µg/g in the raw form to 618.08±1.11 µg/g and 270.70±0.95 µg/g after 72 h respectively. p-Coumaric acid content increased from 7.95±0.06 to 402.63±1.50 µg/g upon germination to 72h. Pradeep and Sreerama (2015) have observed that raw proso millet contained good quantities of ferulic and vanillic acids. Chandrasekara and Shahidi (2011) have also reported the presence of ferulic acid, gallic acid, vanillic acid, apigenin, syringic acid in proso millet extracts. Reports on individual flavonoids present in proso millet is limited and the present study confirmed the presence of compounds like apigenin, naringenin, kaempferol, myricetin, luteolin and catechin.

When we compare the total polyphenol fraction of all the millet samples, it is found that there is a significant increase in the polyphenol composition of all grains upon germination and the values varied from sample to sample. Shikimic acid was found to be the major compound in all the millets studied expect for finger millet. There was several fold increase in the shikimic acid content of all the grains upon germination with barnyard millet for 72 h having 32125.33±2.15 µg/g, followed by sorghum (25098.28±2.25 µg/g,

72 h) and finger millet (22111.01±2.80 µg/g, 72 h). Upon germination, the highest gallic acid content was found in finger millet (68.36±0.95 µg/g), followed by sorghum (53.81±0.10 µg/g) and pearl millet (51.08±0.25 µg/g). In accordance with the present results, Das and Singh (2016) had reported the predominance of vanillic, ferulic and p-coumaric acids as bound phenolics in the pericarp, germ and endosperm fractions of specialty maize genotypes.

Among phenolic acids present in millets, derivatives of cinnamic acids, hydroxyl cinnamic acids (HCA) were more common than the hydroxyl benzoic acids (HBA) and consisted chiefly of p-coumaric, caffeic, ferulic acid, and sinapic acids. These acids are rarely found in the free form. Ferulic acid is one of the abundantly found hydroxycinnamic acids (Hithamani and Srinivasan, 2014) in millets. Besides the monomeric compounds, ferulate dimers exhibiting higher antioxidant activity are reported in in the millet grains especially hydroxycinnamic acids (Chandrasekera and Shahidi, 2011). Derivatives of cinnamic acids are reported to exhibit strong antioxidant activities in millets (Shahidi & Chandrasekara, 2010). These phenolic acids are attracting the interest from both researchers and food manufacturers, as they possess antioxidative, antimutagenic, anti-inflammatory and anticarcinogenic properties and modulate some key cellular enzymes (Arts & Hollman, 2005). In addition, there is an increased commercial interest in phenolic acids because they aid the maintenance of food taste, color, fresh flavor and prevention of oxidative deterioration (Maqsood, Benjakul, & Shahidi, 2013). Depending on the technological necessities, milled fractions of millets, which are particularly enriched with specific phenolic acids, may be used as ingredients in functional foods. It has been shown that depending on the variety, millet phenolic

extracts contain variable amounts of hydroxycinnamic and hydroxybenzoic acids and their derivatives as well as flavonoids (Chandrasekara and Shahidi, 2011). Furthermore, major hydroxycinnamic acids identified were sinapic, ferulic, *p*-coumaric, caffeic, and chlorogenic acids. Hydroxybenzoic acids reported were gallic, protocatechuic, *p*-hydroxybenzoic, gentisic, vanillic and syringic acids. In comparison to the raw millets, germination caused significant ($p \leq 0.05$) increase in gallic, vanillic, chlorogenic, ferulic, sinapic acids, subtotal benzoic acid and subtotal cinnamic acids as observed by Pradeep and Sreerama (2015).

A decrease in polyphenol content was observed for certain millets during germination. p-coumaric acid content in foxtail millet reduced after 24h from 71.35±0.10 μg/g to 50.00±0.90 μg/g and again increased after 72 h to 389.50±1.05 μg/g. Epicatechin content of foxtail millet reduced from 68.32±0.90 after 48 h to 63.15±1.20μg/g after 72 h of germination. There was a slight decrease in the ferulic acid content of proso millet after germination to 72 h. This reduction of polyphenol after germination may be associated with the presence of polyphenol oxidase or may arise due to the hydrolysis of tannin protein and tannin enzymes complexes which promotes the elimination of tannin or polyphenols (Rani et al., 2018). Overall, the changes in polyphenolic contents could be due to hydrolysis of polymeric polyphenols due to the enzyme glucosidase or esterases that yields free or extractable polyphenolic compounds, formation of insoluble or unextractable complex with proteins, polymerization of some polyphenols with low molecular weight, decluming, leaching during grain soaking or antioxidant synthesis (Kim et al., 2016).

From the present study we could confirm the presence of 23 polyphenols in both native and germinated nine millet varieties using LC-MS/MS and the effect of germination on these compounds. Shikimic acid had the biggest peak in the LC-MS/MS chromatogram indicating that it was the bioactive found in the highest concentration in millet varieties. This was followed by other polyphenols like vanillic acid, syringic acid, ferulic acid, cinnamic acid, p-coumaric acid and ellagic acid belonging to the group of hydroxycinnamic acids. As mentioned earlier, such a comprehensive report on phytochemicals with respect to millets are not reported so far.

Table 2.9 Analytical parameters for 28 polyphenols carried out using LC-MS/MS (Abraham et al., 2020)

Sl. no	Standards	Retention time (min.)	Ion monitoring (m/z)		Ion mode	Calibration equation	Collision energy (CE) (V)	R^2	Linear Range (µg/L)	DL/QL (µg/L)
			Precursor ion	Molecular ion						
1	Catechol	1.87	111.20	78.95/64.10	+ve	y = 387.253x+7.0483e+006	-9/-25	0.986	5-50	0.07/0.23
2	Catechin	6.75	291.20	139.10/165.05	+ve	y = 10393.1x-16953.5	-15/-13	0.993	5-150	2.20/6.68
3	Quinine	6.88	325.20	307.10/184.05	+ve	y = 70839.3x+3503.38	-24/-28	0.996	5-150	0.27/0.81
4	Naringenin	7.28	273.20	153.05/147.15	+ve	y = 5753.24x-13117.0	-17/-21	0.992	5-150	0.78/2.37
5	Tocopherol	12.87	429.50	163.15/205.05	+ve	y = 12257.5x-27446.3	-22/-23	0.993	5-150	2.31/7.01
6	Gallic acid	1.91	169.20	125.05/81.00	-ve	y = 2779.60x+1214.12	17/17	0.992	5-150	3.20/9.70
7	Chlorogenic acid	6.81	353.00	191.20/92.90	-ve	y = 10282.0x+16136.9	16/43	0.997	5-150	1.27/3.85
8	Epicatechin	6.77	289.00	245.20/205.20	-ve	y = 2231.57x-3359.76	14/16	0.993	5-150	7.08/21.46
9	Syringic acid	7.20	197.20	182.20/123.05	-ve	y = 941.096x+12146.4	14/24	0.996	5-150	10.44/31.65
10	Vanillic acid	6.79	167.20	152.10/108.10	-ve	y = 8887.775x+2823.33	17/19	0.991	5-150	14.35/43.48
11	Caffeic acid	6.91	179.20	135.15/134.10	-ve	y = 31106.3x-2363.27	16/30	0.995	5-150	0.49/1.49
12	Epigallocatechin	2.01	456.90	169.15/125.05	-ve	y = 904.191x-524.176	17/40	0.987	5-50	2.90/8.79
13	Ferulic acid	7.36	193.20	134.00/178.00	-ve	y = 2014.91x+171906	15/10	0.992	5-50	4.37/13.23
14	Myricetin	7.65	317.00	151.20/179.20	-ve	y = 15064.0x-14109.0	25/19	0.995	5-150	0.25/0.75
15	Quercetin	7.92	301.20	151.05/179.00	-ve	y = 48446.6x+52582.0	22/18	0.991	5-150	0.62/1.88

16	p-Coumaric acid	7.34	163.00	119.15/93.10	-ve	y = 12379.7x+41113.6	15/33	0.992	5-150	2.13/6.46
17	Luteolin	7.92	285.20	151.10/175.05	-ve	y = 29060.1x+177101	25/25	0.991	5-150	0.27/0.83
18	Apigenin	8.18	269.20	149.05/151.00	-ve	y = 26104.3x-13101.5	23/25	0.993	5-150	0.48/1.45
19	Kaempferol	7.83	285.20	159.15/187.05	-ve	y = 1243.29x-487.739	31/27	0.993	5-150	1.44/4.36
20	Rutin	7.34	609.20	300.00/301.15	-ve	y = 25186.6x+9234.48	38/34	0.995	5-150	0.08/0.23
21	Daidzein	7.91	252.90	208.20/224.15	-ve	y = 8324.16x-13781.5	29/25	0.981	5-150	0.71/2.16
22	Hesperetin	7.86	301.20	164.10/286.05	-ve	y = 17857.8x-14187.8	25/18	0.993	5-150	0.57/1.73
23	Shikimic acid	1.76	172.90	111.20	-ve	y = 864.091x+63809.9	11	0.969	5-150	5.79/17.55
24	Ellagic acid	7.56	300.90	185.10/145.20	-ve	y = 648.613x+9964.71	31/40	0.992	5-150	2.28/6.92
25	Morin	7.74	301.20	151.00/149.15	-ve	y = 49085.8x+113300	20/25	0.994	5-150	0.59/1.79
26	Genistein	7.81	269.20	133.20/132.05	-ve	y = 11955.6x-6477.21	31/46	0.990	5-150	0.59/1.79
27	Cinnamic acid	7.93	147.00	103.05	-ve	y = 628.783x+1513.72	13	0.993	5-150	6.60/20.01
28	Chrysin	8.39	252.90	62.95/143.20	-ve	y = 20464.6x-17508.2	32/27	0.996	5-150	0.41/1.26

Table 2. 10 Polyphenol composition of raw and germinated millet grains (µg/g)

Sl. no.	Polyphenol standards	GT	Pearl millet	Sorghum	Barnyard millet	Foxtail millet	Finger millet	Kodo millet	Little millet	Proso millet	Amaranth
1	Catechol	0th	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
		24th	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
		48th	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
		72nd	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
2	Catechin	0th	4.66±1.25^{b}	2.29±0.95^{a}	2.18±0.20^{a}	2.41±0.30^{a}	2.01±0.10^{a}	2.17±0.20^{a}	2.54±0.05^{a}	2.19±0.25^{a}	2.57±0.25^{a}
		24th	6.78±0.60^{c}	4.72±0.70^{b}	2.56±0.80^{a}	2.78±0.50^{a}	2.06±0.20^{a}	2.21±0.20^{a}	2.75±0.25^{a}	2.26±0.10^{a}	2.05±0.08^{a}
		48th	8.68±0.45^{d}	6.54±0.08^{c}	3.09±0.45^{b}	2.41±0.08^{a}	7.32±0.10^{c}	2.28±0.30^{a}	3.85±0.20^{b}	2.20±0.20^{a}	2.58±0.03^{a}
		72nd	17.65±0.27^{d}	13.37±0.25^{c}	69.73±0.04^{f}	2.78±0.02^{a}	21.30±0.20^{e}	2.75±0.45^{a}	4.29±0.15^{b}	2.56±0.30^{a}	2.89±0.20^{a}
3	Quinine	0th	1.23±0.07^{c}	0.22±0.02^{b}	0.06±0.02^{a}	0.24±0.15^{b}	0.10±0.02^{a}	0.11±0.02^{a}	0.33±0.06^{b}	0.18±0.03^{a}	0.11±0.01^{a}
		24th	1.87±0.55^{b}	0.31±0.01^{a}	0.18±0.25^{a}	0.43±0.10^{b}	0.16±0.05^{a}	0.15±0.02^{a}	0.42±0.05^{b}	0.38±0.20^{b}	0.23±0.05^{b}
		48th	1.92±0.45^{c}	2.47±0.03^{d}	0.19±0.70^{a}	0.92±0.08^{b}	0.25±0.07^{a}	0.17±0.03^{a}	0.47±0.07^{b}	0.42±0.05^{b}	1.09±0.02^{c}
		72nd	3.22±1.20^{c}	5.42±0.60^{d}	0.23±0.70^{a}	1.31±0.20^{b}	0.32±0.06^{a}	0.26±0.01^{a}	1.10±0.97^{b}	0.90±0.02^{a}	1.73±0.05^{b}
4	Naringenin	0th	5.12±0.65^{c}	4.92±0.75^{b}	4.54±0.15^{b}	5.43±0.15^{c}	3.93±0.25^{a}	4.71±0.15^{b}	4.08±0.08^{b}	4.22±0.15^{b}	4.70±0.25^{b}
		24th	8.96±0.75^{a}	11.00±0.25^{d}	5.49±0.16^{a}	8.18±0.50^{e}	8.00±0.20^{c}	5.52±0.25^{a}	7.01±0.40^{b}	6.33±0.40^{b}	6.87±0.30^{b}
		48th	9.77±0.80^{d}	11.69±0.20^{f}	5.52±0.42^{a}	8.77±0.70^{c}	10.45±0.40^{e}	7.08±0.70^{b}	11.17±0.50^{f}	6.87±0.50^{b}	8.62±0.20^{c}
		72nd	11.34±0.25^{c}	19.81±0.55^{e}	46.11±0.80^{g}	12.74±0.80^{d}	22.47±0.30^{f}	7.32±0.45^{a}	11.65±0.50^{c}	11.39±0.25^{c}	9.56±0.50^{b}
5	Tocopherol	0th	2.86±0.35^{a}	2.99±0.09^{a}	3.29±0.15^{a}	3.00±0.30^{a}	3.14±0.75^{a}	2.93±0.35^{a}	2.86±0.06^{a}	2.97±0.30^{a}	2.94±0.20^{a}
		24th	3.15±0.67^{a}	3.38±0.07^{a}	3.84±0.65^{b}	3.81±0.09^{b}	3.17±0.25^{a}	2.98±0.65^{a}	3.03±0.07^{a}	3.39±0.20^{a}	3.78±0.25^{b}
		48th	3.26±0.30^{a}	3.31±0.06^{a}	3.81±0.50^{a}	4.24±0.60^{b}	3.37±0.30^{a}	3.10±0.20^{a}	3.16±0.50^{a}	3.63±0.40^{a}	4.74±0.05^{b}
		72nd	3.53±0.45^{a}	3.00±0.06^{a}	4.24±0.50^{b}	9.64±0.25^{d}	3.95±0.20^{a}	3.24±0.30^{a}	4.20±0.60^{b}	5.99±0.02^{c}	5.25±0.09^{c}

6	Gallic acid	0th	3.86±0.95a	5.10±0.15c	5.11±0.15c	4.01±0.30a	4.88±0.20b	5.55±0.60c	5.14±0.35c	4.99±0.35b	5.08±0.20c
		24th	10.31±0.35f	5.68±0.18b	7.16±0.40d	4.16±0.05a	8.20±0.09e	7.15±0.25d	5.20±0.50b	5.99±0.25c	6.14±0.25c
		48th	14.82±0.80d	9.27±0.14b	8.83±0.20a	7.48±0.60a	8.78±0.08a	13.97±1.20f	10.28±0.09c	8.08±0.45a	8.10±0.09a
		72nd	51.08±0.25f	53.81±0.10g	11.48±0.80c	9.64±0.55b	68.36±0.95h	16.21±0.90e	14.15±0.60d	8.25±0.20a	9.72±0.09b
7	Chlorogenic acid	0th	1.72±0.08a	3.55±0.08b	<0.001	<0.001	<0.001	<0.001	4.83±0.10c	1.65±0.03a	1.97±0.02a
		24th	1.85±0.25a	3.57±0.45c	<0.001	<0.001	<0.001	<0.001	<0.001	2.48±0.06b	2.04±0.05a
		48th	5.68±0.25c	7.16±0.70d	<0.001	<0.001	<0.001	<0.001	1.55±0.07a	2.59±0.05b	2.11±0.05b
		72nd	8.63±0.18d	11.79±0.90e	<0.001	<0.001	<0.001	<0.001	1.76±0.04a	3.97±0.10c	2.71±0.11b
8	Epicatechin	0th	32.61±0.15f	62.50±0.95h	37.40±0.25g	11.53±0.30b	27.14±0.25e	8.92±0.04a	11.49±0.06b	13.54±0.09c	25.63±0.17d
		24th	95.29±0.25f	107.45±1.15g	37.38±0.20d	28.55±0.20c	37.41±0.30d	46.92±0.07a	23.66±0.50b	16.88±0.02a	57.35±0.18e
		48th	132.53±0.40i	120.96±1.20h	87.33±0.30g	30.99±0.75b	68.32±0.90d	76.80±0.70f	38.39±0.06c	19.22±0.09a	74.54±0.25e
		72nd	157.29±0.55h	154.04±0.18g	82.31±1.30e	45.21±0.08b	63.15±1.20d	90.18±0.50a	42.73±0.36a	51.50±0.30c	90.00±0.80f
9	Syringic acid	0th	312.60±0.30g	67.87±1.09a	403.10±0.80i	336.49±0.25h	116.12±1.10c	84.36±1.20b	429.74±0.75d	241.07±0.95f	232.18±1.10e
		24th	370.32±0.25e	418.29±1.11f	524.64±1.10i	397.76±0.35d	148.96±0.95b	92.88±0.90a	429.46±0.80g	463.72±0.85h	303.07±0.95c
		48th	540.25±1.65g	476.29±1.26a	632.82±1.30h	525.27±0.95f	461.20±1.50b	305.22±1.25a	483.15±0.95d	464.44±1.20c	509.06±1.02e
		72nd	1501.38±1.80h	497.24±1.00b	659.88±1.08g	562.43±0.90d	518.11±2.25c	385.92±0.90a	1773.47±1.28i	618.08±1.11e	620.50±2.18f
10	Vanillic acid	0th	155.56±0.95f	14.49±0.07c	13.25±0.50b	28.93±0.09e	26.37±0.08d	17.10±0.09b	9.30±0.15a	26.58±0.45d	215.01±0.25g
		24th	221.16±0.80g	42.97±0.25f	14.12±0.20a	39.14±0.15e	37.76±0.95d	22.87±0.20c	19.44±0.15b	36.82±0.25d	299.93±0.10h
		48th	335.08±1.20g	289.85±0.35f	21.00±0.15a	65.19±0.35e	49.63±0.90c	54.52±0.20d	30.62±0.20b	53.32±0.25d	359.52±0.25h
		72nd	294.86±0.90d	772.16±0.50h	283.89±1.10b	534.21±1.25g	262.44±1.40b	305.22±0.95e	117.36±1.20a	270.70±0.95c	482.80±0.80f
11	Caffeic acid	0th	2.51±0.09b	2.26±0.26b	2.75±0.07b	2.03±0.07b	3.38±0.09c	1.40±0.15a	2.14±0.10b	2.05±0.07b	3.52±0.30c
		24th	2.89±0.25b	3.23±0.03c	3.62±0.08c	2.41±0.15b	2.02±0.15a	2.58±0.25b	1.86±0.07a	2.53±0.10b	2.33±0.20b
		48th	11.80±0.23d	7.04±1.17c	3.98±0.03b	3.60±0.05b	2.44±0.25a	2.93±0.25a	4.21±0.05b	2.98±0.20a	3.37±0.15b
		72nd	13.97±0.60f	9.88±0.50e	5.88±0.09d	2.80±0.02a	4.35±0.75c	9.44±0.45e	35.62±0.18h	3.26±0.05b	31.22±0.95g

12	Epigallocatechin	0th	0.79±0.25a	0.75±0.20a	0.70±0.01a	0.68±0.02a	0.64±0.08a	0.69±0.05a	0.79±0.10a	0.62±0.06a	0.72±0.25a
		24th	0.80±0.30b	0.70±0.21a	0.69±0.02a	0.70±0.03a	0.75±0.01a	0.68±0.10a	0.82±0.10b	0.74±0.15a	0.74±0.20a
		48th	0.89±0.15b	0.70±0.09a	0.79±0.01a	0.72±0.02a	0.72±0.30a	0.75±0.06a	0.74±0.09a	0.69±0.10a	0.74±0.09a
		72nd	1.21±0.20b	0.73±0.15a	0.70±0.05a	0.79±0.03a	0.76±0.15a	0.98±0.15b	0.77±0.03a	0.72±0.10a	1.17±0.15b
13	Ferulic acid	0th	70.08±1.15b	217.68±1.12e	110.29±1.50d	20.18±0.05a	387.88±2.80h	77.24±0.85b	287.03±1.02g	265.03±1.10f	107.93± 1.25c
		24th	218.08±0.90d	273.03±0.98e	179.01±0.50c	287.05±0.95f	208.12±0.90d	166.50±0.90b	218.07±1.05b	140.99±0.65a	311.10±2.15g
		48th	324.76±1.20e	333.63±1.20g	320.12±1.25d	350.00±0.90h	215.54±1.10a	231.21±1.20b	368.71±2.15b	328.30±1.15f	301.58±1.14c
		72nd	375.03±0.85c	473.38±2.15e	411.57±2.01d	565.61±1.10g	973.20±1.20i	519.90±2.05f	309.03±1.14b	253.57±0.90a	926.10±2.07h
14	Myricetin	0th	1.22±0.10a	1.11±0.08a	0.97±0.11a	0.98±0.05a	1.15±0.06a	1.15±0.06a	1.24±0.10a	1.08±0.09a	1.01± 0.27a
		24th	1.25±0.15a	1.22±0.11a	1.48±0.25a	1.03±0.25a	1.15±0.02a	1.08±0.02a	1.02±0.07a	1.02±0.08a	1.01±0.05a
		48th	1.19±0.25a	1.07±0.05a	1.07±0.30a	1.12±0.10a	0.96±0.11a	1.00±0.03a	1.29±0.40a	0.98±0.25a	1.24±0.02a
		72nd	1.06±0.10a	1.06±0.15a	1.04±0.25a	1.10±0.05a	1.25±0.19a	0.98±0.01a	1.12±0.05a	1.00±0.06a	1.28±0.05a
15	Quercetin	0th	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
		24th	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
		48th	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
		72nd	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
16	p-Coumaric acid	0th	42.58±0.55e	24.15±0.55c	98.21±0.35i	71.35±0.10g	64.41±0.50f	31.95±0.15d	9.33±0.09b	7.95±0.06a	91.88 ± 1.10h
		24th	154.52±0.70h	107.63±0.95f	99.43±0.25e	50.00±0.90a	91.22±0.60d	79.74±0.50b	85.99±0.55c	114.30±0.87g	85.95±0.10c
		48th	140.24±0.45d	389.17±1.10h	173.64±1.20e	193.67±0.95g	176.75±1.25f	118.00±0.95a	409.93±0.95i	121.25±0.50b	130.41±0.90c
		72nd	180.66±1.02a	429.68±1.20f	338.44±2.10c	389.50±1.05d	489.98±1.20i	474.81±1.10h	463.28±0.60g	402.63±1.50e	187.89±0.65b
17	Luteolin	0th	0.20±0.04b	0.04±0.14a	0.01±0.01a	<0.001	<0.001	0.02±0.01a	0.002±0.01a	0.07±0.01a	0.13±0.20b
		24th	0.21±0.10b	0.06±0.10b	0.07±0.02b	0.07±0.02b	0.02±0.10b	0.07±0.01b	0.005±0.01a	0.15±0.01b	0.12±0.12b
		48th	0.11±0.20a	0.07±0.20a	0.14±0.02a	0.14±0.01a	0.10±0.03a	0.23±0.02a	0.13±0.01a	0.18±0.01a	0.12±0.12a
		72nd	0.15±0.20a	0.28±0.10a	0.22±0.03a	0.17±0.02a	0.11±0.02a	1.21±0.01b	0.15±0.05a	0.19±0.01a	0.13±0.06a

18	Apigenin	0th	0.96±0.07a	1.06±0.05b	1.69±0.06b	1.67±0.15b	1.16±0.15b	1.50±0.15b	1.48±0.08b	1.20±0.10b	1.31±0.26b
		24th	1.10±0.05a	1.13±0.12a	1.92±0.15a	1.71±0.15a	1.62±0.20a	1.82±0.15a	1.62±0.09a	1.30±0.07a	1.66±0.01a
		48th	1.21±0.12a	1.21±0.06a	2.52±0.25b	1.82±0.20a	1.63±0.20a	2.21±0.10b	2.55±0.06b	1.78±0.09a	2.03±0.12b
		72nd	1.26±0.15a	1.39±0.15a	2.64±0.20b	2.87±0.20b	1.92±0.25a	2.38±0.30b	6.62±0.45c	2.00±0.06b	2.46±0.15b
19	Kaempferol	0th	1.04±0.11a	1.53±0.10a	2.04±0.15b	4.43±0.45c	1.73±0.15a	5.98±0.25d	2.53±0.04b	1.96±0.04a	1.99±0.02a
		24th	1.27±0.08a	1.71±0.15a	9.05±0.40f	4.92±0.15c	2.80±0.10b	7.42±0.30e	2.57±0.05b	2.12±0.02b	5.01±0.02d
		48th	1.67±0.02a	3.78±0.75c	9.49±0.25f	10.93±0.15g	3.32±0.08c	7.65±0.25d	8.49±0.15e	2.71±0.02b	3.32±0.02c
		72nd	3.70±0.25a	5.31±0.25b	9.51±0.20e	16.69±0.60g	16.05±0.07g	11.07±0.45f	9.04±0.20d	5.68±0.08b	8.19±0.06c
20	Rutin	0th	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
		24th	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
		48th	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
		72nd	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
21	Daidzein	0th	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
		24th	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
		48th	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
		72nd	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
22	Hesperitin	0th	0.88±0.15a	0.88±0.06a	0.83± 0.10a	0.86±0.07a	0.81±0.03a	0.83±0.02a	0.83±0.01a	0.83±0.02a	0.83±0.01a
		24th	0.88±0.08a	0.92±0.05a	0.86±0.05a	0.86±0.08a	0.83±0.02a	0.85±0.06a	0.83±0.10a	0.83±0.01a	0.83±0.09a
		48th	0.92±0.08a	0.95±0.10a	0.86±0.20a	0.92±0.08a	0.86±0.02a	0.86±0.02a	0.86±0.07a	0.86±0.08a	0.86±0.06a
		72nd	0.92±0.10a	0.95±0.10a	1.38±0.02a	0.99±0.10a	1.19±0.01a	0.92±0.05a	0.99±0.20a	0.88±0.10a	0.86±0.07a
23	Shikkimic acid	0th	1074.14±1.20i	884.20±1.15g	710.20±0.65e	491.87±0.55a	771.29±0.85f	553.15±1.15d	493.82±0.90b	506.95±1.10c	971.57±0.70h
		24th	6668.92±1.50h	4257.54±2.15g	742.5±0.85c	612.17±1.20a	957.58±0.95d	643.68±1.20b	1105.09±1.10e	1101.89±2.25e	1128.31±1.12f
		48th	8834.53±1.85f	24732.62±1.20h	1064.50±2.40b	701.40±1.25b	13508.49±2.50g	653.88±1.30a	2608.44±1.25d	1120.41±2.50c	4912.48±1.25e
		72nd	10166.13±1.90f	25098.28±2.25h	32125.33±2.15i	2543.49±2.20a	22111.01±2.80g	2840.38±2.20c	2642.98±1.08b	3726.52±1.20d	5249.48±1.50e

24	Elagic acid	0th	3.60±0.09e	5.43±0.05c	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	27.66±0.25g
		24th	24.82±0.50d	11.99±0.54c	<0.001	11.17±0.17c	<0.001	2.43±0.50a	4.31±0.06b	<0.001	43.64±0.50e
		48th	29.62±0.85d	58.37±0.86f	26.41±0.10c	17.68±0.09b	26.41±0.35c	18.96±0.90b	69.73±0.25g	2.56±0.09a	50.48±0.25e
		72nd	141.89±0.99g	181.97±1.16h	27.57±0.15c	27.45±0.05c	42.21±0.55d	24.63±0.25b	136.54±0.98f	13.03±0.75a	96.54±0.50e
25	Morin	0th	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
		24th	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
		48th	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
		72nd	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
26	Genistein	0th	0.65±0.25a	0.66±0.15a	0.62±0.30a	0.63±0.20a	0.64±0.10a	0.67±0.05a	0.67±0.05a	0.62±0.06a	0.64±0.04a
		24th	0.64±0.09a	0.73±0.04a	0.66±0.07a	0.64±0.50a	0.67±0.10a	0.68±0.10a	0.67±0.04a	0.64±0.10a	0.66±0.02a
		48th	0.70±0.06a	0.77±0.06a	0.67±0.03a	0.67±0.02a	0.73±0.02a	0.70±0.10a	0.73±0.09a	0.66±0.05a	0.68±0.04a
		72nd	0.80±0.08a	0.78±0.10a	0.68±0.05a	0.74±0.03a	1.20±0.02b	0.72±0.05a	0.77±0.02a	0.68±0.09a	0.69±0.01a
27	Cinnamic acid	0th	236.94± 1.20i	207.45±1.11h	26.13±0.25a	31.97±0.15b	47.82±0.60c	93.06±0.45g	73.43±0.45e	75.40±0.50f	51.34±0.25d
		24th	249.39±0.07h	227.33±0.71g	85.37±0.50b	58.14±0.50a	59.86±0.40a	122.44±0.95d	155.85±1.10f	121.87±0.95c	141.44±0.90e
		48th	309.45±1.20i	267.25±0.24h	118.89±0.90c	41.89±0.25a	61.26±1.20b	148.65±1.05e	230.09±0.95g	128.37±0.85d	177.84±0.75f
		72nd	482.30±1.35i	285.61±0.25g	188.34±0.95d	370.68±1.20h	106.43±1.25a	152.51±0.95b	267.92±0.90f	163.76±0.45c	241.90±0.90e
28	Chrysin	0th	0.95±0.10a	1.00±0.07a	0.91±0.10a	0.89±0.05a	0.91±0.04a	0.91±0.10a	0.94±0.02a	0.92±0.07a	0.93±0.01a
		24th	0.95±0.08a	1.04±0.06b	0.94±0.05a	0.91±0.15a	0.90±0.05a	0.94±0.04a	0.97±0.01a	0.93±0.05a	0.91±0.09a
		48th	0.99±0.07a	1.93±0.20b	0.98±0.20a	0.93±0.20a	0.94±0.03a	0.95±0.10a	0.95±0.07a	0.96±0.02a	0.93±0.06a
		72nd	1.02±0.10a	1.92±0.15a	0.99±0.01a	0.95±0.10a	1.11±0.02a	0.96±0.10a	0.99±0.02a	1.00±0.02a	0.97±0.07a

*Values are means ± SD (n = 3), followed by different superscript letter are significantly different ($p \leq 0.05$). The letter "a" represents the least value.

†Abbrevations : GT-Germination time

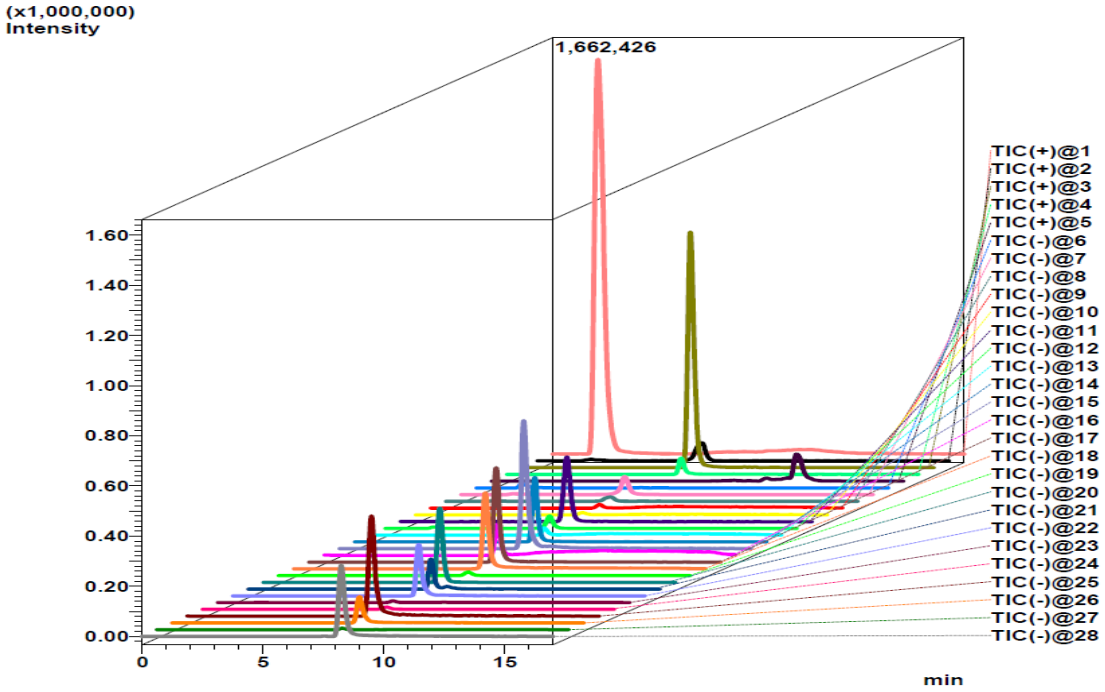
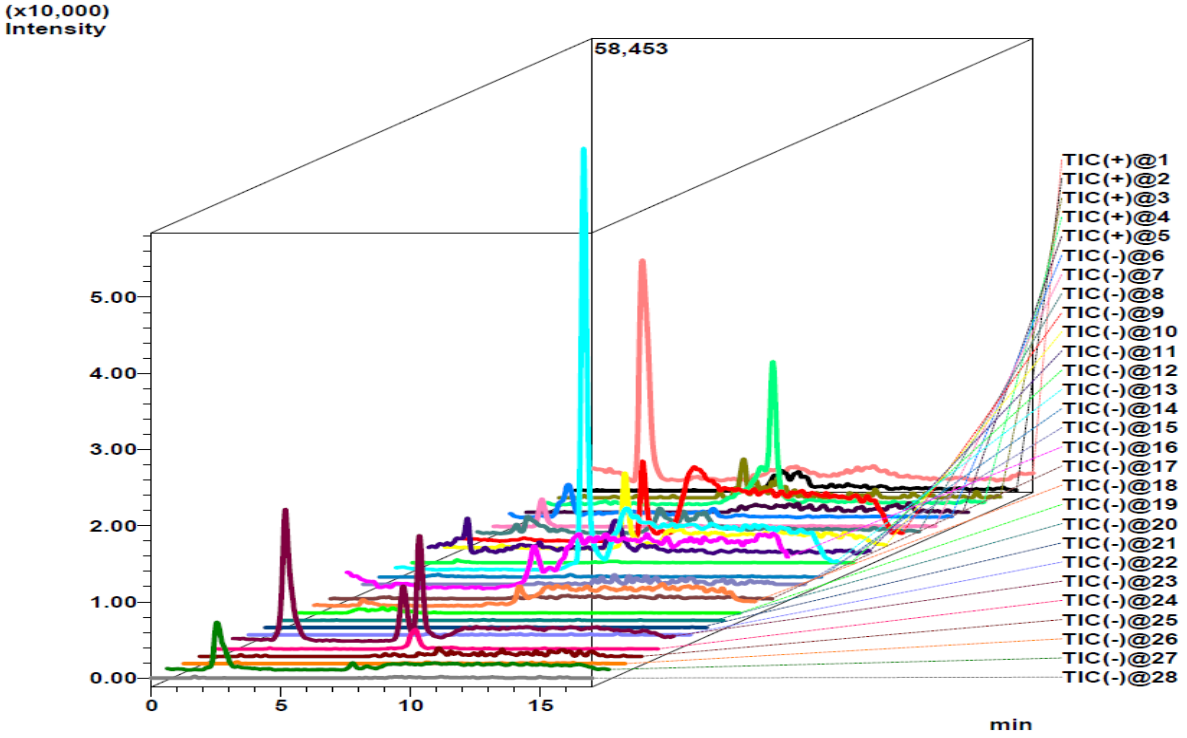


Fig 2.11 Representative LC-MS/MS chromatogram showing standard polyphenols (150 ppb mix)

*****1 – Catechol, 2 – catechin, 3 – quinine, 4 – naringenin, 5 – tocopherol, 6 – gallic acid, 7 – chlorogenic acid, 8 – catechin, 9 – syringic acid, 10 – vanillic acid, 11 – caffeic acid, 12 – epigallocatechin, 13 – ferulic acid, 14 – myricetin, 15 – quercetin, 16 – p-coumaric acid, luteolin, 17 – luteolin, 18 – apigenin, 19 – kaempferol, 20 – rutin, 21 – daidzein, 22 – hesperetin, 23 – shikimic acid, 24 – ellagic acid, 25 – morin, 26 – genistein, 27 – cinnamic acid, 28 - chrysin

a


(x10,000)
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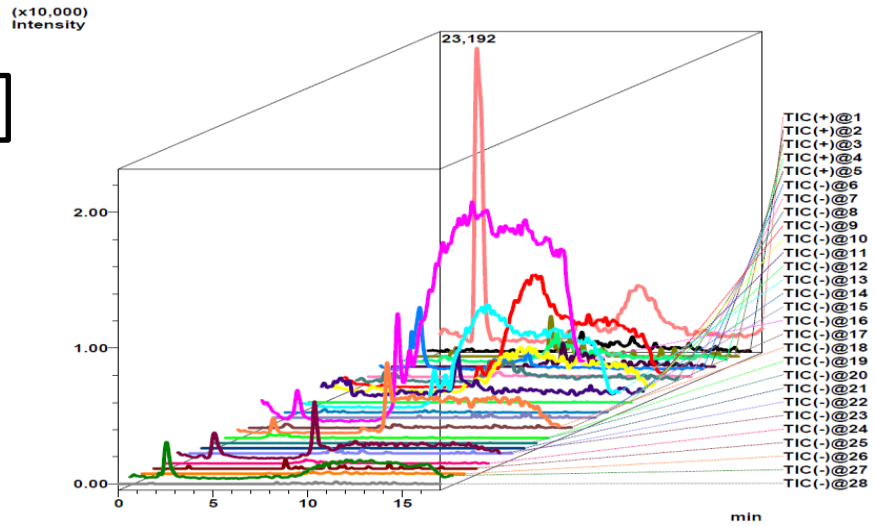
3.00
2.00
1.00
0.00
0
5
10
15
min
TIC(+)@1
TIC(+)@2
TIC(+)@3
TIC(+)@4
TIC(+)@5
TIC(-)@6
TIC(-)@7
TIC(-)@8
TIC(-)@9
TIC(-)@10
TIC(-)@11
TIC(-)@12
TIC(-)@13
TIC(-)@14
TIC(-)@15
TIC(-)@16
TIC(-)@17
TIC(-)@18
TIC(-)@19
TIC(-)@20
TIC(-)@21
TIC(-)@22
TIC(-)@23
TIC(-)@24
TIC(-)@25
TIC(-)@26
TIC(-)@27
TIC(-)@28

b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10
15
min
TIC(+)@1
TIC(+)@2
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TIC(+)@5
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TIC(-)@7
TIC(-)@8
TIC(-)@9
TIC(-)@10
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TIC(-)@12
TIC(-)@13
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TIC(-)@20
TIC(-)@21
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TIC(-)@23
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TIC(-)@25
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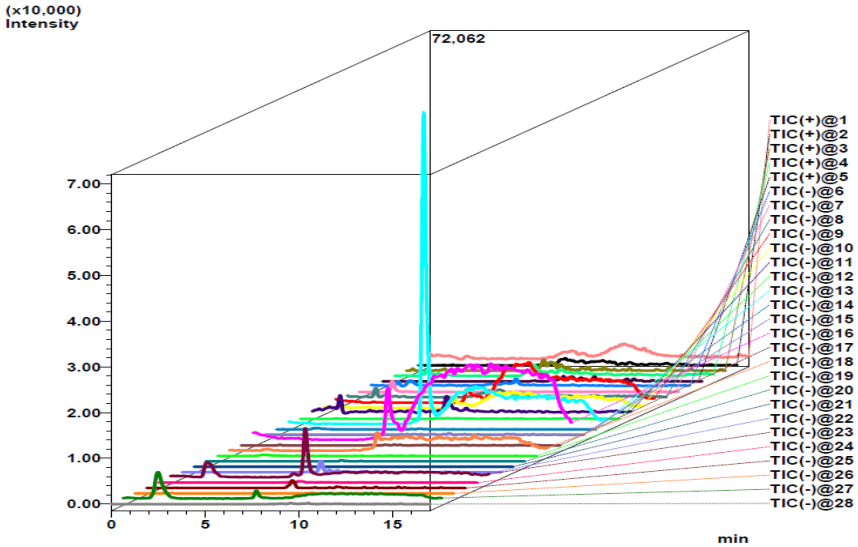
TIC(-)@27
TIC(-)@28

c


(x10,000)
Intensity
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6.00
5.00
4.00
3.00
2.00
1.00
0.00
0
5
10
15
min
TIC(+)@1
TIC(+)@2
TIC(+)@3
TIC(+)@4
TIC(+)@5
TIC(-)@6
TIC(-)@7
TIC(-)@8
TIC(-)@9
TIC(-)@10
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d

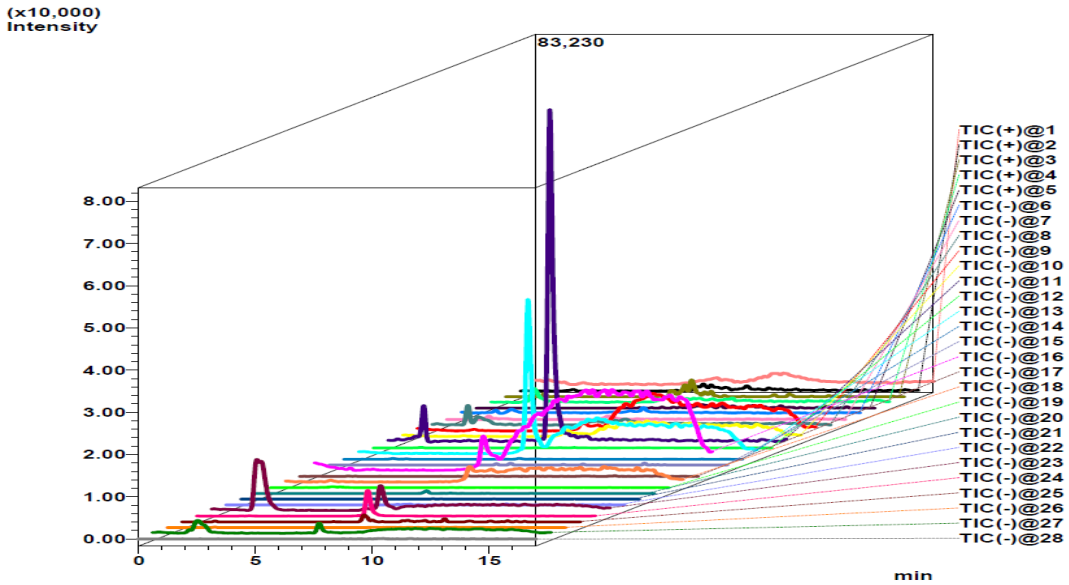

Fig 2.12 Representative LC-MS/MS chromatogram showing polyphenol concentration in sample (little millet): **a** – raw sample, **b** – 24 h germinated, **c** – 48 h germinated, **d** – 72 h germinated.

***1** – Catechol, 2 – catechin, 3 – quinine, 4 – naringenin, 5 – tocopherol, 6 – gallic acid, 7 – chlorogenic acid, 8 – catechin, 9 – syringic acid, 10 – vanillic acid, 11 – caffeic acid, 12 – epigallocatechin, 13 – ferulic acid, 14 – myricetin, 15 – quercetin, 16 – p-coumaric acid, luteolin, 17 – luteolin, 18 – apigenin, 19 – kaempferol, 20 – rutin, 21 – daidzein, 22 – hesperetin, 23 – shikimic acid, 24 – ellagic acid, 25 – morin, 26 – genistein, 27 – cinnamic acid, 28 - chrysin

2.3.8 Antioxidant analysis by 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay

The extracts from the raw and germinated millet samples were further investigated for its antioxidant potential in terms of DPPH radical scavenging activity and ABTS assay. The measurement of antioxidant activity using the DPPH radical scavenging method is a better *in vitro* model to quickly assess the effectiveness of the millet samples within a short time period which is based on electron transfer (Sharma et al., 2016). A stronger radical quenching agent yield lower IC_{50} value (Ibidapo et al., 2019). In the DPPH scavenging assay, the yellow color of DPPH radical is brought down in the presence of an antioxidant which donates hydrogen to non-radical DPPH–H (Ragaee, 2006). Figure 2.13 represents the IC_{50} values of raw and germinated millet grains. IC_{50} value shows the concentration of the extract required for scavenging 50% of radicals present in the test medium and hence a lower value represents a higher activity. When germinated, the millet extracts showed better radical scavenging activity which is in accordance with the phytochemical analysis. Following barnyard millet, the IC_{50} values of raw millets followed the order of proso millet > sorghum > foxtail millet > amaranth > little millet> kodo millet > finger millet> pearl millet. Barnyard millet germinated for 72 h had the highest radical scavenging activity (IC_{50} - 2.95±0.85 µg/mL GAE). Pearl millet in its raw form had the least IC_{50} values (21.65±1.50 µg/mL GAE) which improved to 8.44±0.25 µg/mL GAE (24h), 4.46±0.20 µg/mL GAE (48 h) and 3.64±0.58 µg/mL GAE (72 h) upon germination. Similar observations were made by Pradeep and Sreerama, (2015), who observed that the radical scavenging activity of millet grains improved upon germination.

Ofosu et al., 2020 also found out that the radical scavenging activity of millets improved upon germination by DPPH and ABTS assays. The results obtained indicated that the millet extracts are capable of scavenging the free radicals and prevent the initiation of free radicals by stabilizing them to participate in any deleterious reactions. Anastasia et al., (2012) reported that the total polyphenolic content of whole grain barley was significantly correlated (r^2 = 0.99) to their total antioxidant capacity. Therefore, antioxidant activity enhances due to the increase in the total polyphenolic content in seed during germination (Sharma et al., 2017). In the germinated brown rice, the bound extract had significant antioxidant capacity, which was higher than the raw seeds (Ti et al., 2014).

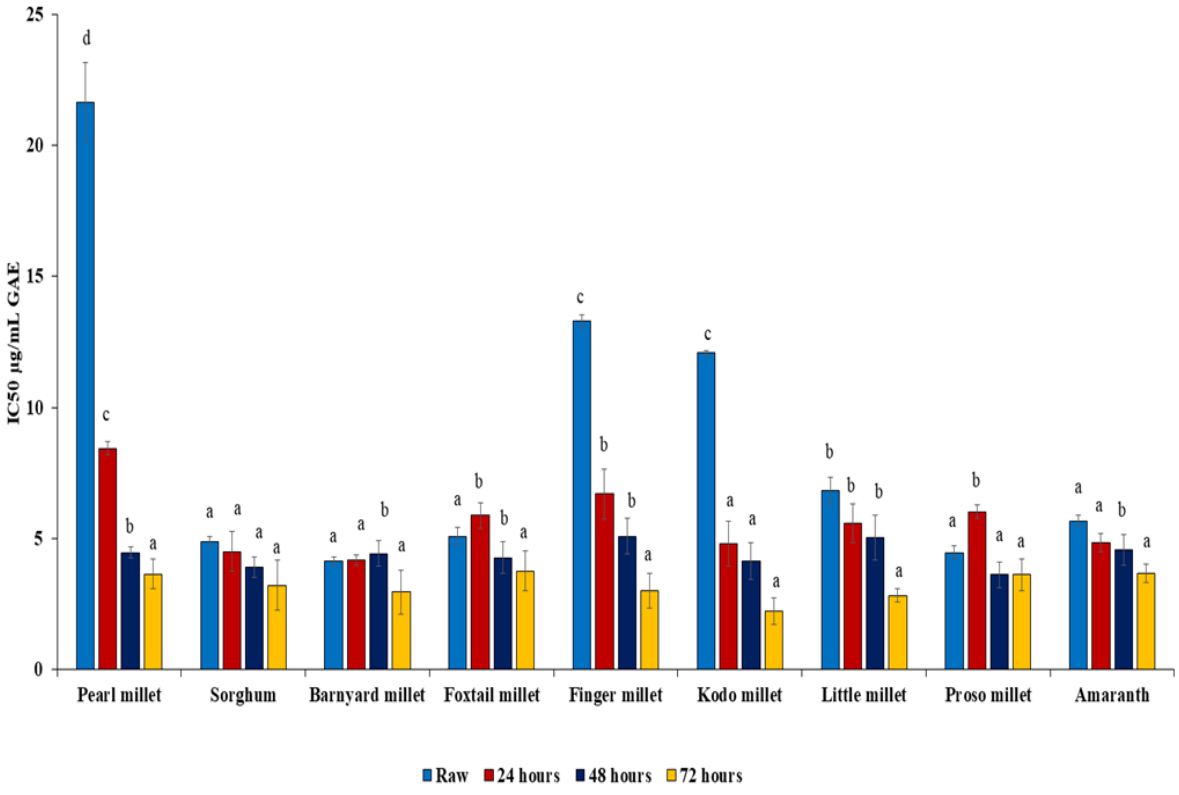

Fig 2.13 IC_{50} values of native and germinated grains using DPPH radical scavenging activity (µg/mL GAE). (*Values are means ± SD (n = 3), followed by different superscript letter are significantly different ($p \leq 0.05$). The letter "a" represents the least value).

ABTS radical scavenging activity has been widely applied to evaluate the antioxidant capacity of food extracts, and it is based on hydrogen-donating antioxidants against nitrogen radicals (Huang et al., 2005). The % inhibition of ABTS assay is represented in Figure 2.14.

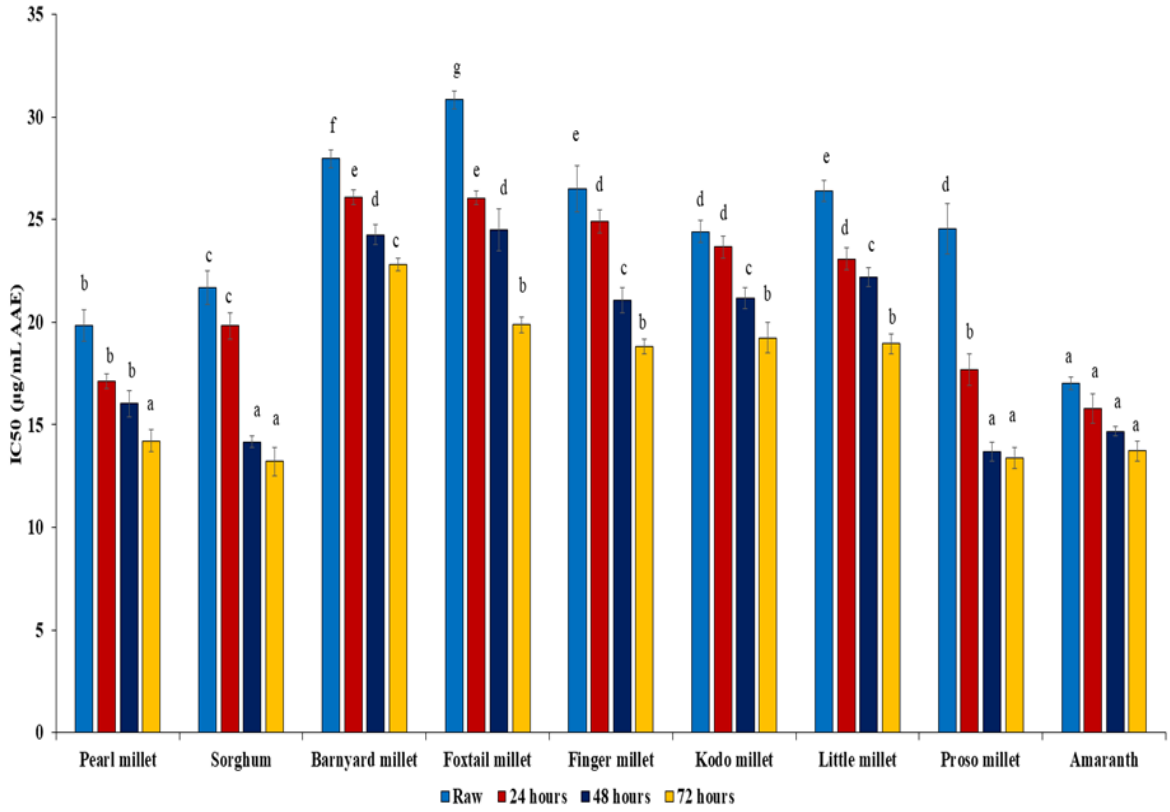

Fig 2.14 IC_{50} values of native and germinated grains using ABTS assay (µg/mL AAE). (*Values are means ± SD (n = 3), followed by different superscript letter are significantly different ($p \leq 0.05$). The letter "a" represents the least value).

Similar to IC_{50} values of DPPH assay, in ABTS assay, the samples germinated for 72 h showed good scavenging properties. Amaranth showed the best scavenging property with an IC_{50} value of 17.03±0.32 μg/mL AAE in its raw form to 13.72±0.47 μg/mL AAE after 72 h of germination. The decrease in IC_{50} is independent of the type of grain (Niroula et al., 2019). The DPPH and ABTS activity of methanolic extracts of brown proso millets were found to improve after germination (Azeezz et al., 2022). Phenolic antioxidant activity depends on phenolic ring position, and the degree of hydroxylation. Various other structural features further influence the antioxidant activity in millet grains (Liang and Liang, 2019). Duenas et al., (2009) reported that germination is attributed to the biochemical metabolism of seeds during germination resulting in increased scavenging activity. Hydrolytic enzymes change the endosperm during germination and may free some of the bound components that have a role in antioxidant activity (Singh et al., 2019; Tarasevičienė et al. 2019). The phytochemical analysis noted in the study also revealed an increase in phytochemicals after germination. All the millet extracts possessed potent and distinct DPPH and ABTS radical quenching activities with wide variations in the IC_{50} values between the processed samples. Therefore, the higher radical scavenging activities of germinated millet samples could be attributed to the higher phenolic indices like TPC and TFC of the samples (Ferreira et al., 2019). The increase in antioxidant activity with the germination bioprocess is one of the many metabolic changes that take place upon germination of seeds, mainly due to an increase in the content of phenolic compounds by the action of the endogenous hydrolytic enzymes (Alveraz-Jubete et al., 2010). Extraction solvents and extraction methods are reported to affect the final concentration of phenolics and antioxidant capacities of extracts (Meenu et al., 2016).

2.4 Conclusion

From the study, it can thus be concluded that ancient grains are highly nutritious grains with a good amount of protein, essential amino acids and vitamins. They are nutritionally superior in terms of almost all the macronutrients present in them. These traditional grains contain a balanced amino acid and are also rich in essential amino acids like methionine, valine and tryptophan. They also contain good amounts of ascorbic acid and B vitamins. The study reports a comprehensive evaluation of phenolic compounds in nine millet varieties comprising of pearl millet, sorghum, proso millet, finger millet, little millet, kodo millet, barnyard millet, foxtail millet and amaranth during germination using LS-MS/MS analysis. The phenolic, flavonoid and antioxidant activity of millet grains were affected by germination time. Polyphenols like shikimic acid, cinnamic acid, ferulic acid etc. were found in good amounts in all the samples and the presence of a total of 23 polyphenols were identified in the grains, for the first time. There was several fold increase in many of these phytochemicals on germination which was also reflected in the antioxidant activity. The identified phytochemicals are reported to have potential bioactivity in prevention and management of many chronic health conditions. Thus, the findings of this study indicate that incorporating germinated millets into convenience food products can improve their bio-functionality, making them suitable for use in the development of functional foods. The conclusions drawn from this study hold significance since the cereal-based industries are searching for such substitutes to replace conventional wheat-based products in various food preparations such as bakery, extrusion, and indigenous preparations. It is therefore evident that the use of germinated millets can provide the natural health advantages of phytochemicals to consumers. These

findings can also be used to investigate the potential use of germinated millet grains in food formulations and product development, specifically in the production of functional/health foods and gluten-free products.

The key findings of this chapter are summarized below:

- Millet grains are good sources of micro as well as macro nutrients and their amount improve with germination up to 72 h.
- They are also found to be fair sources of essential amino acids and water-soluble vitamins.
- The phenolic, flavonoid and antioxidant activities of millet grains vary with the variety and its contents increase with respect to germination time.
- The individual polyphenols, as well as the cumulative phenolics and flavonoids, synergistically contribute to the overall increase in antioxidant activity observed in germinated millet grains.
- They also contain good amount of minerals when compared to the other staple cereals consumed.

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Chapter 3

Isolation of starch from millet grains, size reduction and its characterisation based on structural, functional, thermal and rheological properties

3.1 Introduction

Emulsions are fabricated through the dispersion of one immiscible liquid phase into a continuous phase consisting of a second fluid, manifesting as droplets. These systems exhibit an inherent lack of thermodynamic stability, rendering them prone to rapid phase separation unless augmented with interfacial active constituents, including small molecular surfactants, amphiphilic polymers, or solid particles. These additional components form interfacial layers, affording the emulsions kinetic stability. A notable proportion of processed food and beverage products are characterized by oil-in-water (o/w) emulsions, wherein the preservation of their shelf life is frequently contingent upon the maintenance of their kinetic stability (Berton-Carabin, C., & Schroën, 2019). Across the world, the food colloids research groups are looking for alternative emulsifiers to address the limitations of conventional emulsifiers.

In the above context, use of solid colloidal particles to stabilize emulsions is gaining momentum in the recent times. These interface-stabilizing solid particles are known as the '*Pickering particles'* and the emulsions stabilized using them are called *'Pickering emulsions'*. Pickering particles offer manifold advantages over conventional emulsifiers. With partial dual wettability, the solid particles can spontaneously and irreversibly adsorb onto the oil-water interface and then around the droplets to form stable Pickering emulsions (Xiao et al., 2016). The colloidal particles form a dense adsorption layer/film around the droplets to provide them with a physical barrier in space and prevent interactions between the adjacent droplets that eventually lead to emulsion instability (Chen et al., 2020). They are more stable against coalescence and Ostwald ripening than emulsions stabilized by conventional molecular surfactants (Aveyard et al., 2003).

Besides the above merits, Pickering particles can stabilize emulsions at a much lower concentration compared to the conventional emulsifiers. Also, most of the Pickering particles are derived from natural sources. The resultant biocompatibility facilitates their applications in the development of Pickering emulsions that can be used as carriers for the delivery of bioactive substances (Yang et al., 2017).

Starch, especially modified starches are established Pickering particles with potential food applications. Starch is GRAS (Generally Recognized As Safe), non-allergenic, abundant, and cost-effective food ingredient. The structure and properties of native starches from different plant sources vary. The well-known sources of plant starches are potatoes, corn, legumes, yams, and sweet potatoes. Millet varieties are recent additions to the above list, wherein starch constitutes approximately 70% of the grain weight. Millet is a generic term describing a range of small-seeded grains in two tribes *Paniceae* and *Chlorideae* of the family Poaceae (true grass) which became the staple food for humans 10000 years ago even before the rise of wheat and rice (Lu et al., 2009).

Owing to their size and composition, native starch granules from any plant source have the potential to function as food-grade Pickering particles, and millet starch is no exception to this. However, native starch suffers from the limitations of poor stability, insufficient hydrophobicity, and large particles, which prevent their adsorption at the oil-water interface. This hinders their emulsifying ability to stabilize Pickering emulsions (Chen et al., 2020; Tavernier et al., 2016), thus necessitating their modification by physical or chemical means. Physical modification methods are more viable than chemical treatments as they are economical, non-toxic, and natural (Chen et al., 2015). Moreover, their chemical-free process permits the physically-modified starch to be used

a clean-label ingredient in food products. Thus, physical modified starch have an edge in terms of food safety and consumer preference.

Physical modification of starch alters its morphology (size and shape) and three-dimensional structure, governed by physical stimuli such as milling, moisture, temperature, pressure, pH, radiation, pulse-electric field, and ultrasonic waves. The size of Pickering particles is the key to their emulsion stabilizing function. Recently, the trend in food research and development is inclined towards the *'micronization'* or particle size reduction of food ingredients. This is because of the proven research findings that the reduction of particle size of food materials not only alters their structure and surface area but also exhibits a positive effect on their functional properties (Raghavendra et al., 2004; Sangnark & Noomhorm, 2003). *Micronization* can be defined as the particle size reduction to less than ten microns which can be attained through various techniques (Joshi, 2011). As a result of micronization, the internal links of the material are broken down by hydrodynamic and mechanical methods. The preparation of micro-particles can be done either through the "top-down" (method of reducing the size of larger particles) or the "bottom-up" (method of modifying molecules in a solution to create micro-particles) approaches (Rodríguez-Meizoso & Plaza, 2015). In the food industry, size reduction is commonly done by grinding. However, using micronization as an approach to design Pickering starch particles is an uncharted concept.

In the above background, the present work focusses on the physical modification of native millet starch granules by micronization and validates its ability to function as a stable colloidal particle to stabilize Pickering emulsions. This research work is built on the hypothesis that micronized millet starch particles may have superior Pickering activity

than the native starch. The objective of this work is to validate and establish the proof-of-concept on the Pickering particle function of micronized millet starch from different millet varieties. This is accomplished by assessing the various characteristics that ascertain the Pickering ability of solid particles, i.e., particle size, surface charge, contact angle, surface morphology, degree of crystallinity, and surface activity of micronized millet starch vis-à-vis their native counterparts. Finally, the proof-of-concept is validated by applying the micronized millet starch particles as Pickering particles in an appropriate emulsion system.

3.2 Objective

To validate and establish the proof-of-concept on the Pickering particle function of micronized millet starch from different millet varieties by size reduction and its characterization based on physical, structural, thermal, and rheological properties.

3.3 Materials and Methods

3.3.1 Materials

Grain samples (pearl millet, sorghum, kodo millet, finger millet, little millet, barnyard millet, and foxtail millet) used in the study for starch isolation were received as gift samples from Indian Council of Agriculture Research – Indian Institute of Millet Research, Hyderabad, India. Proso millet and amaranth were purchased from authenticated dealers in Trivandrum, Kerala, India. The grains were ground to a powder form using a mixer grinder (Prestige Dry masala grinder PDMG 02; India) and stored in airtight containers in room temperature (30±5°C) prior to their use in the actual experiments. Sodium hydroxide (NaOH) pellets and glycerol were procured from Sisco

Research Laboratories Pvt. Ltd. (SRL), Mumbai, India. Analytical grade hydrochloric acid (HCl) was purchased from Rankem. The water used in all procedures was produced with a Milli-Q water purification system (Millipore, Billerica, MA). Roasted coffee oil (Arabica) was purchased from Proderna Biotech Pvt Ltd., Zamrudpur, New Delhi, India.

3.3.2 Experimental design

The outline of the study is presented in the figure below:

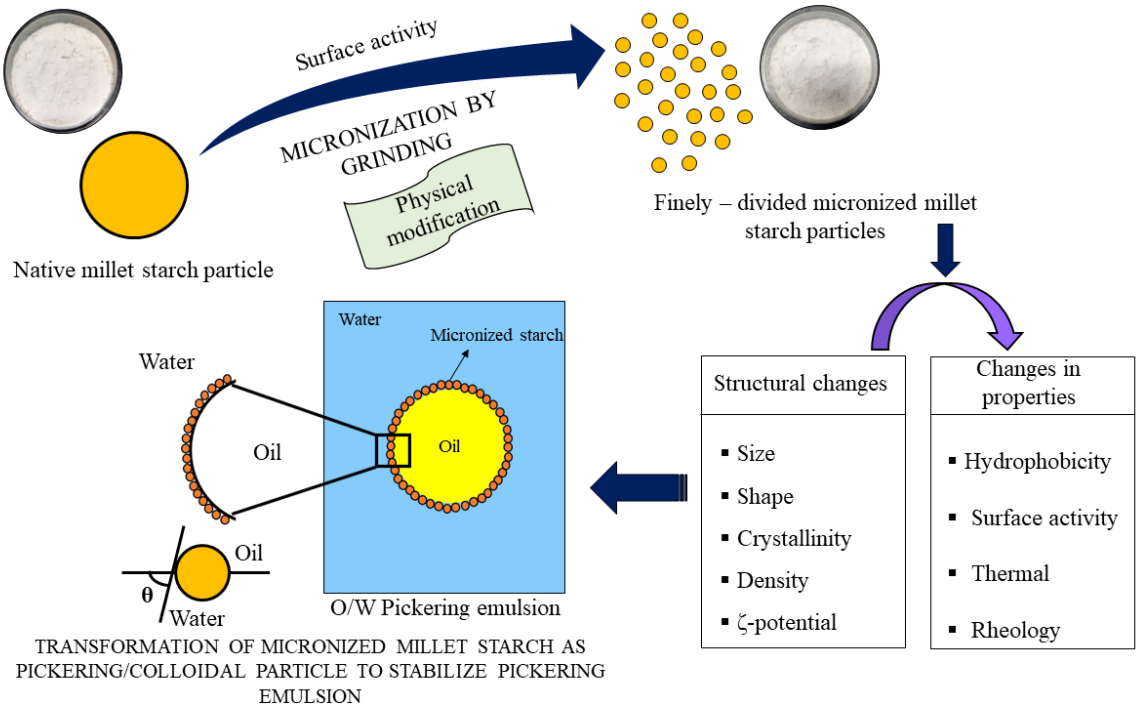


Fig 3.1 Outline of the chapter

3.3.3 Isolation of starch

Starch isolation from millets was carried out using the alkaline steeping method described by Fan et al., (2019) with some modifications. The grain flour was added to 0.5 M NaOH solution and stirred for 4 h. The slurry was then centrifuged at 3000 rpm for 15 min. The

supernatant was discarded, and the top yellowish protein layer was scrapped off. The residue was again extracted with 0.5 M NaOH for 2 h. Distilled water was added to sediment the residue and it was kept overnight at 4°C. The slurry was centrifuged again till the yellowish layer was completely removed. The residue was filtered using a muslin cloth and the filtrate was neutralized with 1M HCl to pH 7. The resultant slurry was again centrifuged at 3000 rpm for 15 min. The supernatant was discarded, and the remaining residue was washed for three to four times using double distilled water and centrifuged. The resultant starch obtained was dried at 40 °C in an oven for 48 h. The dried starch cake was ground to powder using a blender, passed through a sieve (30 mm), and stored at 4° C until further use. The starches were named as follows: Pearl millet starch (S1), proso millet starch (S2), little millet starch (S3), finger millet starch (S4), kodo millet starch (S5), amaranth starch (S6), barnyard starch (S7), foxtail millet starch (S8) and sorghum starch (S9).

3.3.4 Micronization of starch

Starch samples were ground in a grinder (Prestige Dry masala grinder PDMG 02; India) for 25 s, 1 min and 2 min. The particle size of the samples was then analyzed. They were passed through a 50 mm mesh sieve and stored at 4° C in airtight containers until further analysis.

3.3.5 Characterization of native and micronized starch

3.3.5.1 Physical characterization

3.3.5.1.1 Particle size and zeta potential

The particle size and zeta potential (ζ-potential) of the native and micronized starch suspensions (refractive index: 1.50) were analyzed using the Malvern Zetasizer (Zeta Nano-ZS; Malvern Instruments, UK) by using water (refractive index: 1.33) as the dispersant and employing the dynamic light scattering (DLS) technique. Determination of volume distributions in DLS-based particle size analysis depends on the assumption that all the molecules are homogenous and spherical in shape (Stetefeld et al., 2016). The Z-average of the samples were measured which is the intensity weighted mean hydrodynamic size of the ensemble collection of particles measured by dynamic light scattering. The Z average is derived from a cumulants analysis of the measured correlation curve, wherein a single particle size is assumed, and a single exponential fit is applied to the autocorrelation function. The analysis was carried out in triplicates and the mean diameter and zeta potential values were calculated.

3.3.5.1.2 Morphology

Native and micronized starch particles (10% w/v) were added to 10% glycerol and then observed under microscope (Olympus IX 83, Tokyo, Japan) in brightfield and polarized light for their morphology analysis in 40X magnification. Further morphological analysis of starch particles was carried out using SEM analysis (EVO 18; Zeiss, Oberkochen, Germany). The starch powders were spread on the metallic plate and coated with a thin layer of gold for an hour and micrographs obtained.

3.3.5.1.3 Contact angle measurement

Starch suspensions (25%) were prepared and heated at 60 °C for 3 min. The resultant solution was poured on cover slips to make starch films. Contact angle was determined

by direct observation of a liquid drop of water on smooth, horizontal, solid surfaces mounted on the cover slips. Contact angles were measured at room temperature (30±5 °C) using a Drop Shape Analysis System (Kruss Drop Shape Analyzer; Model: DSA30E, Germany) by the sessile drop method. Water droplets were dropped carefully onto the films and measurements were taken. The contact angle was calculated by the computer software (DSA3 software) from the droplet images. Readings were taken on both the left and right sides of the droplet profile.

3.3.5.2 Determination of chemical fingerprint by Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra of all the starch samples were carried out using a FT-IR spectrometer (Bruker Alpha-T, Germany) at room temperature (30±5°C) in the absorbance region from 4000 to 400^{cm-1} of 32 scans at 4^{cm-1} resolution.

3.3.5.3 Surface activity

3.3.5.3.1 Determination of Critical Micelle Concentration (CMC) by conductometry

Native and micronized starch suspensions were prepared in various concentrations ranging from 0.01% to 5 % and its conductivity was measured using a conductivity meter (Labtronics, Labman, LT-51, Haryana). The CMC of starch was calculated from the graph plotted using conductivity against surfactant concentration. The conductivity of any solution is directly proportional to the concentration of its ions. The point, where the micelle formation starts, is indicated on the concentration dependence of specific conductivity (k) as a breaking point. The breaking point gives the CMC of the surfactant (Bhattarai et al., 2014).

3.3.6 Determination of the degree of crystallinity by X-Ray Diffraction analysis (XRD)

The XRD measurements of the native and micronized starch granules were conducted using a XEUSS SAXS/WAXS system from Xenocs (Grenoble, France). The X-ray diffraction was recorded in the 2θ range 2 – 50 using Cu Kα radiation at room temperature.

3.3.7 Rheology

The rheological measurements were performed on a controlled stress rheometer (MCR 102 Rheometer, Anton Paar GmbH, Ostfildern-Scharnhausen, Germany) with cone and plate geometry (25-mm diameter; 0.105 mm gap). Dispersions of 25% starch (w/v) were stirred at 300 rpm for 15 min and their flow behavior was determined using flow curve at variable shear rate ranging from 0 to 100^{s-1}. The apparent viscosity (in Pascal second) was determined from the slope of the flow curve. For the determination of dynamic viscoelastic properties, two steps of rheological measurements were performed: (1) amplitude sweep to determine the maximum deformation (% strain) attainable by the sample in the linear visco-elastic range (LVE range) performed at strain of 0.01-2% and fixed frequency of 1 Hz , and (2) frequency sweep at constant deformation within the linear visco-elastic range which were carried out at frequency of 0.1- 100 Hz and constant strain of 0.2% to evaluate the dynamic rheological properties such as G' and G".

3.3.8 Thermal properties

3.3.8.1 Differential Scanning Calorimetry (DSC)

The thermal properties of the native and micronized starch samples were determined using a differential scanning calorimeter (DSC Q2000, TA Instruments, USA). Starch samples (3 mg) were carefully weighed into an aluminium pan and 12 µL of distilled water was added. The pan was then sealed. The thermogram of the samples were recorded and an empty pan was used as reference. The scanning temperature was in the range from 30° to 120 °C at heating rate of 10° C/min.

3.3.8.2 Thermogravimetric analysis (TGA)

Thermal analysis of the samples was carried out in a Perkin Elmer ST6000, TG-DTA Analyzer in the temperature range of 0 to 600 °C with a heating rate of 10 °C/min in nitrogen atmosphere.

3.3.9 Validation of the proof-of-concept: Emulsion preparation and characterization

3.3.9.1 Preparation of emulsion stabilized by micronized millet starch particles

O/W emulsions were prepared using both native and micronized pearl millet and proso millet starches (0.4% w/v). The starch was dispersed in water using a magnetic stirrer at 500 rpm for 30 min. To this hydrated starch roasted coffee bean oil at 3.0% of weight of starch added and homogenized at 20,000 rpm for 5 min (Prasad et al., 2019) using a homogenizer (Omni International Tissue Master 125, United States). After preparation, the emulsions were immediately transferred into graduated test tubes (15 mL) and kept for 24 h at room temperature.

3.3.9.2 Droplet size, zeta potential and turbidity measurements

The droplet size and zeta potential of the emulsions were calculated before and after 24 h using the Malvern Zetasizer (Zeta Nano-ZS; Malvern Instruments, UK). The transmittance of the emulsion was taken at 600 nm on Shimadzu ultraviolet-visible 2600 (UV) spectrophotometer (Kyoto, Japan) for analyzing the turbidity of the emulsions.

3.3.9.3 Morphology observation

Morphology of the prepared Pickering emulsions were observed using fluorescence microscopy (Olympus IX-83, Japan). An aliquot of 100 µL of emulsion was stained with Nile red (to stain oil) and safranine (to stain starch). The dyed emulsions were placed on concave slides and covered with coverslips. The fluorescence image from the samples were then viewed in 10x and 20x magnifications.

3.3.10 Statistical analysis

All experiments were conducted at least in triplicate, with mean values and standard errors determined for these experiments. The means of all the parameters were examined for significance by analysis of variance (ANOVA) at a confidence level of 95%, using the Data Analysis ToolPak Add-in of Microsoft Office Excel, 365 Version 2204, USA.

3.4 Results and discussion

Starch was obtained from nine millet varieties with its extraction yield ranging between 60.58 – 83.31% (Table 3.1) which was in the range reported by Zhu (2014) and Mahajan, et al., 2021. Isolated starches were micronized to tailor their particle size and surface properties such that they position themselves at the oil-water interface and stabilize an emulsion system. The subsequent sections would present a comparative analysis of the properties of micronized millet starch particles vis-à-vis their native counterparts. The

inferences would provide insights into the influence of micronization on the particle properties of starch (mainly, morphology: particle size and shape, surface charge, amphiphilicity) and assess its suitability as Pickering particle.

Table 3.1 Extraction yield (%) of starch samples from millet grains

Starch sample	Extraction yield (%)
Pearl millet (S1)	78.85 ± 0.02^{d}
Proso millet (S2)	77.52 ± 0.05^{d}
Little millet (S3)	83.31 ± 0.04^{g}
Finger millet (S4)	64.55 ± 0.04^{b}
Kodo millet (S5)	60.58 ± 0.02^{a}
Amaranth (S6)	68.23 ± 0.02^{c}
Barnyard millet (S7)	80.25 ± 0.05^{e}
Foxtail millet (S8)	81.44 ± 0.02^{f}
Sorghum (S9)	60.95 ± 0.07^{a}

*Values are means ± SD (n = 3), followed by different superscript letter are significantly different ($p \leq 0.05$). The letter "a" represents the least value.

3.4.1 Characterization of native and micronized starches

3.4.1.1 Physical characterization

3.4.1.1.1 Particle size and zeta potential (ζ) properties of millet starch

Particle size is a vital parameter with respect to Pickering functionality, as it governs the timescales during which particle adsorption occurs at the oil–water interface during emulsification (Duffus et al., 2016). The research investigation involved subjecting all starch samples to particle size reduction through grinding, wherein the grinding time was methodically varied. Following this, a meticulous optimization procedure was undertaken to ascertain the most favorable final grinding time. Notably, the starch samples ground

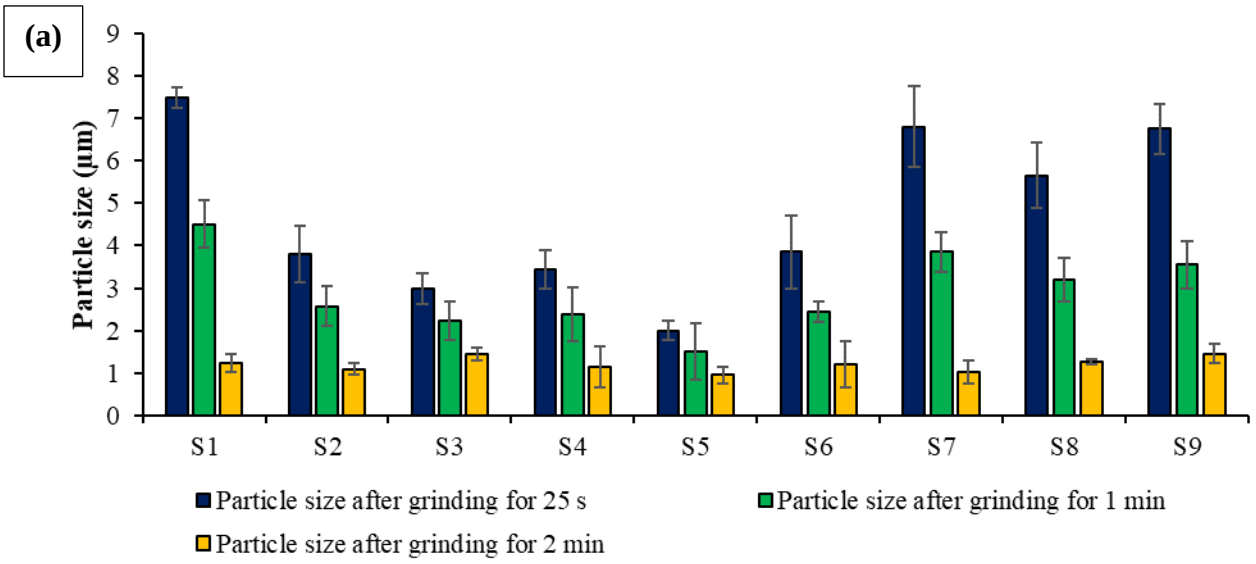
for 2 min produced the smallest particles (1.46±0.23 - 0.95±0.21 µm) (Fig. 3.2a) and hence considered for further analysis. The native starches and their micronized counterparts were analyzed based on the principle of dynamic light scattering to understand the extent of size reduction. The average particle size of native starch ranged between 2.43 ± 0.45 µm (kodo millet) and 8.30 ± 0.33 µm (pearl millet). The variation in particle size of starch between different millet varieties may be attributed to their chemical composition, for instance, the morphological difference of starches (Jhan et al., 2020). The higher the particle size of native starch granules, greater will be the resistance to mechanical force due to intra-molecular and intermolecular hydrogen bonding forces (Bhat et al., 2016). There was a significant reduction in the particle size of all the starch granules upon micronization (Fig. 3.2b). The micronized starch grains had a particle size in the range of 0.95±0.21 - 1.46±0.23 µm, irrespective of its origin. The highest level of size reduction was observed in pearl millet (84.93%) and barnyard millet (86.67%), and the lowest was seen in kodo millet (60%). The lowest percentage reduction in the particle size of kodo millet could be related to the difference in branching pattern of amylopectin within the starch (Ahmad et al., 2020). From our current study, it is evident that the particle size of micronized starch particles obtained are comparable to that reported in other studies related to food-grade Pickering particles (Costa et al., 2018; Qian et al., 2020; Hu et al., 2020; Noor et al., 2022, Zhai et al., 2019).

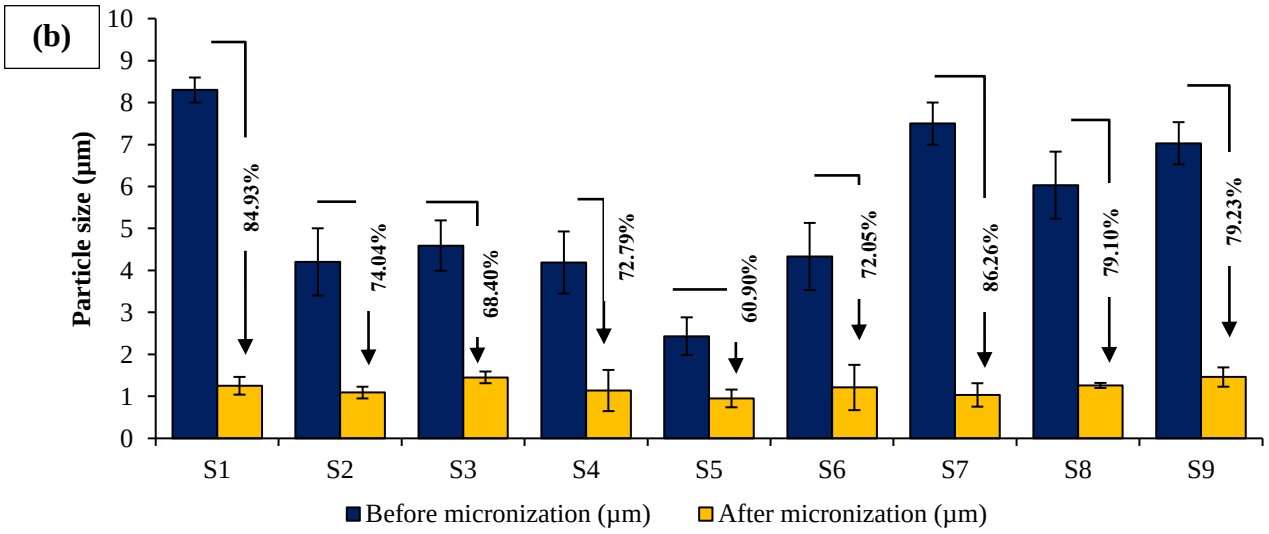
The lower particle size of micronized millet starch particles is relevant from the perspective of its application as Pickering particle. The higher stability of Pickering emulsions relative to conventional emulsions is attributed to the size of their stabilizing Pickering particles (> 10 nm), which provides a steric hindrance/barrier against close

contacts of emulsion droplets and prevent coalescence (Dickinson, 2010). Small particles tend to form denser layers at the interface, thus effectively improving the interface performance. Also, they favor the formation of stable interfacial layers, since they exhibit faster adsorption kinetics and higher efficiency in reducing interfacial tension (Li et al., 2013; Pei et al., 2017). Moreover, smaller Pickering particles are preferred over the larger ones that require more time to adsorb at the oil-water interface, ultimately producing emulsions with large droplets. This higher stability is due to the large particle sizes.

On the other hand, zeta potential (ζ) is a parameter that changes with the surface charge of a particle, though it is not directly related to the surface charge density (Weiner et al., 1993). Surface charge is the distribution of ions on the particle surface that affects the interfacial region of the surrounding medium. There was significant change ($p \leq 0.05$) in the zeta potential of all the starch granules upon micronization (Fig. 3.2c). The most evident change was seen in pearl millet starch whose zeta potential value changed from -2.92 ± 0.08 to -25.74 ± 0.05 mV. The enhanced net negative charge on all the samples could be due to the naturally occurring phosphate esters in amylopectin molecules (Blennow et al., 2001). The increased volume of fine particles modifies the surface composition and increases the specific charge of particles (Hemery et al., 2009). The high zeta potential increases the electrostatic repulsions between the particles, which results in the decrease of Van der Waals forces of attraction. (Schäfer et al., 2010). Generally, colloidal systems with zeta potential values of less than −30 mV and more than +30 mV are known to have adequate repulsive force to attain better physical stability (Joseph & Singhvi, 2019). Specifically, studies related to emulsion stabilization infer that higher

values of ζ-potential cause electrostatic repulsion between droplets and enhance the emulsion stability (Wang et al., 2022; Griffin et al., 2020; Huang et al., 2020).





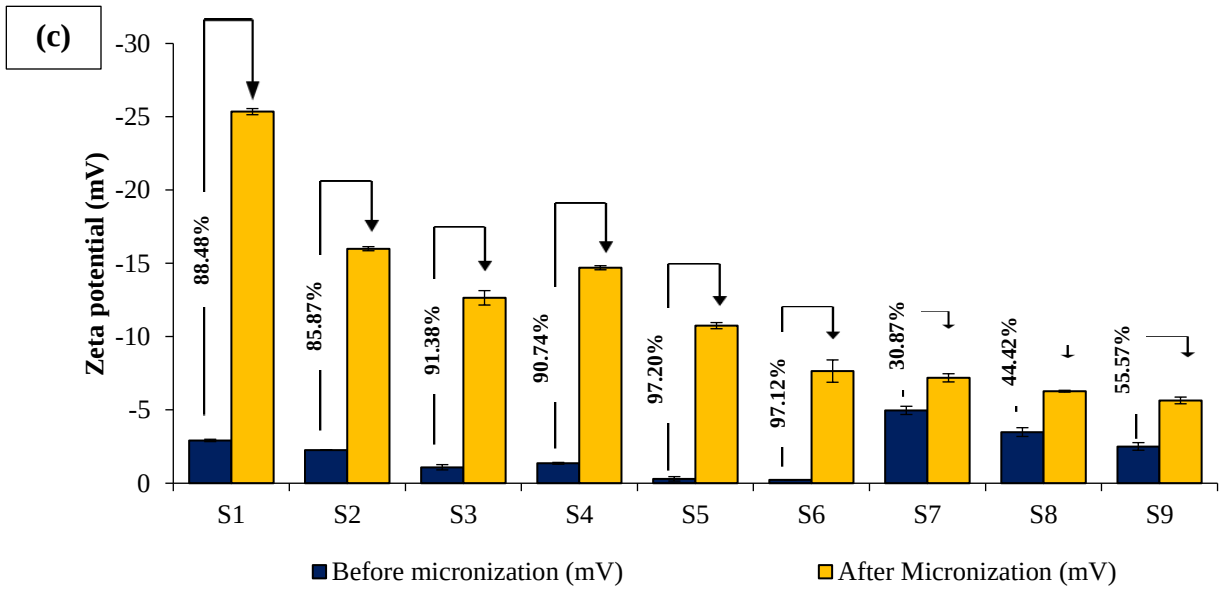


Fig 3.2 Effect of micronization on the physical properties of starch particles: **(a)** change in particle size with respect to micronization time **(b)** particle size (x-y fold reduction in the particle size of starch particles after micronization) **(c)** zeta potential (the negative surface charge of starch particles increases with the reduction in particle size)

3.4.1.1.2 Morphology of starch granules

Bright field microscopy was used for the observation of the morphology of starch granules in their native and micronized form. As the particle size decreased, the shape of the particles seemed more spherical and uniform, whereas the native starch particles presented a more polygonal scheme (Fig. 3.3a). Similar observations were reported by Protonotariou et al., 2014. The same was confirmed with the SEM analysis of the starch granules (Fig. 3.3b). In alignment with the results of DLS-based particle size analysis, the micrographs showed that the diameter of starch granules reduced after micronization (Liu et al., 2009). The structural integrity of micronized starch granules was more intact

compared to the native form. The round shape and smoother surface of these granules will enhance the stabilizing capacity of emulsions compared with granules that are polyhedral and sharp-edged (Timgren et al., 2011, Saari et al., 2016). In addition to being able to stabilize foams and emulsions, small particles of different shapes and morphology can also adsorb strongly at liquid/liquid and gas/liquid interfaces (Velikov and Velev, 2007).

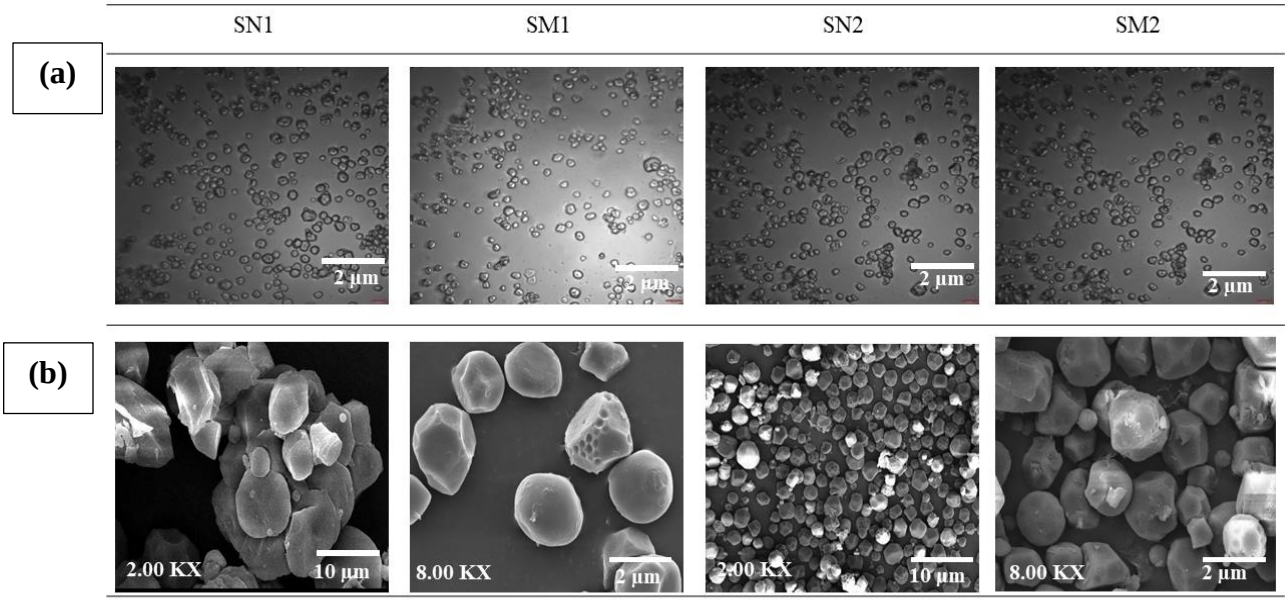


Fig 3.3 Effect of micronization on the morphology of starch granules: **(a)** bright field microscopy images **(b)** SEM images; SN1 – Pearl millet native starch, SM1 – Pearl millet micronized starch, SN2 – Proso millet native starch, SM2 – Proso millet micronized starch

3.4.1.1.3 Contact angle

Starch particles of different botanical origins have different surface compositions, which significantly influence their wettability at the interface (Xu et al., 2020). Generally, starch

is known to stabilize oil-in-water emulsions due to its hydrophilicity (Xu et al., 2020), except for the acetylated starch nanoparticles (Lam et al., 2014). Potato starch particles have a larger desorption energy owing to their larger size but cannot form emulsions (Chen et al., 2019), whereas relatively small and unimodal particles such as rice starch and quinoa starch have superior emulsion performance (Chen et al., 2019; Marefati et al., 2017). Particle wettability defined by a three-phase contact angle (θ) is a key parameter in Pickering-type stabilization, which regulates the distribution of particles at the oil-water interface. Thermodynamically, θ is interrelated with the balance of surface free energies among particle water, particle-oil, and oil-water interfaces (Huang. et al., 2019). In the present study, the contact angle measurements of native and micronized starch granules showed that the millet starch granules are hydrophilic. The contact angle values of native starch granules ranged from 63 ± 0.30° (barnyard millet) to 78.1 ± 0.64° (proso millet) (Table 3.2). The hydrophilicity of the granules increased after micronization. The contact angle of micronized starch granules ranged from 55.4±0.4° (pearl millet) to 74.9 ± 0.6° (sorghum). For most particles, θ increases with the particle sizes till a plateau value (Maestro et al., 2014). McBride & Law (2012) pointed out that the growth of θ with the particle size was because of the line tension that decreased as the diameter increased. Pickering particles contribute to droplet stabilization by dual wettability towards both phases which can be characterized by contact angle θ. The adsorbed particle will cause the interface to bend towards the phase with lower affinity and therefore particles that are more hydrophilic (<90°) are more suitable for O/W emulsions and particles with more hydrophobic traits (>90°) are more suitable for W/O emulsion (Marefati et al., 2017).

Thus, due to their θ value being less than 90°, the micronized millet starch particles would be more suitable as Pickering particles for the stabilization of O/W emulsion.

Table 3.2 Effect of micronization on the hydrophobicity of starch granules

Samples	Contact angle of native starch granules (°)	Contact angle of micronized starch granules (°)
S1	68.00 ± 0.25^{c}	55.40 ± 0.35^{a}
S2	78.10 ± 0.64^{h}	58.70 ± 0.50^{c}
S3	77.80 ± 0.70^{g}	57.40 ± 0.45^{b}
S4	72.30 ± 0.45^{e}	69.67 ± 0.70^{g}
S5	71.60 ± 0.55^{d}	61.70 ± 0.20^{e}
S6	67.70 ± 0.56^{b}	65.70 ± 0.45^{f}
S7	63.00 ± 0.30^{a}	61.36 ± 0.50^{e}
S8	68.40 ± 0.60^{c}	60.40 ± 0.40^{d}
S9	76.00 ± 0.70^{f}	74.90 ± 0.60^{h}

*Values are means ± SD (n = 3), followed by different superscript letter are significantly different ($p \leq 0.05$). The letter "a" represents the least value.

3.4.2 Determination of chemical fingerprint by Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra of native and micronized starch are presented in Figs. 3.4(a) and 3.4(b), respectively. Both spectra exhibit nearly identical characteristic bands, with slight variations in peak intensities. Notably, a strong absorption peak within the range of 3290–

3246 cm^{-1} is observed, which is attributed to the -OH stretching vibrations (Ahmad et al., 2020a). The breadth of this peak indicates the extent of inter- and intra-molecular hydrogen bonding. Furthermore, the spectral region spanning wavenumbers 3000 to 3700 cm^{-1} corresponds to N-H stretching vibrations, specifically related to the N-H group involved in the formation of the polypeptide backbone. Additionally, the wavenumbers falling within the range of 1265 to 1530 cm^{-1} are associated with amide II vibrations arising from N-H bending (Sharma et al., 2023).

In the case of micronized starch, Ahmad et al., (2020) observed a shift of the -OH stretching peak towards higher wavelengths. Other characteristic bands were identified at 2923 cm^{-1}, attributed to -CH2 stretching vibrational modes, and at 1147, 1078, and 990 cm^{-1}, associated with the stretching vibrations of the C-O bond, C-O-H, and C-O-C groups in the anhydrous glucose ring, respectively (Hebeish et al., 2014). The presence of moisture content in the amorphous region of starch is represented by peaks at 1630–1660 cm^{-1} (Gautam et al., 2021). Additionally, the characteristic peaks observed at 855, 856, and 867 cm^{-1} in the samples indicate the existence of β-glycosidic bonds (Liu et al., 2017). Shi et al., (2012) reported significant stretching of C-H (asymmetric) at 2971 cm^{-1} and an absorption band at 1458 cm^{-1}, attributed to the binding of water in the starch granules.

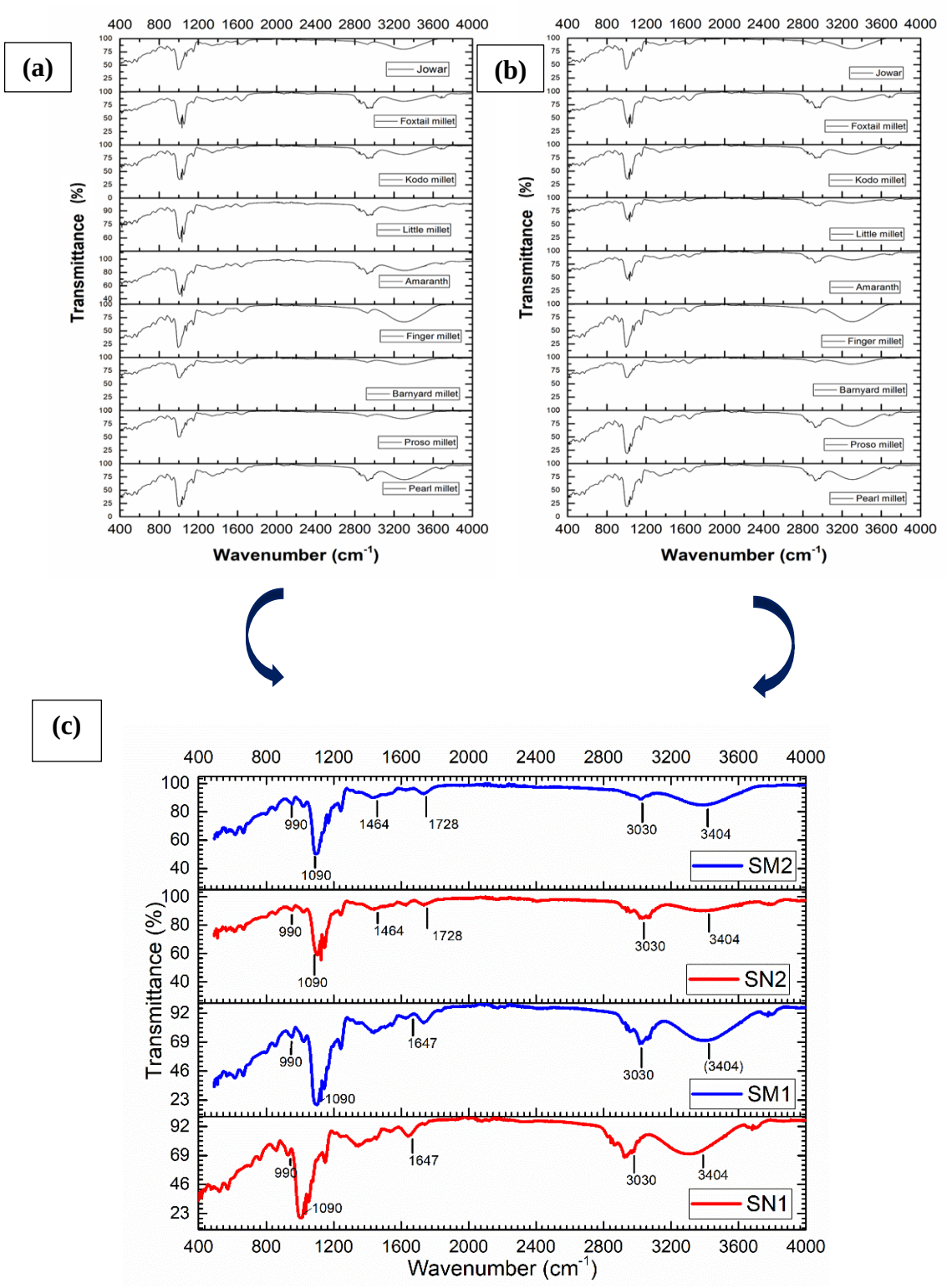

(a)
(b)
(c)
Jowar
Foxtail millet
Kodo millet
Little millet
Amaranth
Finger millet
Barnyard millet
Proso millet
Pearl millet
Transmittance (%)
Wavenumber (cm-1)
990
1090
1464
1728
1647
3030
3404
(3404)
SM2
SN2
SM1
SN1

Fig 3.4 FTIR spectra of: (a) native starch (b) micronized starch (c) enlarged FTIR spectra of native and micronized pearl (SN1, SM1) and proso millet (SN2, SM2) starches

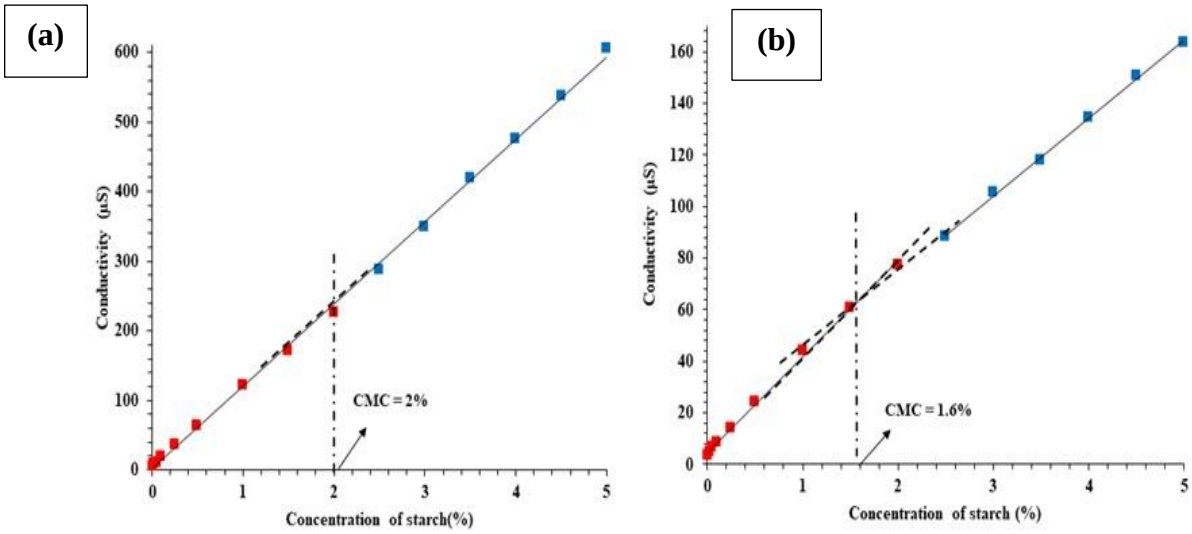
Based on the particle size, zeta potential values and three-phase contact angles of all the starches, it was found that pearl and proso millet granules were more suitable for acting as good emulsifiers and Pickering particle. Therefore, for further detailed studies these two starches in their native and micronized form were selected.

3.4.3 Surface activity

3.4.3.1 Determination of Critical Micelle Concentration by conductometry

One of the most significant parameters working with micellar phases is the surfactant critical micelle concentration (CMC) i.e., the concentration above which micelles start to form. Lower the CMC better is the surfactant property. When the conductivity of solutions with increasing concentration of surfactant is measured, the specific conductivity–surfactant concentration plots show two straight lines with different slope. The first one corresponds to the concentration range below the CMC, when only monomers of surfactant exist in solution. At higher concentrations of surfactant, micelles start to form, and a change of slope appears because the conductivity increases in a different manner. The intersection of these two straight lines is taken as the CMC value of the surfactant (Mukerjee & Mysels, 1971). In this study, the CMC of micronized pearl millet starch was 1.6% and that of micronized proso millet starch was found to be 1.0% (fig. 3.5) which was lesser when compared to the CMC of native starch (S1-2.0% and S2-2.5%). The smaller particle size resulted in a significant increase in the specific surface

area, which resulted in an increase in the surface activity of starch granule (Wang et al., 2018). It has been proved that when using surfactants to stabilize emulsions, as the surfactant concentration increases, the size of the droplets decreases, and the emulsion stability is improved. When the concentration of the surfactants reaches the critical micelle concentration, the droplet size remains constant (Wu & Ma, 2016). Therefore, micronized starch granules can be used as possible emulsifiers for preparation of emulsions by taking an amount that is less than the CMC found in the study.



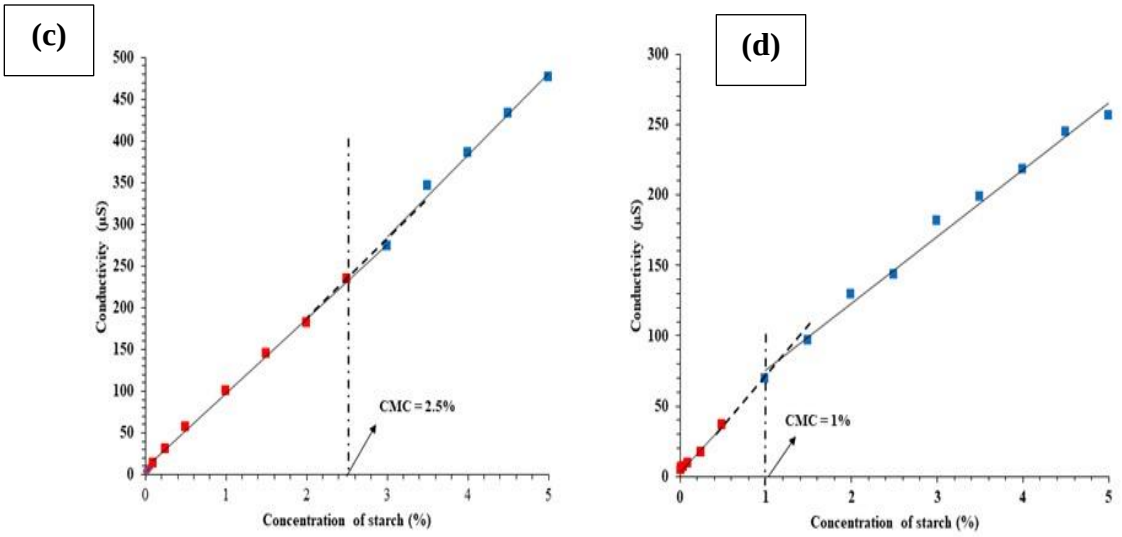


Fig 3.5 Conductometry based determination of Critical Micelle Concentration for starch (a) native pearl millet (b) micronized pearl millet (c) native proso millet (d) micronized proso millet

3.4.4 Determination of the degree of crystallinity by X-Ray Diffraction analysis (XRD)

The crystallographic characteristics of both native and modified starch granules have been depicted in Fig. 3.6 through X-ray diffraction (XRD) analysis. The XRD patterns for millet starch samples exhibited prominent peaks corresponding to Bragg angles at approximately 15°, 17°, 18°, and 23.5°. Starch granules are known to possess a semi-crystalline nature, consisting of regions with varying degrees of crystallinity and amorphousness. Different types of starches exhibit distinct XRD patterns (Karwasra et al., 2017). X-ray diffraction serves as a valuable tool to investigate the presence and characteristics of the crystalline structure within starch granules. The crystalline regions within starch display sharp peaks, while the amorphous regions exhibit dispersive peaks

(Gernat et al., 1990). Based on these observations, starch can be classified into A, B, and C types.

The diffraction patterns of both native and micronized starches showcased a Type A crystalline structure primarily containing dispersed α-1,6 branch linkages within the amorphous and crystalline domains. These characteristic peaks observed at Bragg angles (2θ) around 15°, doublet peaks at 17° and 18°, and a single peak at 23° are typical of cereal starches (Mahajan et al., 2021; Mahajan et al., 2022). This X-ray diffraction pattern for millet starch aligns with previous findings reported by Sun et al. (2014). Upon subjecting the starch to micronization, a decrease in crystallinity was generally observed, indicative of a transformation from a crystalline to an amorphous state (Wu et al., 2022). The reduced intensity of certain diffraction peaks can be attributed to the disruption of the ordered 3-D structure of amylopectin and the increased amorphous character resulting from the particle size reduction during the grinding process (Ahmad et al., 2020). The mechanical forces generated during grinding may lead to the relaxation of crystalline clusters, the emergence of disorder and defects in the crystals, as well as the breakdown of starch granule structure (Zhang et al., 2010; Lu et al., 2018). Consequently, the proportion of the crystalline structure diminishes while the non-crystalline fraction increases in the case of millet starch (Lu et al., 2018). Thirumdas, Trimukhe, et al. (2017) and Sun et al., (2022) have reported that physical treatments primarily influence the amorphous regions, which are more sensitive than the crystalline components.

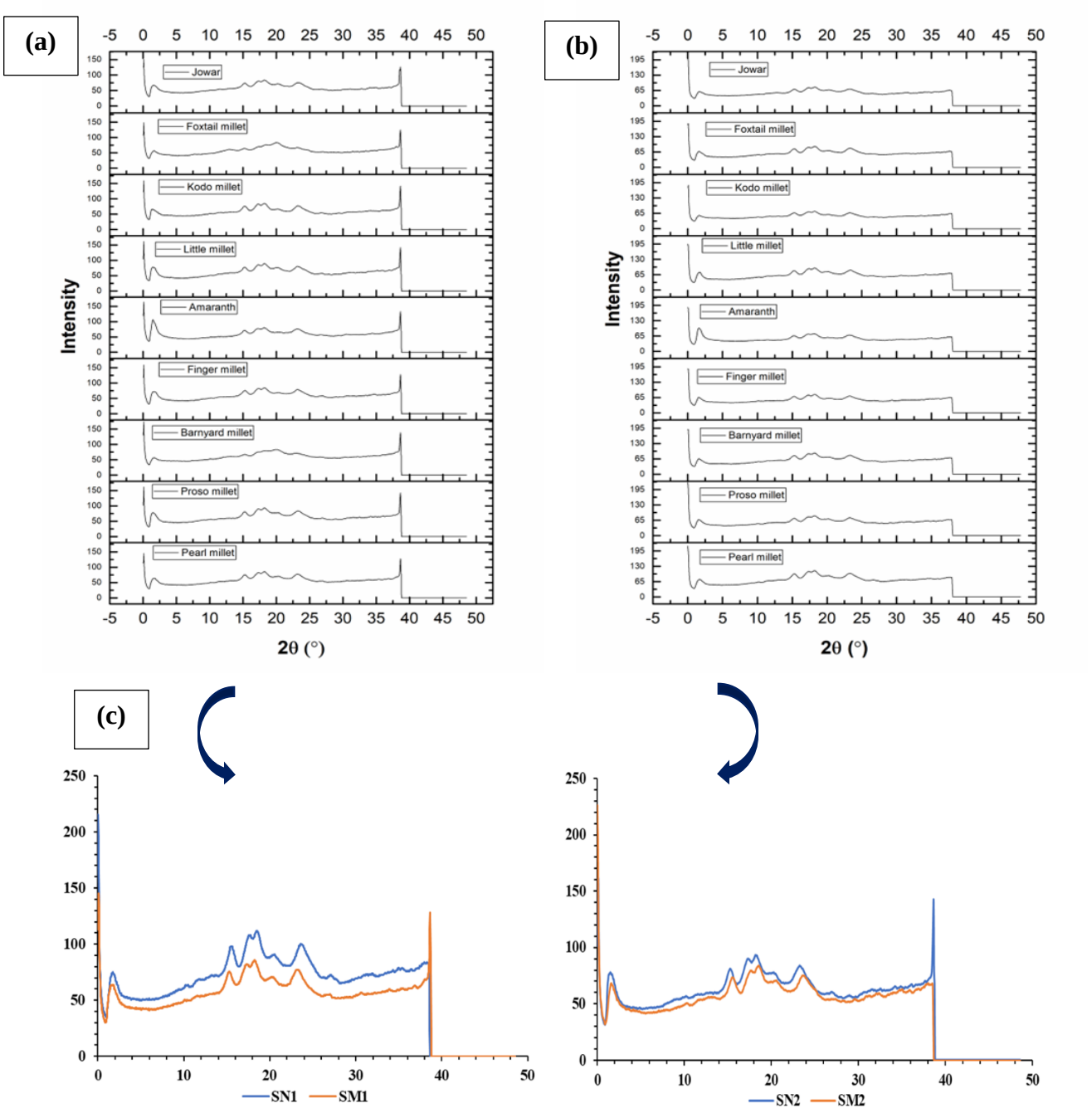
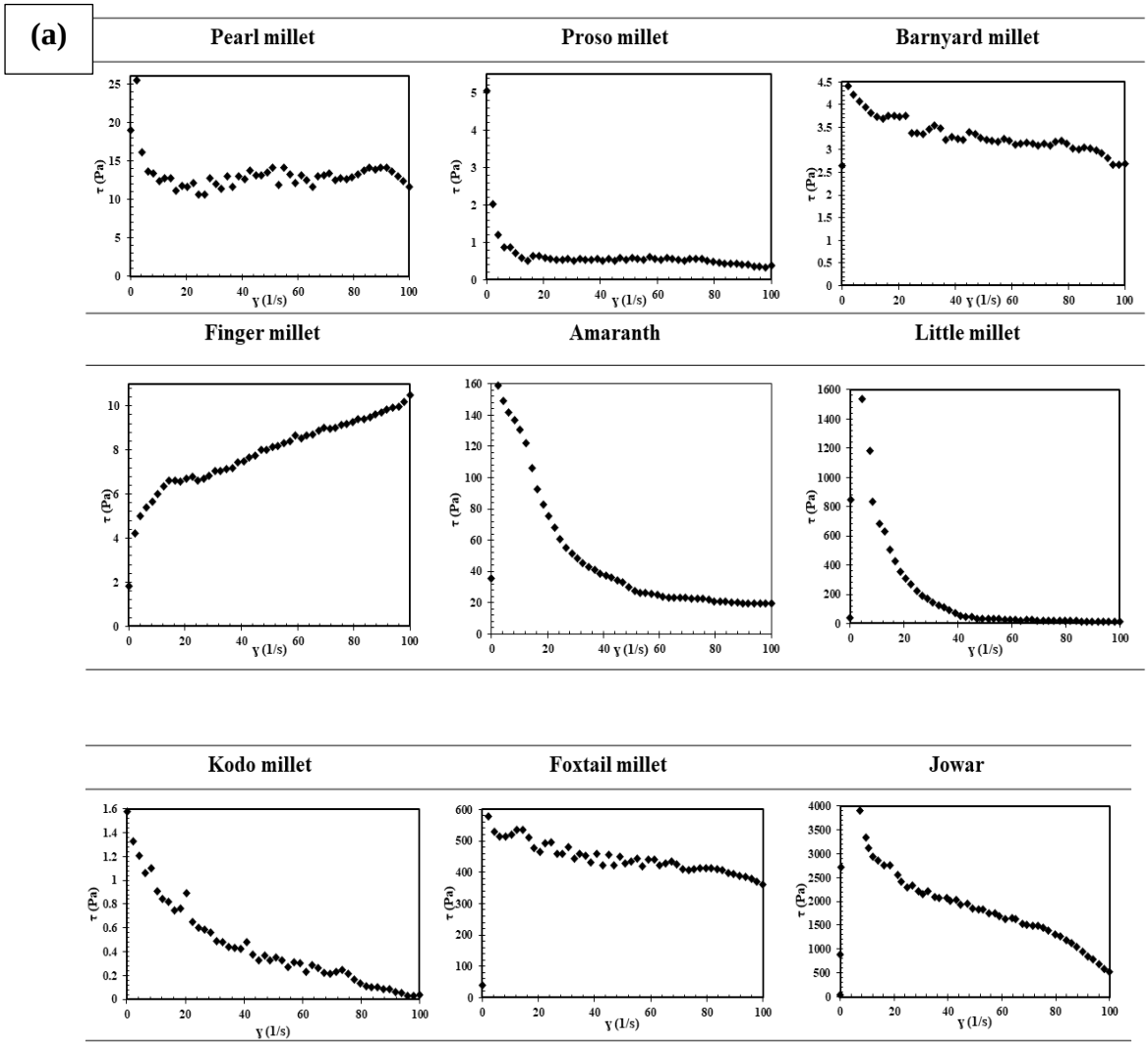


Fig 3.6 XRD spectra of: **(a)** native starch **(b)** micronized starch **(c)** enlarged XRD spectra of native and micronized pearl (SN1, SM1) and proso millet (SN2, SM2) starches.

3.4.5 Rheology

Rheological properties of a material pertain to its deformation and flow behaviors in response to applied stress (Tabilo-Munizaga & Barbosa-Cánovas, 2005). Flow curves, represented by the relationship between shear stress (τ) and shear rate (γ), for both native and modified starches are presented in Figs. 3.7(a) and 3.7(b), respectively. The flow behavior index (n) indicates the degree of proximity of the sample's flow behavior to that of a Newtonian fluid. The flow curves obtained for the various starches in this study resemble those reported by Zhou et al., (1998) for oat starch. The viscosity of starch paste exhibits shear rate dependence, revealing a non-Newtonian nature of the material. Starch pastes also demonstrate thixotropic characteristics, displaying gel-like properties in quiescent conditions and fluid-like behavior when subjected to shearing forces (Shuey & Tipples, 1982).

The size of starch granules significantly influences the pasting properties, wherein larger granules exhibit reduced molecular bonding, resulting in faster swelling and higher viscosities compared to smaller granule fractions. Notably, pearl millet starch is reported to possess a higher pasting temperature, indicating increased resistance towards swelling, a trait attributed to its smaller granule size (Siroha et al., 2020). Generally, the micronization treatment of starch leads to a lower apparent viscosity, and the starch paste displays weakened shear-thinning gel-like behavior with enhanced elasticity (Wu et al., 2022). Similar shear-thinning behavior has been observed for native barley and sorghum starches (Punia et al., 2019; Siroha et al., 2019). Bhandari et al. (2002) attributed the shear-thinning behavior to a higher degree of breakage of the intra- and inter-molecular associative bonding system in starch network micelles due to shearing at high rates.


(a)
Pearl millet
Proso millet
Barnyard millet
Finger millet
Amaranth
Little millet
Kodo millet
Foxtail millet
Jowar
τ (Pa)
γ (1/s)

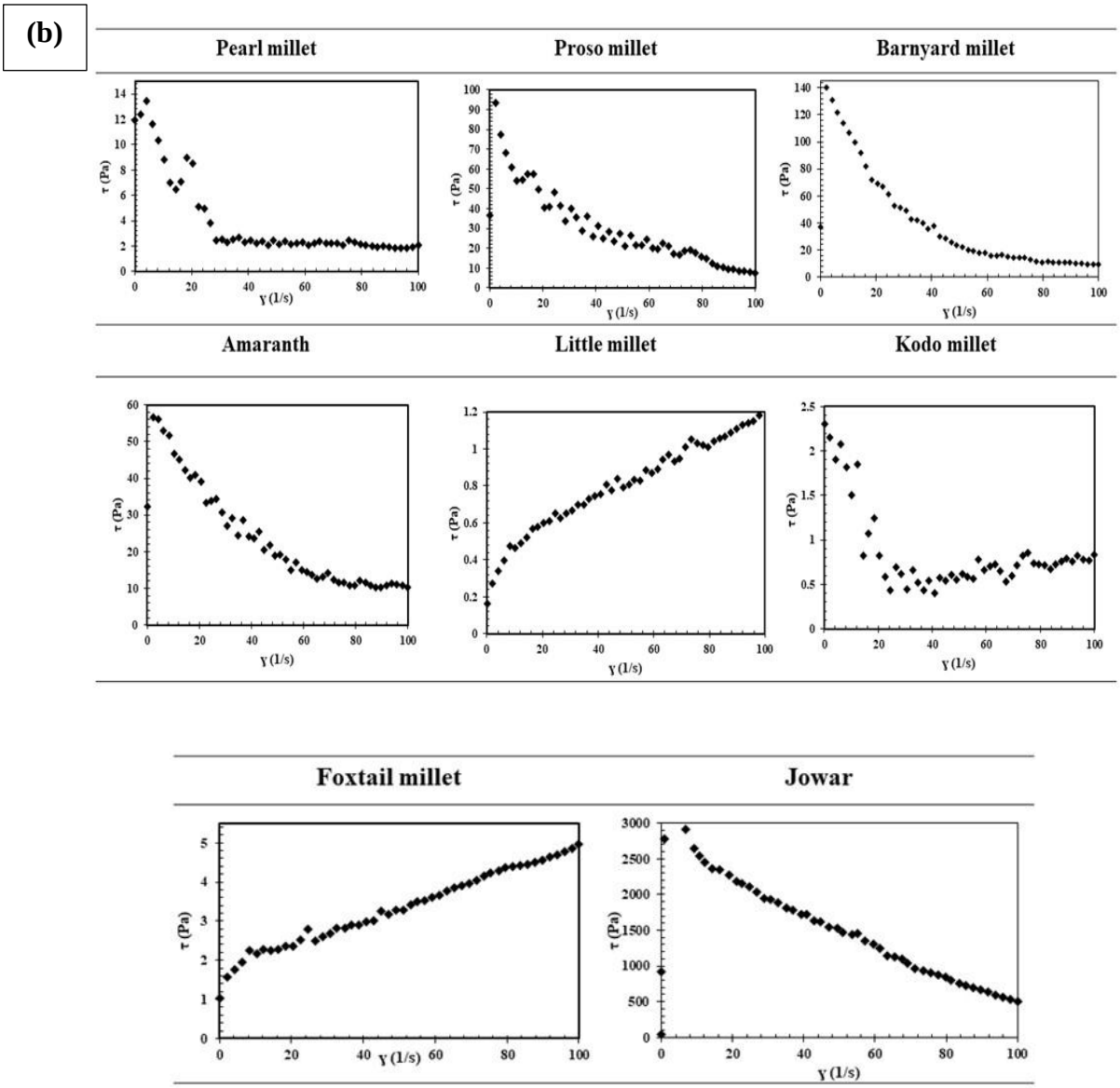


Fig 3.7 Effect of micronization on the flow behavior of starch suspensions: (a) native starch; (b) micronized starch

The dynamic shear rheology (Fig. 3.8) is essential for the information pertaining to relative viscous and elastic character of materials. Storage modulus (*G'*), Loss modulus (*G''*) and tan δ obtained during dynamic testing signify the extent of elastic character, viscous character, and physical state of the material, respectively. *G'* gives information

about energy stored, *G″* reveals energy dissipated by the sample and tan δ is the ratio of two moduli (*G″/ G′*) (von Borries-Medrano et al., 2019).

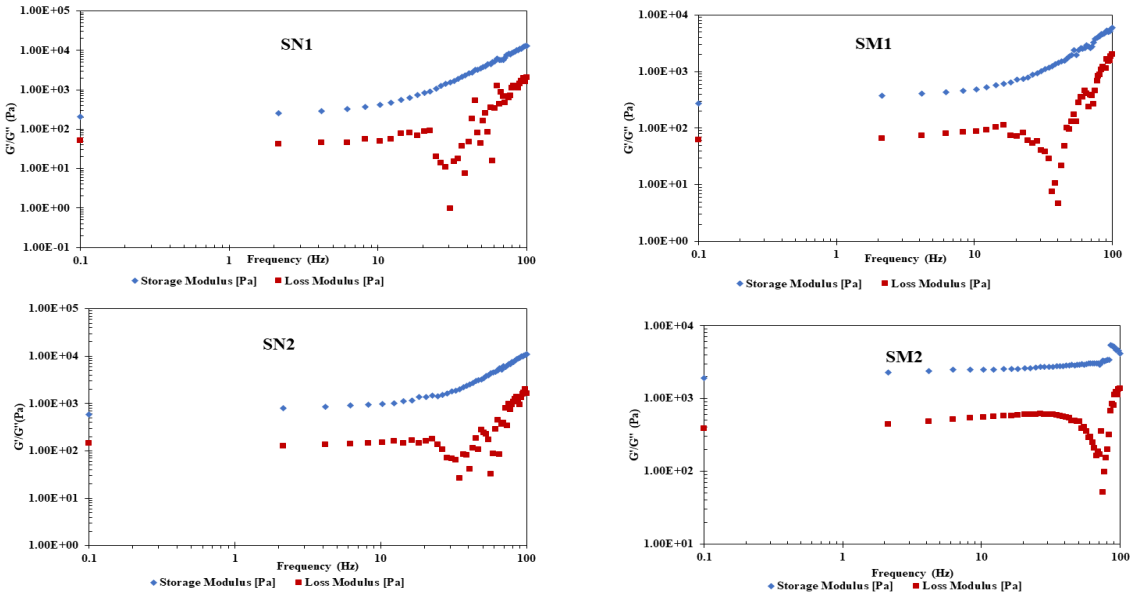


Fig 3.8. Effect of micronization on the storage modulus and loss modulus of starch samples

The critical strain value, denoted as γc, represents the threshold strain beyond which the storage modulus (G') and loss modulus (G") deviate from being constant. Concurrently, the corresponding stress at this limit within the linear viscoelastic (LVE) region is termed the critical stress (τγ) (Bharadwaj et al., 2019). For the starch samples under investigation, the LVE region was determined to be at 0.2% strain. Notably, no crossover was observed between G' and G" within the evaluated frequency range of 0.1 to 100 rad s^{-1}, indicating the stability of the starch pastes. The G' value was found to be higher than the G" value, suggesting that the starch paste exhibits predominantly elastic behavior over viscous behavior (Siroha et al., 2020). This characteristic indicates that the starch particles function as highly active filler particles, substantially enhancing the gel strength of

emulsions prepared from millet starch (Jo et al., 2021). Furthermore, it was observed that no cross-over occurred between G' and G" across the assessed frequency range, further confirming the stability of the starch dispersions within this range (Punia et al., 2021).

3.4. 6 Thermal properties

3.4. 6.1 Differential Scanning Calorimetry (DSC)

When heated with sufficient amount of water over a range of temperature, granular starch undergoes an order–disorder phase transition termed gelatinization. Water uptake by the amorphous region, radial swelling of the granules, breakdown of crystalline region with the disruption of double helices, and starch molecule leaching are the characteristics of gelatinization (Hoover, 2001). The gelatinization can be quantified by various methods with DSC being most used for millet starch analysis where onset (T_o), peak (T_p), and conclusion (T_c) temperatures and enthalpy change (ΔH) are regularly recorded (Zhu, 2014). External chains of amylopectin are packed together in clusters and during the gelatinization process, uncoiling and melting of chains occur (Li et al., 2016). Starches with low gelatinization temperature (an average of 70 °C) show better cooking quality (Waters et al., 2006). Gelatinization temperatures are also significant for selecting specific properties of starches according to requirements in various food applications (Morales-Martínez et al., 2014).

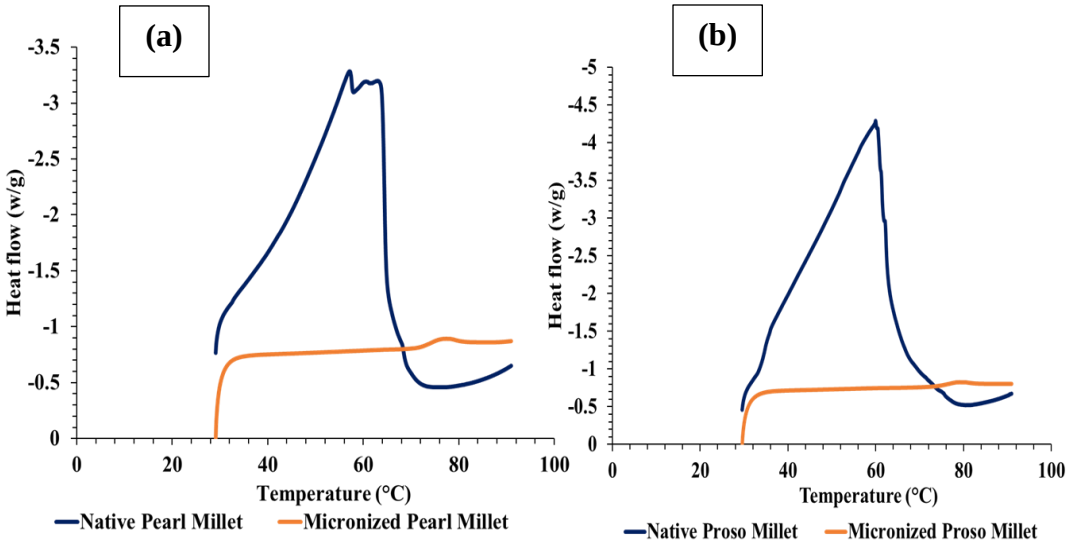


Fig 3.9 DSC thermograms of (a) pearl millet starch and (b) proso millet starch

The gelatinization temperature of native starch granules was in the temperature range of 55 - 60 °C as observed from fig. 3.9. After size reduction the gelatinization temperature increased to the range of 65 – 76°C. The diversity in gelatinization may be attributed to the molecular structure and content of amylopectin and amylose, the presence of lipids, the physical association and orientation of these chemical components in the granules (Srichuwong & Jane, 2007). Starches with higher gelatinization temperatures have higher stability to temperature and granule swelling because to initiate gelatinization process, more energy is required. Higher crystallinity results in higher transition temperatures which change the gelatinization enthalpy due to the loss of molecular arrangements in the starch granules (Tester & Morrison, 1990). Noda et al. (1998) postulated that the T_o, T_p, and T_c values were influenced by the molecular architecture of the crystalline region, which corresponds to the distribution of amylopectin shorter chains. Physicochemical properties (pasting & gelatinization), enzyme susceptibility, and crystallinity of starches are affected by starch granule size (Lindeboom et al., 2004).

3.4.6.2 Thermogravimetric Analysis (TGA)

The TGA curves of native and micronized starches are shown in Fig. 3.10. TGA and DTG thermograms were used to describe the thermal stability caused by the heating method (Moreira et al., 2013).

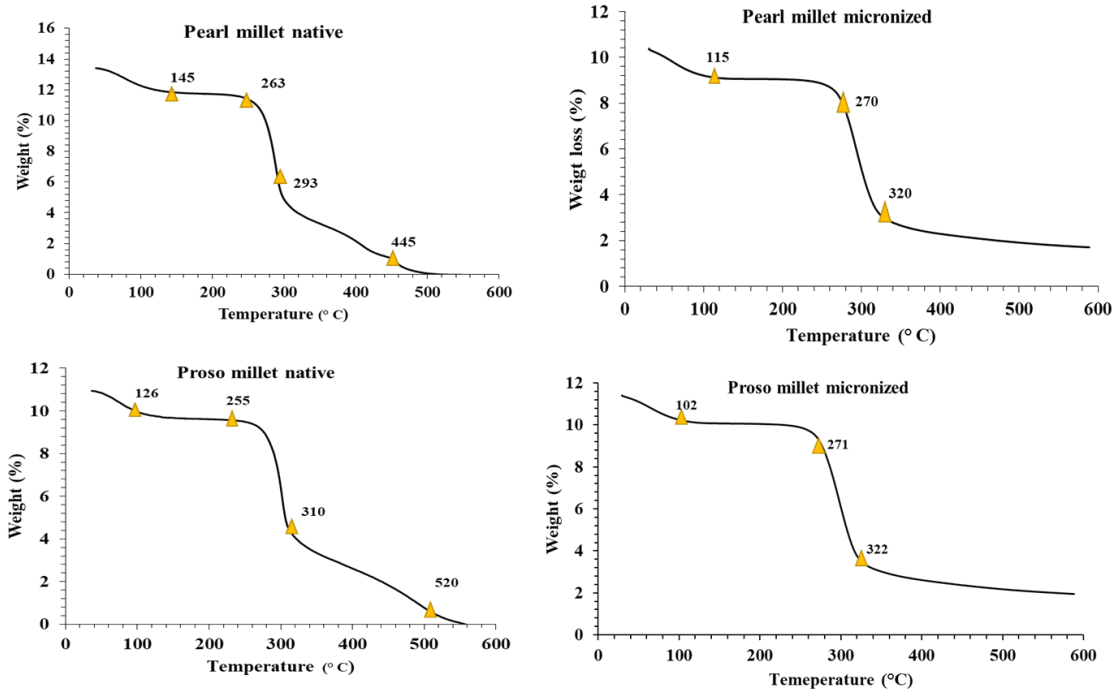


Fig 3.10 Thermogravimetric analysis curves of native and micronized pearl and proso millet starches

Three distinct regions can be seen in these thermogravimetric curves. The initial mass loss is generally ascribed to the decomposition of free water in the samples (Tian et al., 2011). The second stage is the main degradation zone of starch, and the final stage is generally carbonization decomposition. The onset decomposition temperature of the native starch was around 290 °C while that of micronized starch samples were in the range of 220-250°C. The results of our study are in accordance with that observed by Sun et al.

(2014). The native starch had a higher onset decomposition temperature than the micronized starch and the micronized starch showed a more stable thermal degradation therefore making the starch granule suitable for use in many thermally processed foods. Most of the food systems operate within the range of 250 °C. Therefore, emulsions prepared using modified starch granules can be incorporated to food systems that operate in the temperature range till 220 – 300°C.

3.4.7 Validation of the proof-of-concept: Emulsion preparation and characterization

3.4.7.1 Droplet size, zeta potential and turbidity measurements

To understand the emulsion stabilization potential of native starch and micronized starch, emulsions were prepared using native and micronized pearl and proso millet starches. The droplet size and zeta potential of the fresh emulsions and that after 24 h are presented in table 3.3. The size distribution of droplets is one of the major characteristics that strongly influences emulsions stability towards gravitational separation, flocculation, and coalescence, as well as its rheological and sensorial properties (McClements, 2015). The droplet size is usually reported by its diameter. If all droplets in an emulsion have the same size there are referred to as “monodisperse” whereas, if the droplets have a wide range of sizes, there are referred to as “polydisperse”. Food emulsions fall in the polydisperse category (Dickinson, 2012).

Table 3.3 Droplet size (μm) and ζ-potential (mV) of physically modified starch emulsions. Data represented as mean ± SD, n = 3, $p \leq 0.05$. Letter 'a' represents least value

Starch emulsions	Droplet size (μm)	Zeta potential (mV)
Pearl millet native (SN1)	1.83 ± 0.20^{b}	-66.0 ± 0.01^{a}
Pearl millet micronized (SM1)	1.01 ± 0.25^{a}	-76.1 ± 0.02^{b}
Proso millet native (SN2)	1.58 ± 0.20^{b}	-65.7 ± 0.02^{a}
Proso millet micronized (SM2)	0.94 ± 0.30^{a}	-74.4 ± 0.02^{b}

The droplet size of the emulsions decreased with the addition of micronized starch granules. Also, this study confirms that physically modified starch granules can be used as an emulsifier for the preparation of Pickering emulsions. Smaller particles could stabilize emulsions with small droplet size and high stability at a lower concentration (Zhang et al., 2021). The zeta potential values showed a decrease when micronized granules were added in case of both pearl (-66.0±0.01 to -76.0±0.02 mV) and proso millet (-65.7±0.01 to -74.4±0.02 mV). The negative ζ-potential of oil droplets confirmed the adsorption of micronized millet starch particles at the O/W interface. This shows that the surface of millet starch particles did not vary after combining with oil during the emulsification process (Ridel et al., 2016). A colloidal system with ζ potential ranging between-41 to -60 mV has *'fairly good stability'* and that with values varying from -61 to -80 and -81 to -100 has *'very good stability'* and *'extremely good stability'*, respectively. Thus, based on their ζ potential values, the micronized millet starch stabilized emulsions can be considered to have very good stability (Riddick, 1968).

The turbidity storage and stability of the prepared emulsions for 24 h in room temperature was also analyzed (Fig. 3.11) (Table 3.4).

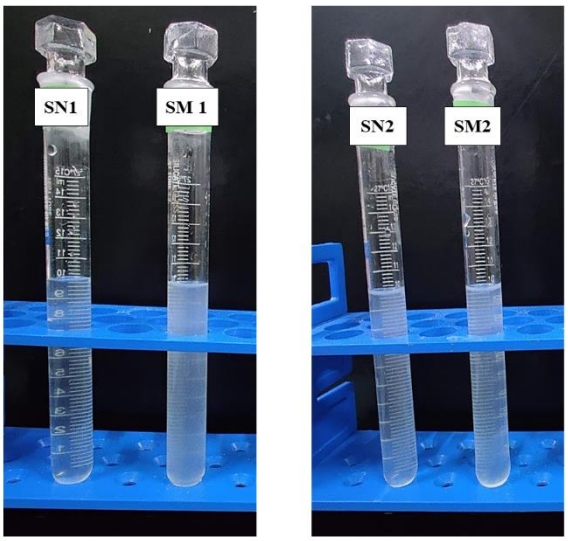


Fig 3.11 Emulsions prepared using native and micronized starch (SN1-native pearl millet starch, SM1- micronized pearl millet starch, SN2-native proso millet starch, SM2-micronized proso millet starch)

Table 3.4 Turbidity of the prepared emulsions (@ 600 nm) (Data represented as mean ± SD, n = 3, $p \leq 0.05$) Letter 'a' represents least value.

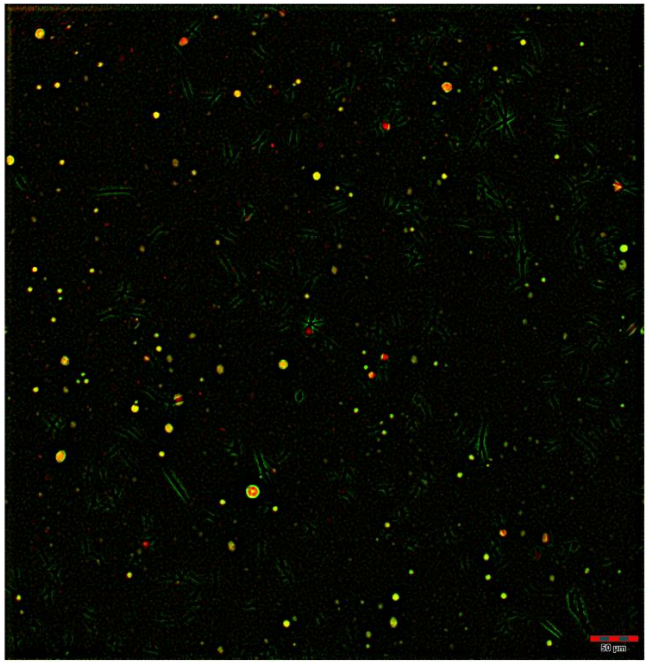
Starch emulsions	Turbidity (%)	
	Fresh emulsion	24 hours
Pearl millet native	45.97±0.01[a]	42.92±0.02[a]
Pearl millet micronized	81.23±0.01[d]	77.89±0.01[d]
Proso millet native	60.28±0.01[b]	58.37±0.01[b]
Proso millet micronized	66.73±0.01[c]	62.90±0.02[c]

Turbidity is generally believed to be associated with particle size and number (Ru et al., 2012; Yan et al., 2013). The coalescence of emulsion droplets leads to the change in the appearance of the system since the light scattering capability of a large droplet is weaker as compared to that of a smaller droplet. As a result, the emulsion may become less turbid and more intensely colored (McClements, 2002). In that sense, the measurement of the turbidity of a Pickering emulsion can be considered for determining the coalescence stability of the Pickering emulsion system (Low et al., 2020). Saari et al. (2016) reported that small starch particles could produce much smaller droplet size of emulsions than large particles. The results indicated that smaller particles of the modified starch granules had better emulsifying properties, consistent with the previous report by Timgren et al., (2013). The hydrophilic-lipophilic balance (HLB) value of surfactants also plays an important role in the determination of its functionality. Emulsions with surfactants of HLB < 6 tend to be oil soluble and stabilize water-in-oil, whereas emulsions with surfactants of HLB > 10 tend to be water soluble and stabilize oil-in-water (Feng et al., 2018). Emulsions which have HLB values above 10 forms milky white emulsions which is seen in the present study too. It also proves that the starch granules acted as surfactant which made it suitable to form O/W emulsions (El-Sayed and Mohammed 2020).

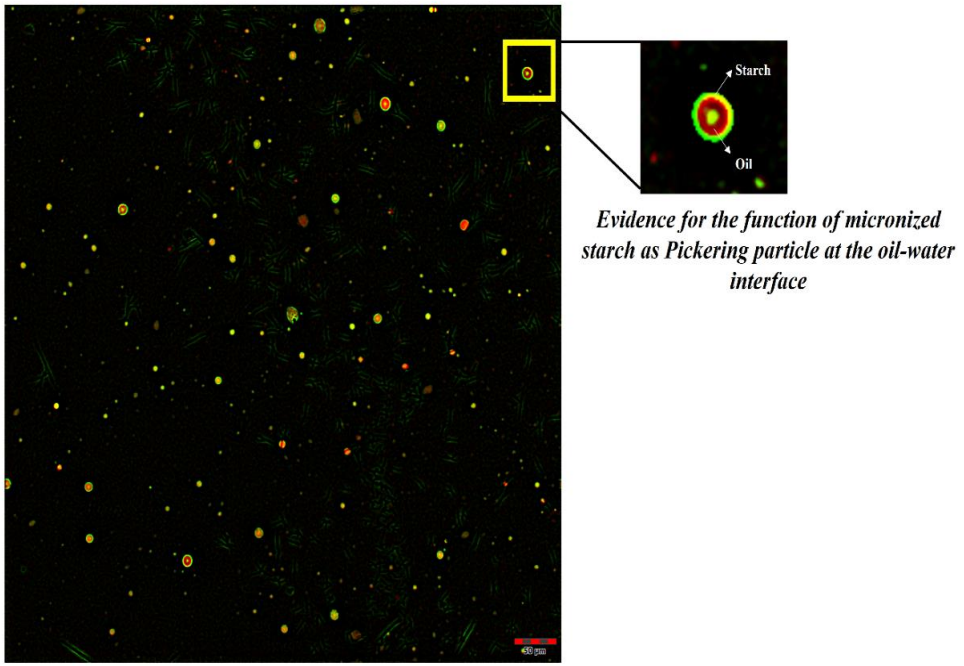
3.4.7.2 Morphology of millet starch—stabilized Pickering emulsion

Fig. 3.12 shows the fluorescence micrographs of freshly prepared emulsions. The bright and red regions indicate oil droplets. The green color layer at the interface of emulsion corresponds to Safranin O that has the capacity to bind with starch (Chouët et al., 2019), thus suggesting that the emulsion was stabilized by starch particles at the oil-water

interface. The fluorescence image clearly shows that a green halo was present surrounding the red oil droplets. This result reveals that the emulsion was stabilized by the starch particles at the interface between the oil and aqueous phases (Lu et al., 2018), thus affirming the Pickering activity of micronized millet starch.

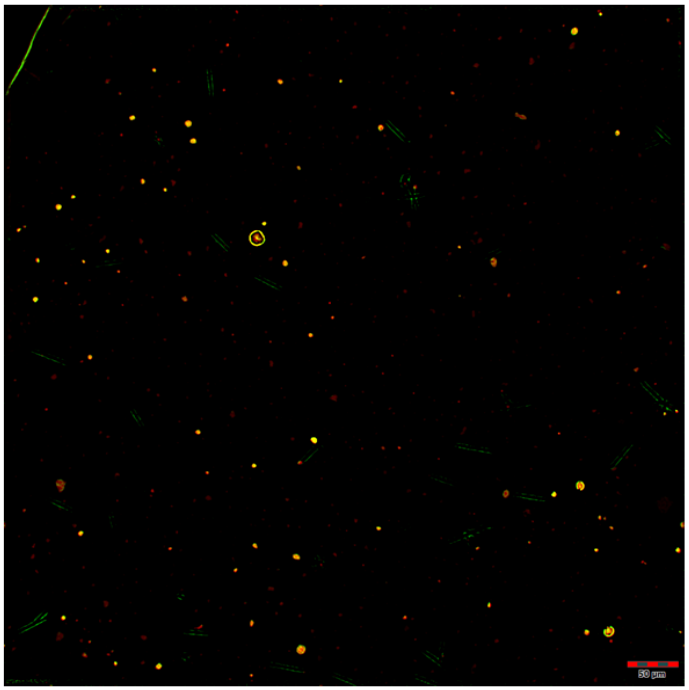


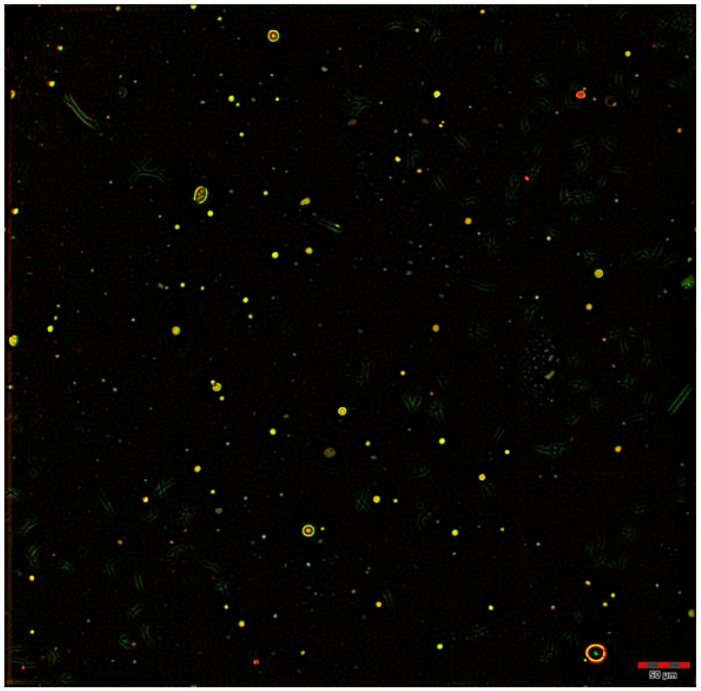
(a)



Evidence for the function of micronized starch as Pickering particle at the oil-water interface

(b)





(c)

(d)

Fig 3.12 Fluorescence micrographs of the prepared native and micronized starch Pickering emulsions: (a) native pearl millet starch emulsion (b) micronized pearl millet starch emulsion (c) native proso millet starch emulsion (d) micronized proso millet starch emulsion

3.5 Conclusion

The present study demonstrates that the physically modified starch particulates can act as effective colloidal particles to stabilize oil-in-water emulsions. This opens up a new possibility that particles suitable for acting as emulsifiers can be generated by non-chemical modification of starch granules and confirms the potential of size reduction as the physical modification approach. The preparation of micronized starch granules demonstrated notable advantages in functionality, particularly as Pickering particles, successfully achieving the desired particle size. It was also determined from the obtained data that the starch microparticles were more amorphous having decreased water absorption and oil absorption capacity. These properties could be useful in particular food and drug formulations which may increase the commercial scope of millet starches in both food and pharmaceutical industries. The information gained from this study may shed new lights on the eco-friendly utilization and deep processing of millet starches for industrial applications in the food, nutraceutical, and cosmetic sectors.

The key findings of this chapter are summarized below:

- Starch is the major component of millet grains.
- Physical modification of starch by micronization produced more uniform particles with improved functional, structural, and thermal stability.
- Physically modified starch particulates can act as very effective stabilizers for the preparation of oil-in-water emulsion.
- The preparation of micronized starch granules proved advantageous in terms of functionality, particularly as Pickering particles with desired particle size.

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Chapter 4

Optimisation and characterisation studies on Pickering emulsions of roasted coffee oil stabilised by physically modified pearl millet starch granules

4.1 Introduction

Emulsions, comprising one liquid phase dispersed within another, play vital roles in industries like food and cosmetics (McClements, 2015). Traditionally, they rely on synthetic or animal-based emulsifiers, but these can lead to problems like excessive foaming and skin irritations (Sjöö et al., 2015). With growing demand for natural products, Pickering emulsions have gained traction (McClements & Gumus, 2016). These emulsions use solid plant-derived particles, such as starch and cellulose, for stabilization (Tavernier et al., 2016). Unlike surfactant-stabilized emulsions, Pickering emulsions are notably stable against processes like Ostwald ripening and coalescence (Marku et al., 2012; Matos et al., 2018). This natural approach aligns with the trend for plant-based, label-friendly ingredients in consumer products. This shift emphasizes the importance of innovative emulsion stabilization methods, rendering Pickering emulsions a promising choice for industries seeking stable, eco-friendly formulations. An important aspect in Pickering emulsion formation is the type of oil that will be added as the oily phase. Different types of oils such as Triaglycerols (LCT (Long chains triaglycerols), MCT (Medium chains triaglycerols), SCT (Short chains triaglycerols)), flavor oils, essential oils, mineral oils, waxes, and various other lipophilic components can be used (Liang et al., 2016). In particular, O/W Pickering emulsions with triaglycerol oils display an array of advantages since destabilization phenomena rarely occur, while oils with low water solubility capacity, such as flavor and essential oils, display increased difficulty in emulsification (Chang et al., 2012; Mikulcová et al., 2016).

Starch granules in their native form are not viable candidates for solid-particle stabilization in Pickering emulsions, due to their quite large size (Ge et al., 2017; Saari et

al., 2017). An important factor in determining the stability of a system is particle size, which must be substantially smaller than the emulsion droplet size. The average droplet size of food – grade emulsions falls in the range of 0.1 – 100 μm. Characteristically, reducing particle size leads to the production of smaller emulsion droplets in the dispersed phase, thus allowing the coverage of a larger surface area per unit mass.

Roasted coffee oil (RCO), is generated through the soluble coffee production process. RCO comprises an intricate blend of numerous volatile compounds (Böger et al., (2021), Lucci et al., (2015), Zanin et al., (2020)). Application of roasted coffee oil extends to mitigating the presence of fine particulate matter in soluble coffee and acting as a flavor-enhancing agent within the realm of the food industry (Calligaris et al., (2009); Hurtado-Benavides et al. (2016)). Furthermore, its utility extends to formulations within the cosmetic and pharmaceutical sectors (Calligaris et al., 2009). Nevertheless, it is imperative to acknowledge that exposure of roasted coffee oil to environmental factors may precipitate the oxidation of its intricate constituents, including fatty acids, triacylglycerols, diterpenes, tocopherols, caffeine, and chlorogenic acids (Böger et al. (2021) and Getachew and Chun (2016)). Consequently, to safeguard the nutritional integrity and sensory attributes of RCO, exploration of the microencapsulation methodology is warranted. Microencapsulation, a technique of paramount significance, entails the envelopment of solid, liquid, or gaseous entities within a protective matrix, thereby ameliorating susceptibility to detrimental influences such as oxygen, thermal fluctuations, moisture, and luminous exposure. This principle aligns with the tenets elucidated by Bakry et al. (2016) and Gharsallaoui et al. (2007), rendering it particularly pertinent to materials vulnerable to oxidative processes. A precursor to certain modes of

oil microencapsulation is the formation of emulsions, wherein the oleaginous phase is incorporated into the matrix solution (Carvalho et al. (2014)). In the context of this investigation, the focus resided upon the encapsulation of RCO. The encapsulation of RCO emerges as a proficient avenue to safeguard the integrity of its pivotal constituents (Freiberger et al. (2015) and Zanin et al. (2021).) In the above background, the current chapter focusses on the optimization and characterization of Pickering emulsions comprising RCO encapsulated by micronized pearl millet starch.

4.2 Objective

In the antecedent chapter, we conducted a thorough characterization of millet starches derived from all nine distinct grain varieties. Our investigations have conclusively established that micronized pearl millet starch holds the preeminent position as the most suitable candidate for the formulation of oil-in-water (O/W) Pickering emulsions. Consequently, the principal aim of the current chapter is the systematic optimization and comprehensive characterization of Pickering emulsions of coffee oil, wherein stabilization is achieved through the utilization of physically modified pearl millet starch granules.

4.3 Materials and methods

4.3.1 Materials

Pearl millet grains used in the study for starch isolation were received from Indian Institute of Millet Research, Hyderabad. The grains were ground to a powder form using a mixer grinder (Prestige Dry masala grinder PDMG 02; India) and stored in airtight containers in room temperature (30±5°C) prior to their use in the actual experiments.

Sodium hydroxide (NaOH) pellets were procured from Sisco Research Laboratories Pvt. Ltd. (SRL), Mumbai, India. Analytical grade hydrochloric acid (HCl) was purchased from Rankem. The water used in all procedures was produced with a Milli-Q water purification system (Millipore, Billerica, MA). Roasted coffee oil (Arabica) was purchased from Proderna Biotech Pvt Ltd., Zamrudpur, New Delhi, India. Gellan gum (food grade) was purchased from Urbanplatter, Maharashtra, India.

4.3.2 Experimental design

The outline of the study is depicted in the figure below:

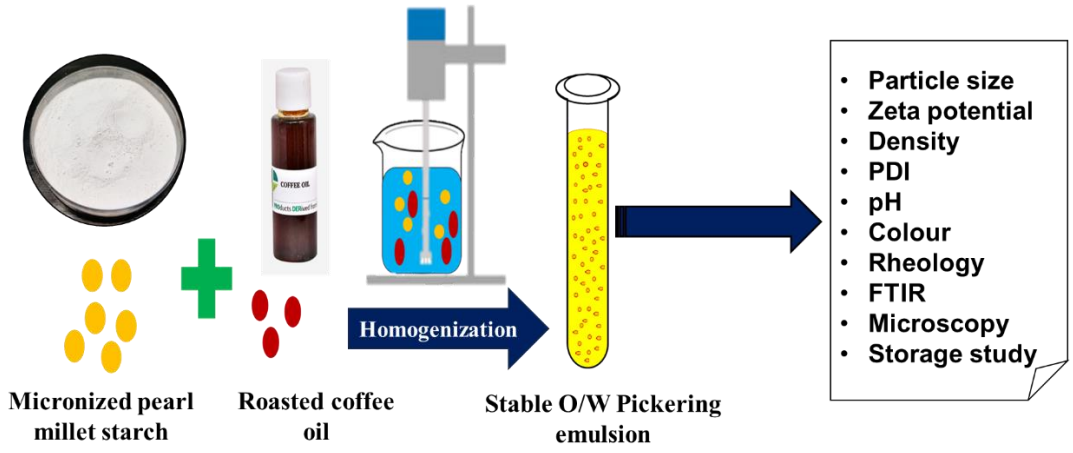


Fig 4.1a Outline of chapter 4

4.3.3 Preparation and optimization of micronized pearl millet starch Pickering emulsions

O/W emulsions were prepared using micronized pearl millet starch particles and roasted coffee oil at different proportions varying the starch and oil content concentration. After optimizing the core and wall material concentrations the homogenization speed and time were optimized. Pickering emulsions were prepared by using pearl millet starch as the

wall material and roasted coffee oil as the dispersed phase and water as the continuous phase. Based on the analysis done in our previous chapter, various trials were attempted varying the parameters. Some of the emulsion trials carried out and the observations are listed in table 4.1. During these trials, the percentage of pearl millet starch, coffee oil, homogenization time and speed were varied. Each sample was analyzed for 24 h stability by measuring the creaming index. After conducting various trials, three stable emulsions were finalized for further analysis and storage studies.

4.3.4 Storage and characterization studies

The emulsions (designated as 40E, 20E, and 20EU), which were fabricated under conditions optimized for this study, underwent an extended storage assessment spanning a duration of 15 days. The storage conditions were maintained at a refrigeration temperature, specifically 4±2°C. Periodically, at intervals of every 3 days, samples were extracted from the storage environment for a comprehensive assessment encompassing particle size analysis, determination of zeta potential, measurement of pH, density quantification, flow curve measurements, analysis of color attributes, FTIR analysis and microstructural investigations via fluorescence microscopy.

4.3.4.1 Particle size, polydispersity index and zeta potential of the prepared emulsions

The droplet size, poly dispersity index (PDI) and zeta potential of the samples were analyzed using the Malvern Setasizer (Zeta ano-ZS; Malvern Instruments, (UK)which works on the principle of Dynamic Light Scattering (DLS). Measurement was done in triplicates for all the analysis.

4.3.4.2 pH and bulk density measurements

The pH of the emulsions was measured using a pH meter, Oakton pH 700 (Benchtop meter Oakton Instruments, USA). Before commencing the pH measurements, the pH meter underwent calibration using a buffer solution with a pH value of 7 for accuracy. The determination of bulk density for the emulsions was executed following the method outlined by Huang et al. (2020), with some minor modifications. In this protocol, 5 mL of the emulsions were incrementally introduced into an empty 10 mL graduated measuring cylinder. The calculation of bulk density was predicated on the assessment of the volume occupied by the emulsion's mass within the confines of the cylinder.

4.3.4.3 Color analysis

The color parameters (L*, a*, b*) of the three emulsions were determined using Hunter lab, Ccolorflex EZ (Hunter Associate Laboratory Inc., Reston, US) spectrophotometer. Where L* denotes lightness/darkness, a* denotes redness (+) and greenness (-), and b* denotes yellowness (+) and blueness (-). The total color difference or change in color between two samples was calculated using the formula given below:

$$\Delta E = [(L_{\circ}^{*} - L^{*})^2 + (a_{\circ}^{*} - a^{*})^2 + (b_{\circ}^{*} - b^{*})^2]^{1/2}$$

Where,

$L^{*}_{\circ}$, $a^{*}_{\circ}$, and $b^{*}_{\circ}$ are the color values of the standard reference type;

L*, a* and b* are the color values of the test sample.

4.3.4.4 Rheology – flow behavior analysis of the emulsions

Rheological assessments were conducted employing a controlled stress rheometer (MCR 102 Rheometer, Anton Paar GmbH, Ostfildern-Scharnhausen, Germany) equipped with

a cone and plate geometry configuration featuring a 25-mm diameter and a 0.105 mm gap. The flow characteristics of the emulsions over the course of storage were evaluated by generating flow curves across a range of variable shear rates extending from 0 to 100 s^{-1}. The determination of the apparent viscosity, at a shear rate of 50 s^{-1} was derived from the gradient of the flow curve.

4.3.4.5 ATR-Fourier Transform Infrared Spectroscopy (FTIR)

The Fourier-transform infrared (FTIR) spectra of micronized pearl millet starch, roasted coffee oil, and the resultant emulsions were acquired using an attenuated total reflection FTIR spectrometer (ATR-FTIR, Perkin Elmer, USA) under ambient conditions at a controlled temperature of 30±5°C. The spectral acquisition encompassed the absorbance range spanning from 4000 to 400 cm^-1, encompassing a total of 32 scans at a resolution of 4 cm^-1.

4.3.4.6 Morphology observation of the prepared emulsions

The morphological characteristics of the prepared Pickering emulsions were examined through the utilization of fluorescence microscopy, specifically employing the Olympus IX-83 microscope from Japan. To facilitate this analysis, a volume of 100 µL of the emulsion was subjected to staining with Nile red (for oil visualization) and safranine (for starch visualization). The stained emulsions were subsequently deposited onto concave slides and enclosed by coverslips. The fluorescence imagery obtained from these samples was subsequently subjected to observation at 10x, 20x and 40x magnifications.

4.3.5 Statistical analysis

All experiments were conducted at least in triplicate, with mean values and standard errors determined for these experiments except for rheology measurements. The means of all the parameters were examined for significance by analysis of variance (ANOVA) at a confidence level of 95% (Duncan's multiple range tests were used to calculate the significance of differences between means), using the Data Analysis ToolPak Add-in of Microsoft Office Excel, 365 Version 2204, USA.

4.4 Results and discussion

4.4.1 Standardization and optimization of core, wall materials and processing conditions for stable O/W Pickering emulsions

An emulsion is a complex two-phase dispersion composed of two immiscible liquid components, with one phase being dispersed as small droplets or globules within the other phase. Emulsions are inherently thermodynamically unstable systems characterized by high energy levels, primarily due to the increased interfacial area between the dispersed phase and the continuous medium. With progressing storage time, emulsions tend to undergo phase separation due to various destabilization phenomena such as flocculation, coalescence, creaming, sedimentation, and Ostwald ripening, among others. Emulsion stability is influenced by a multitude of factors, including interfacial tension, bulk and interfacial rheological properties, droplet size, and more. These factors are regulated by a combination of emulsion composition and the parameters of emulsification process. Thus, a judicious selection of above parameters are critical in the design and development of stable emulsions (Badrudozza, 2023). In the context of Pickering emulsions, their popularity has surged due to their exceptional physical stability. The various emulsification techniques utilized for the preparation of emulsions stabilized by

surfactants can also be applied to craft Pickering emulsions. Nevertheless, rotor-stator homogenization, high-pressure homogenization, and sonication stand out as the most commonly employed methods for formulating Pickering emulsions, as noted by Albert et al. (2019).

In our study we have carried out various trials for optimizing the emulsion preparation conditions, some of which are shown in Table 4.1.

Table 4.1 Emulsion trials carried out to optimize emulsion preparation concentration, homogenization time, and speed.

Trial no.	Oil concentration (w/v)	Starch concentration (w/v)	Homogenization speed	Time	Observations
1	40%	6.25%	12500 rpm	20 min	Curdling and phase separation of emulsion (observed immediately) (Oil layer separates on top)
2	40%	10%	12500 rpm	20 min	Phase separation, curdling (observed immediately)
3	25%	10%	12500 rpm	10 min	Phase separation (observed immediately)
4	10%	10%	12500 rpm	20 min	Phase separation (observed immediately)
5	25%	2.5%	12500 rpm	30 min	Phase separation (observed immediately)
6	40%	2.5%	12500 rpm	20 min	Phase separation (after 3 days)
7	10%	2.5%	12500 rpm	15 min	Phase separation (after 3 days)
8	10%	1%	12500 rpm	15 min	Phase separation (observed immediately)
9	25%	2.5%	15000 rpm	20 min	Curdling and phase separation (after 2 day)

10	25%	2.5%	15000 rpm	15 min	Phase separation (After 4th day)
11	20%	2.5%	15000 rpm	20 min	Slight phase separation (After 7th day)
12	10%	2.5%	15000 rpm	10 min	Phase separation (After 3rd day)
13	10%	2.5%	15000 rpm	15 min	Thin layer of oil separation at the top (After 3rd day)
14	10%	2%	15000 rpm	20 min	Very thin layer of oil at the top (After 4th day)
15	**10%**	**1.5%**	**15000 rpm**	**15 min**	**Stable emulsion (Till 7 days)**
16	10%	1%	15000 rpm	15 min	Slight phase separation (After 7 days)

From the various trials we have confirmed that starch concentration near to the value of 1.5% w/v gives stable emulsions. This was in line with the CMC that we calculated for micronized pearl millet starch in our previous chapter. Therefore, we considered the following three emulsions based on our analysis and prepared them by varying the oil content as explained below:

- **40% Oil + 2% Starch; 20000 rpm for 15 min (40E)**
- **20% Oil + 2% Starch; 20000 rpm for 7 min (20E)**
- **20% Oil + 2% Starch + Ultrasonication for 10 min; 20000 rpm for 7 min (20EU)**

These emulsions were subjected to further analysis.

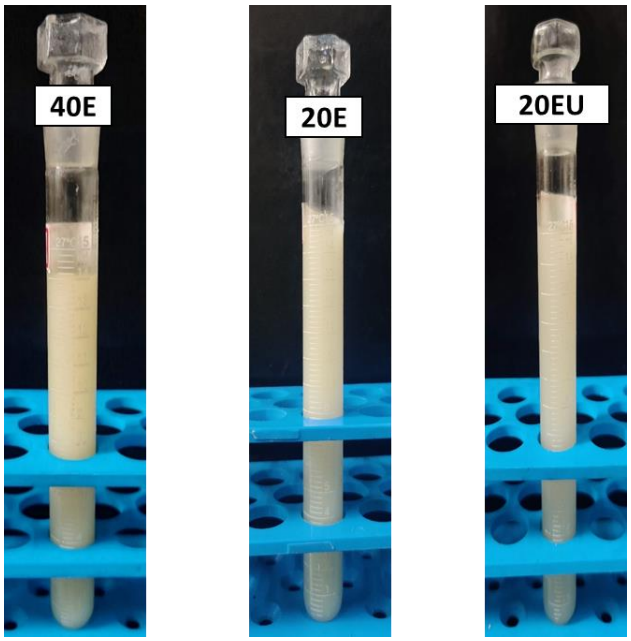


Fig 4.1 Pickering emulsions prepared from micronized pearl millet starch

4.4.2 Storage and characterization studies

4.4.3 Droplet size, polydispersity index and zeta potential of the prepared emulsions

The droplet size of the 3 emulsions ranged from 1.2 to 7.6 µm. Emulsions prepared with 20% w/v oil had the smallest and more gradual increase in droplet size upon storage. It ranged from 1.2 to 5.6 µm (Fig 4.2 a).

Upon the formation of an emulsion, it becomes imperative to ensure its sustained stability throughout its designated shelf-life. Emulsions are susceptible to deterioration due to various mechanisms of destabilization, encompassing phenomena such as creaming, flocculation, and coalescence (McClements, 2015). The characteristics of the emulsifying agent enveloping the droplets can exert significant influence on these instability mechanisms (Piorkowiski, 2014).

Firstly, the size of the droplets generated during the homogenization process plays a pivotal role in determining the propensity for creaming to transpire, as smaller droplets exhibit reduced mobility, thereby mitigating gravitational separation. Consequently, emulsifiers that yield smaller droplets during the homogenization step contribute to enhanced stability against gravitational separation. Secondly, the emulsifier layer profoundly impacts the balance between attractive and repulsive interactions among the droplets. Emulsifiers that engender robust repulsive interactions tend to be more efficacious in thwarting droplet aggregation.

The polydispersity index (PDI) serves as a crucial parameter characterizing the degree of variation or dispersion within a particle size distribution. In practical terms, the PDI value exists within the range of 0 to 1, where colloidal particles exhibiting PDIs less

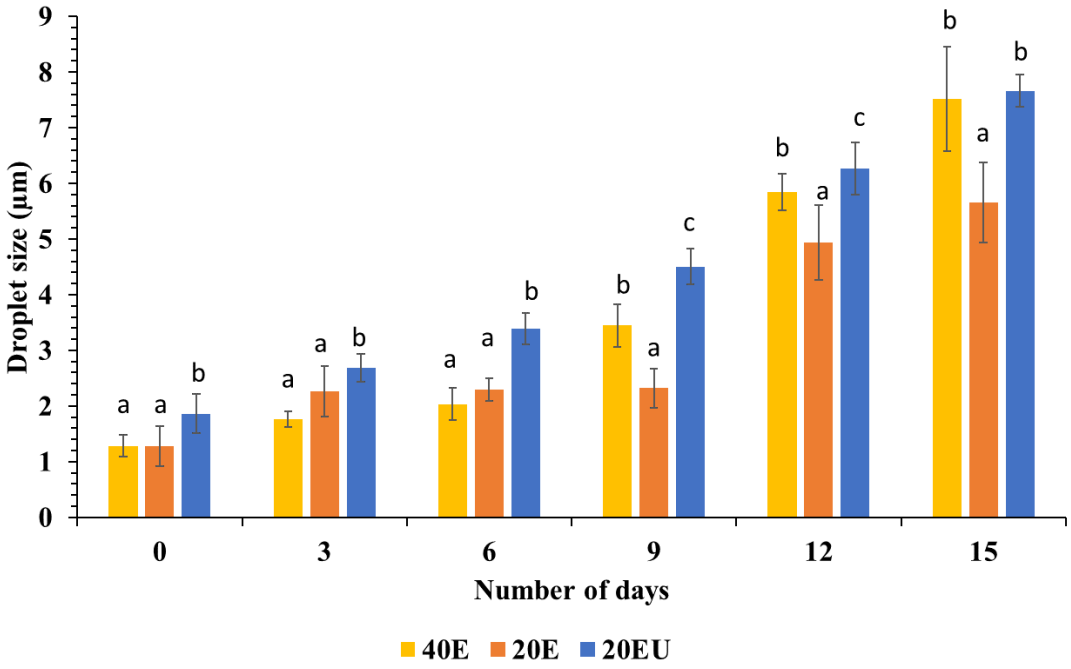
than 0.1 are indicative of a monodisperse nature, while values exceeding 0.1 suggest a polydisperse distribution of particle sizes, as elucidated by Ravel et al. in 2019. The determined PDIs for the emulsions in our study spanned from 0.143 to 0.613 (Fig 4.2 b). It is noteworthy that the PDI values exhibited an increasing trend during the storage period in all three emulsions. However, it is essential to emphasize that these PDI values consistently remained below 1, signifying that the emulsion droplets retained a high degree of homogeneity throughout the storage duration.

Zeta potential represents a crucial parameter closely tied to the stability of emulsions and is influenced by the electrical charge present at the interface. The zeta potential values recorded for the emulsions in this study ranged from -34 to -45 for 40E, -41 to -48 for 20E, and -43 to -40 for 20EU (Fig 4.2 c). A noticeable decline in zeta potential values was observed after the 6^{th} day of storage, aligning with the concurrent increase in droplet size, and this phenomenon is indicative of emulsion instability, as documented by Du et al. in 2020.

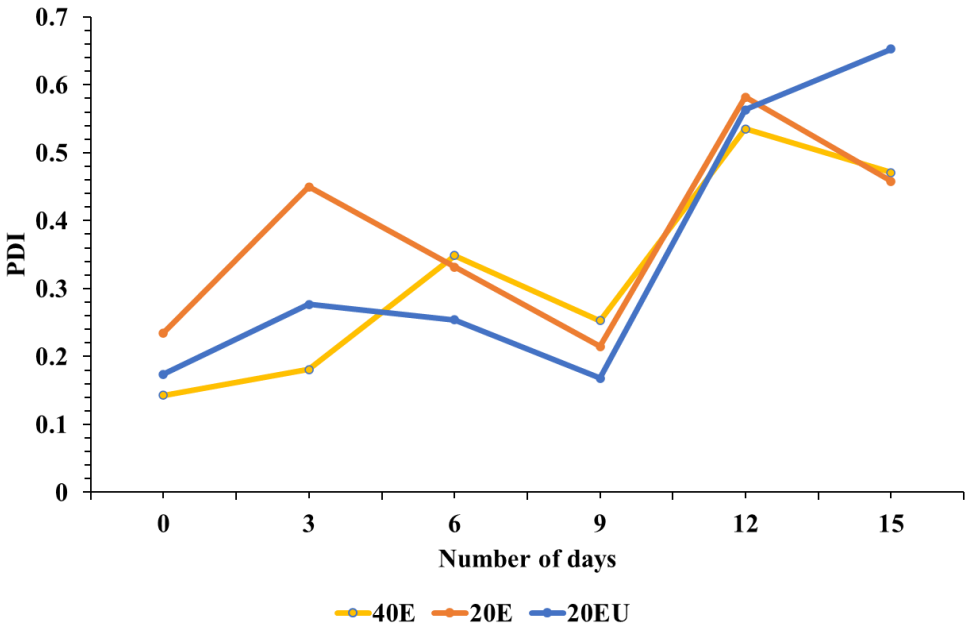
It is worth noting that particles possessing a high value for zeta potential, typically exceeding ±30 mV, exhibits the capacity to effectively stabilize emulsions formed using these particle dispersions, as indicated by Tavernier et al. in 2017. The negative zeta potential values signify the presence of a negative charge on the surface of the starch particles, a characteristic highlighted by Wei et al. in 2014. A heightened zeta potential serves to diminish Van der Waals forces, owing to the electrostatic repulsion between particles, an assertion substantiated by Ahmad et al. (2020). Van der Waals forces are attractive forces responsible for particle agglomeration, ultimately leading to the formation of larger particles, as explained by Shafer et al. in 2010. Consequently, the greater the absolute value of the zeta potential, the more stable the suspension or dispersion tends to be. Additionally, findings from Dai et al. in 2018

support the notion that a higher zeta potential contributes to enhanced stability and a reduced tendency for particle agglomeration.

a



b



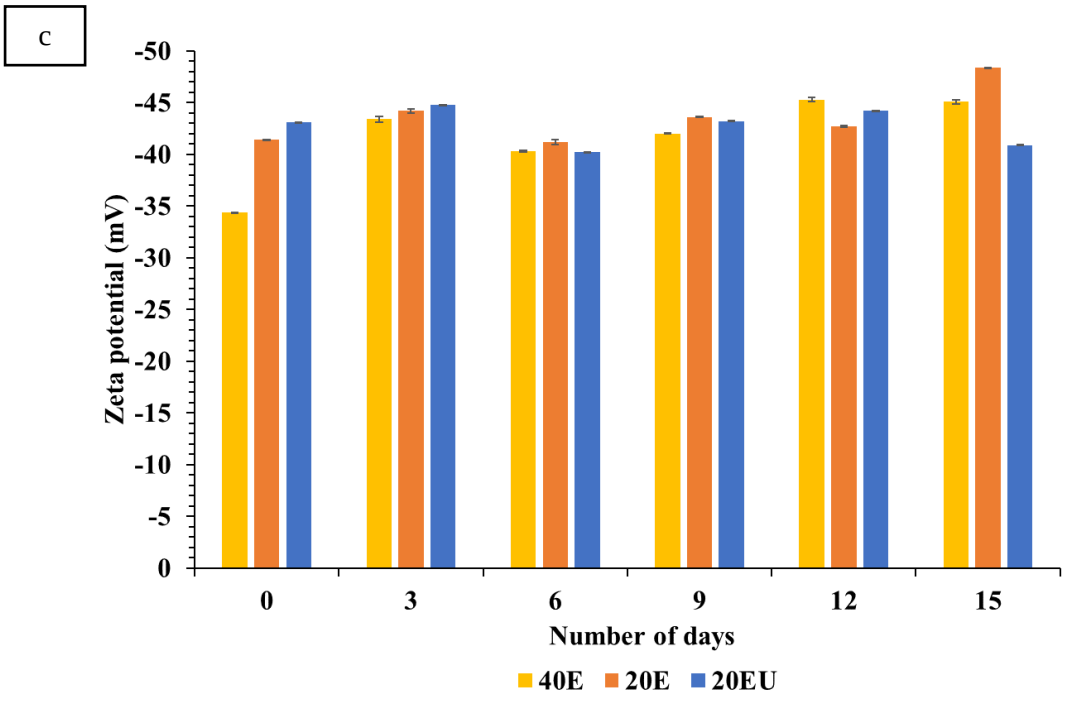


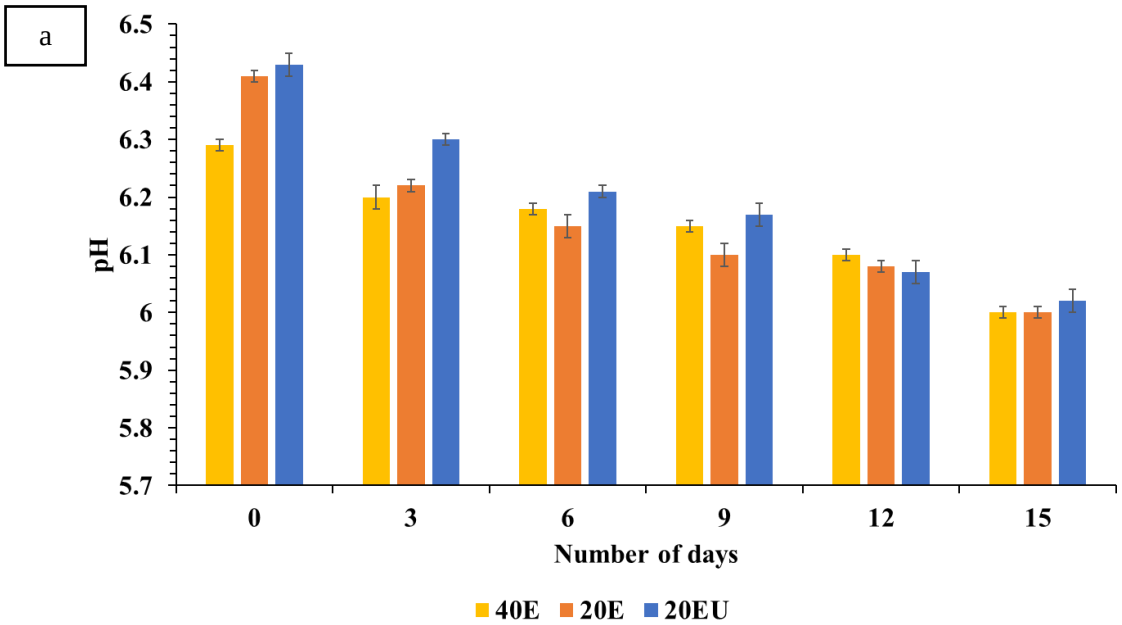
Fig 4.2 (a) Droplet size (b) Polydispersity index (c) Zeta potential of the prepared emulsions. (Values are means ± SD (n = 3), followed by different superscript letter are significantly different ($p \leq 0.05$). The letter "a" represents the least value)

4.4.4 pH and bulk density measurements

pH represents a crucial parameter impacting emulsion stability, as variations in pH can signal potential chemical alterations within the formulation components. In this investigation, the pH levels of the emulsions were promptly determined post-homogenization. The initial pH measurements for the freshly prepared emulsions were as follows: 6.29±0.01 (40E), 6.41±0.01 (20E), and 6.43±0.02 (20EU) (Fig. 4.3 a). Remarkably, after a 15-day storage period, there were no discernible alterations in the pH values of the emulsions, suggesting that the emulsion constituents remained substantially unaltered throughout the observational duration. Typically, food emulsions exhibit pH values ranging from 2.5 to 7.5 (McClements, 2016).

The quantity of surfactant employed stands as a critical parameter significantly influencing both the stability and pH characteristics of the emulsion system (Daaou et al., 2012). The stabilization of lipid droplets predominantly relies on steric repulsion, attributable to their possession of substantial hydrophilic groups that extend into the aqueous phase (Dickinson, 2003). Consequently, emulsions stabilized by polysaccharides tend to manifest notable stability, even when exposed to variations in pH (as illustrated in Figure 4.2a), fluctuations in ionic strength, or alterations in temperature (Ozturk, 2016).

Bulk density exhibits an inversely proportional relationship with volume when the mass remains constant, and thus, we anticipate a comparable correlation between bulk density and droplet diameter. In the case of the fresh emulsions, the bulk densities were measured as 0.96±0.02 g/mL, 0.97±0.02 g/mL and 0.95±0.02 g/mL, respectively for 40EU, 30EU and 20EU (Fig 4.3 b). Notably, there were no statistically significant changes observed in the bulk density of the prepared emulsions ($p>0.05$). This observation underscores the stability of these emulsions.



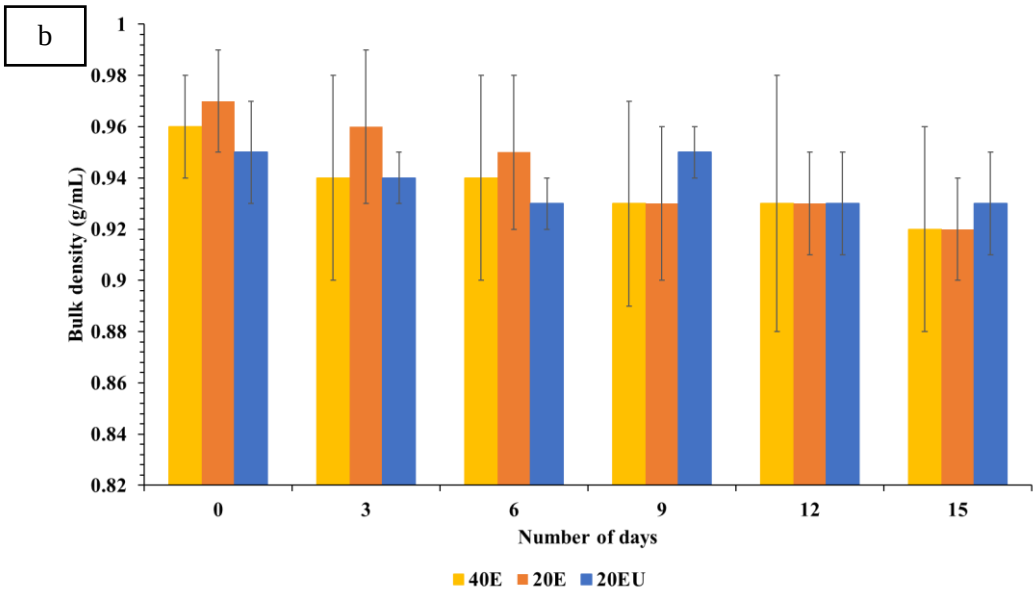
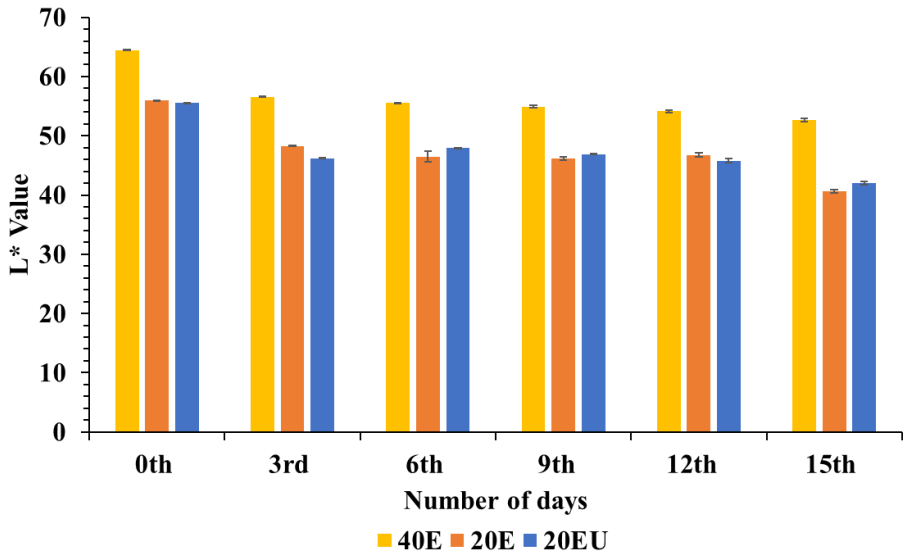


Fig. 4.3 (a) pH and (b) bulk density (g/mL) of the prepared emulsions

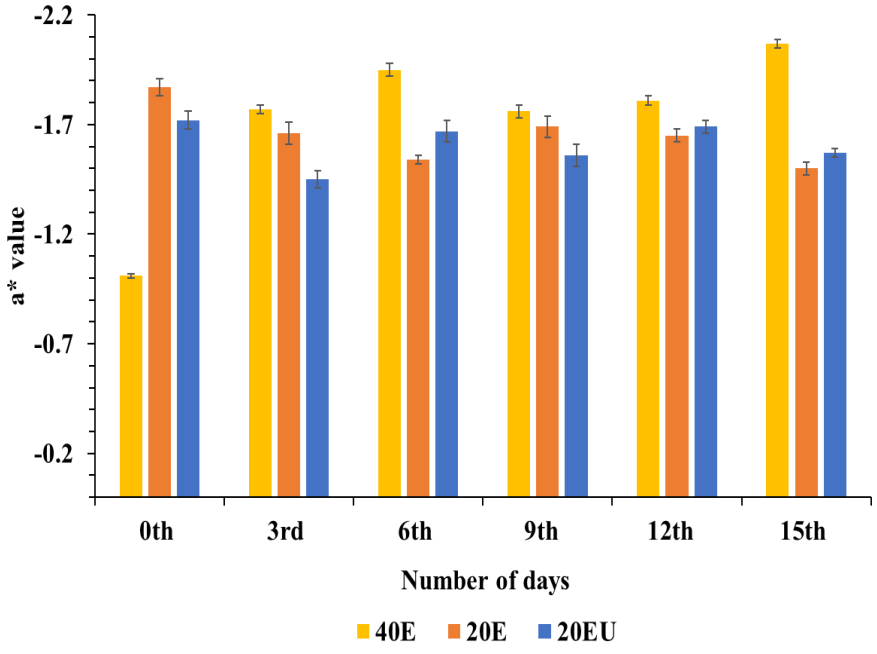
4.4.5 Color analysis

From the L*a*b* values it was clear that the emulsions had a slight greenish-yellow color and the lightness value was highest for the emulsion with 40% oil concentration. The values of color parameters (L*, a* and b*) are expressed as total color change (ΔE). ΔE of the three emulsions in the study are 15.91±0.02 (40E), 16.85±0.03 (20E) and 14.00±0.01 (20EU). There was a significant change in the color of 20E emulsions when compared to the other two (Fig. 4.4). Upon storage, there are chances for emulsion instability due to Ostwald ripening as the droplet size increased upon storage for 15 days. These results confirm that the optical properties of emulsions depend on droplet size. Any physicochemical process that alters the droplet size should change their appearance. The optical properties of emulsions were influenced by Ostwald ripening because the transport of oil molecules from smaller to larger droplets caused a gradual increase in the mean droplet size. Thus, Ostwald ripening not only reduces the stability of the emulsion but also alters the color and appearance of the product (Weiss and McClements, 2001).

a


70
60
50
40
30
20
10
0
L* Value
0th
3rd
6th
9th
12th
15th
Number of days
40E
20E
20EU

b


-2.2
-1.7
-1.2
-0.7
-0.2
a* value
0th
3rd
6th
9th
12th
15th
Number of days
40E
20E
20EU

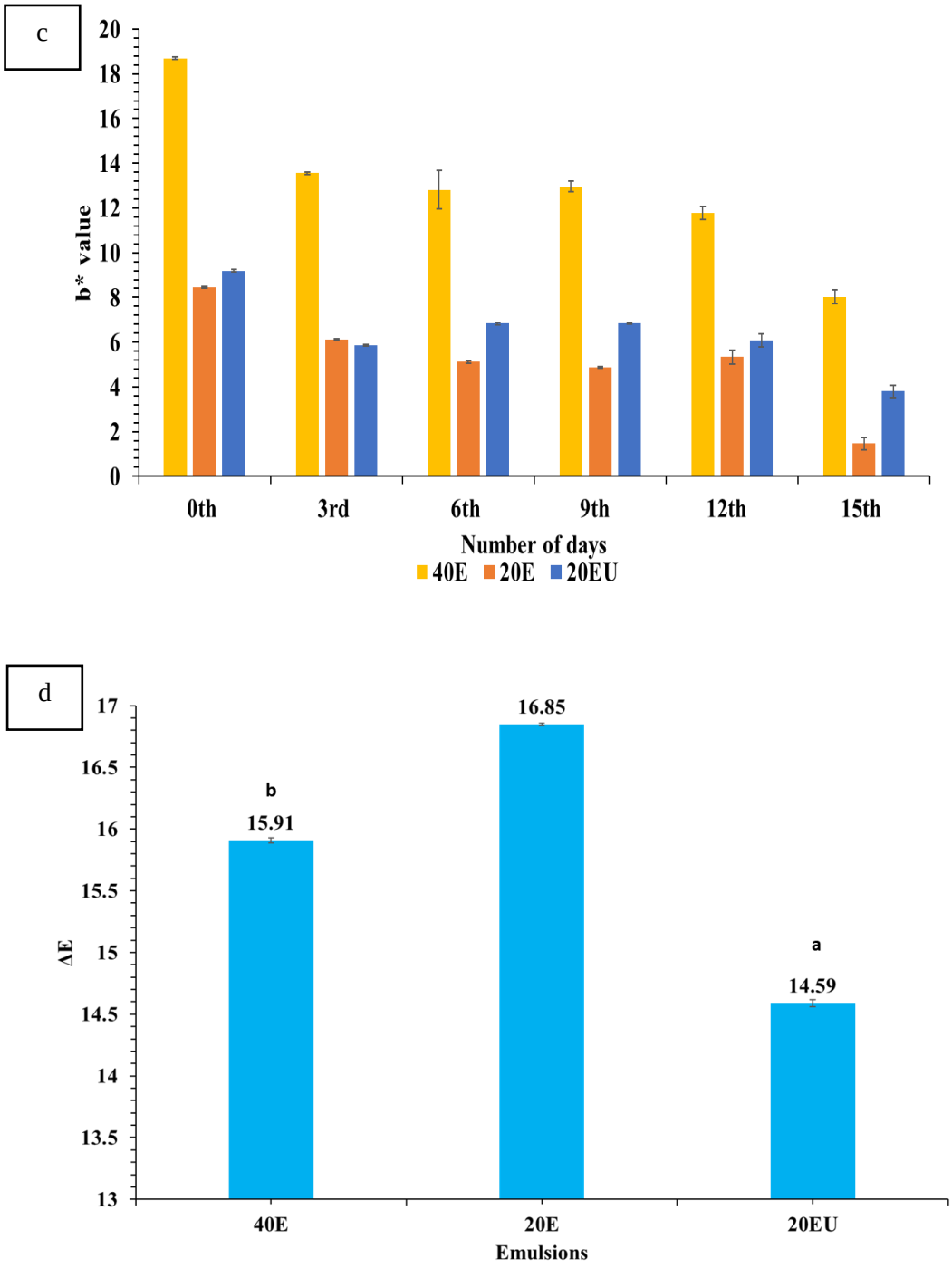


Fig 4.4 Color analysis of the prepared emulsions (a) L* value (b) a* value (c) b* value (d) color change of the samples (ΔE)

4.4.6 Rheology – flow behavior analysis of the emulsions

The flow behavior of all the prepared emulsions is presented in Figure 4.5, 4.6, and 4.7. It was observed that all emulsions exhibited a non-Newtonian flow pattern

characterized by shear-thinning property. In shear-thinning behavior, the viscosity of the fluid decreases as the shear rate increases. This shear-thinning property is associated with a decline in resistance to flow with escalating shear rates, primarily attributed to the disruption of intra- and intermolecular bonding within the starch matrix.

To quantify this behavior, we employed the power law model, a commonly utilized rheological model. This model offers a quantitative measure, where a value closer to zero indicates more pronounced shear-thinning characteristics. It serves to describe materials exhibiting power law behavior, which manifests as a proportional stress response. In technical terms, a power law index, denoted as 'n,' greater than 1 signifies shear-thickening behavior, 'n' less than 1 indicates shear-thinning behavior, and 'n' equal to 1 signifies Newtonian, viscous behavior (Chabbra, 2010). Consequently, we fitted our data into the Power law model to ascertain the flow characteristics of the emulsions (see Table 4.2, 4.3, and 4.4).

Subsequently, we calculated the apparent viscosity of the emulsions over time. A gradual decline in apparent viscosity was observed until the 9^{th} day of storage, followed by an increase. This behavior can be attributed to droplet coalescence. The reduction in viscosity with increased storage time is associated with phase separation or the detachment of starch particles. The extent of coalescence inversely correlates with the viscosity of the continuous phase. Coalescence occurs when the viscous binding component is expelled from the film that separates the droplets, resulting in the dissipation of energy. If the energy loss due to this dissipation surpasses the initial kinetic energy, the droplets coalesce to form larger ones. The inverse relationship between emulsion viscosity and droplet coalescence suggests that increased viscosity may hinder the expulsion of the film between two droplets, thereby delaying

coalescence (Nowak et al., 2016). Towards the end of the storage period, the increase in viscosity may signify a transition in the emulsion instability mechanism, shifting from droplet coalescence to phase separation between water and oil.

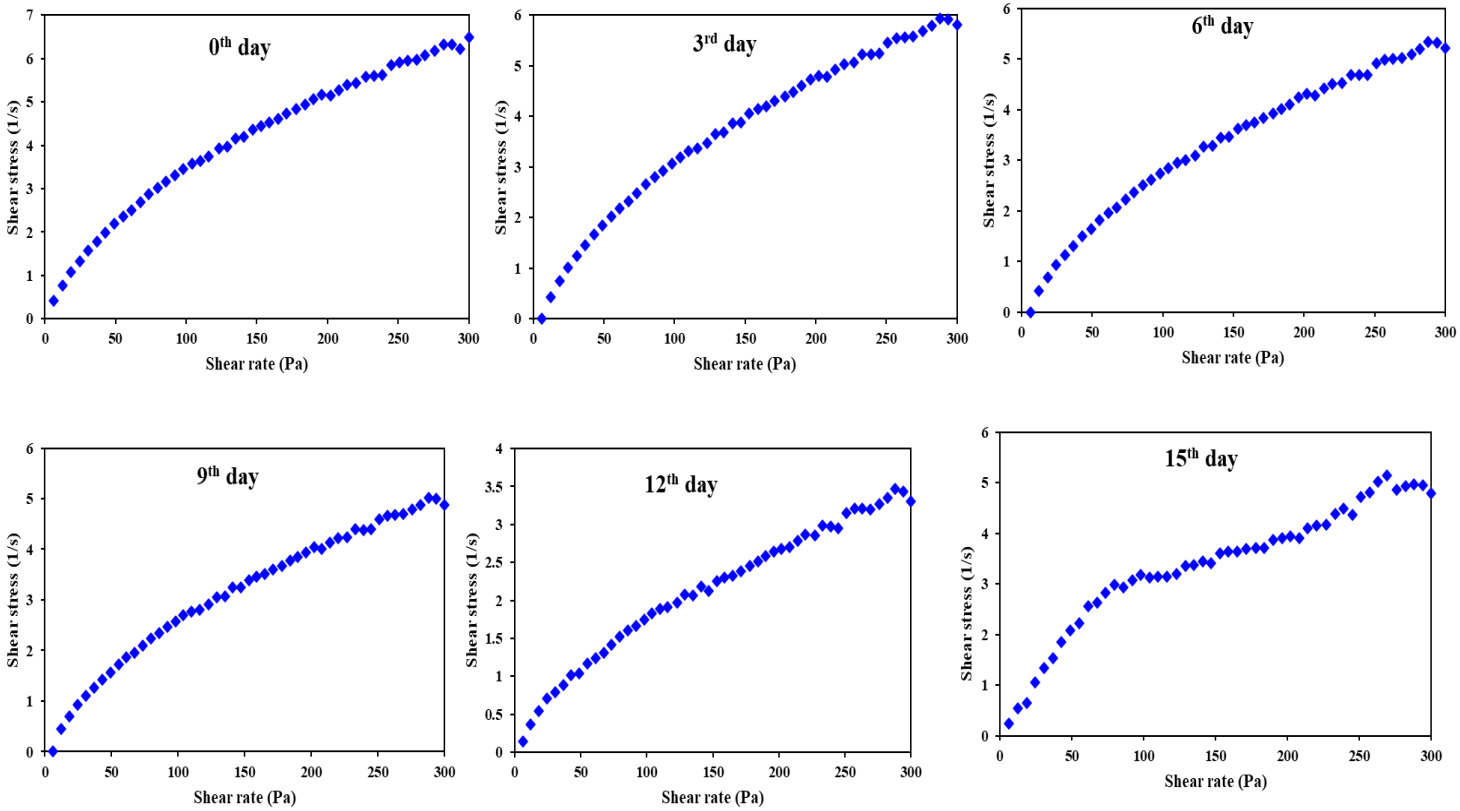


Fig. 4.5 Flow behavior of 40E emulsions

Table 4.2 Apparent viscosity of 40E emulsions

Day	Equation for power trendline	R^2 value of power trendline	Apparent viscosity @ 50 s^{-1}
0th	$y = 0.0641x^{0.84}$	0.977	0.044
3rd	$y = 0.0073x^{1.25}$	0.928	0.038
6th	$y = 0.0113x^{1.14}$	0.946	0.026
9th	$y = 0.0247x^{0.97}$	0.968	0.001
12th	$y = 0.0623x^{0.71}$	0.994	0.015
15th	$y = 0.1261x^{0.66}$	0.951	0.040

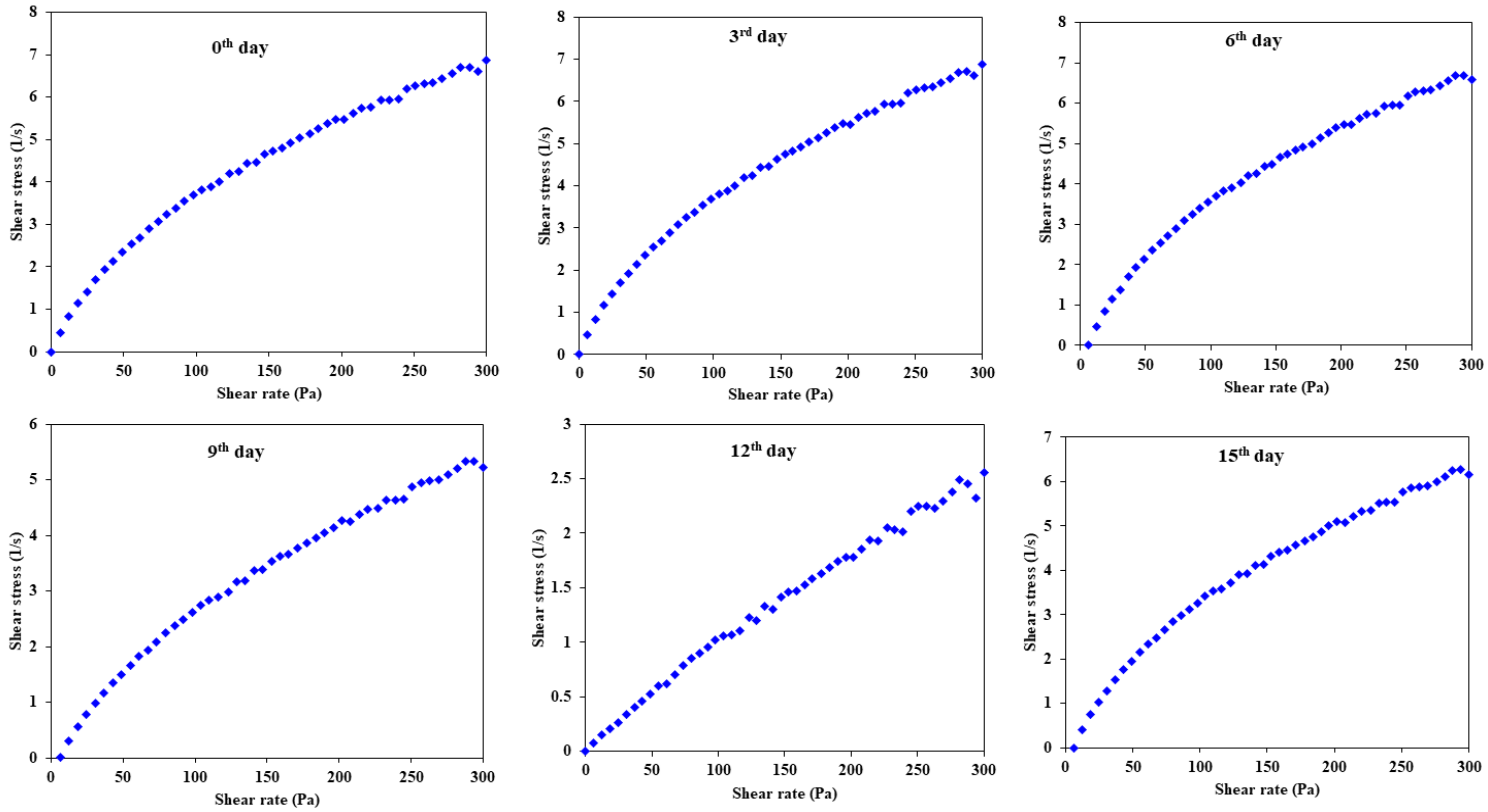


Fig. 4.6 Flow behavior of 20E emulsions

Table 4.3 Apparent viscosity of 20E emulsions

Day	Equation for power trendline	R^2 value of power trendline	Apparent viscosity @ 50 s^{-1}
0^{th}	$y = 0.027x^{1.03}$	0.950	0.041
3^{rd}	$y = 0.0187x^{1.11}$	0.939	0.040
6^{th}	$y = 0.0049x^{1.35}$	0.903	0.033
9^{th}	$y = 0.0052x^{1.29}$	0.934	0.032
12^{th}	$y = 0.0093x^{1.00}$	0.992	0.010
15^{th}	$y = 0.0078x^{1.24}$	0.924	0.030

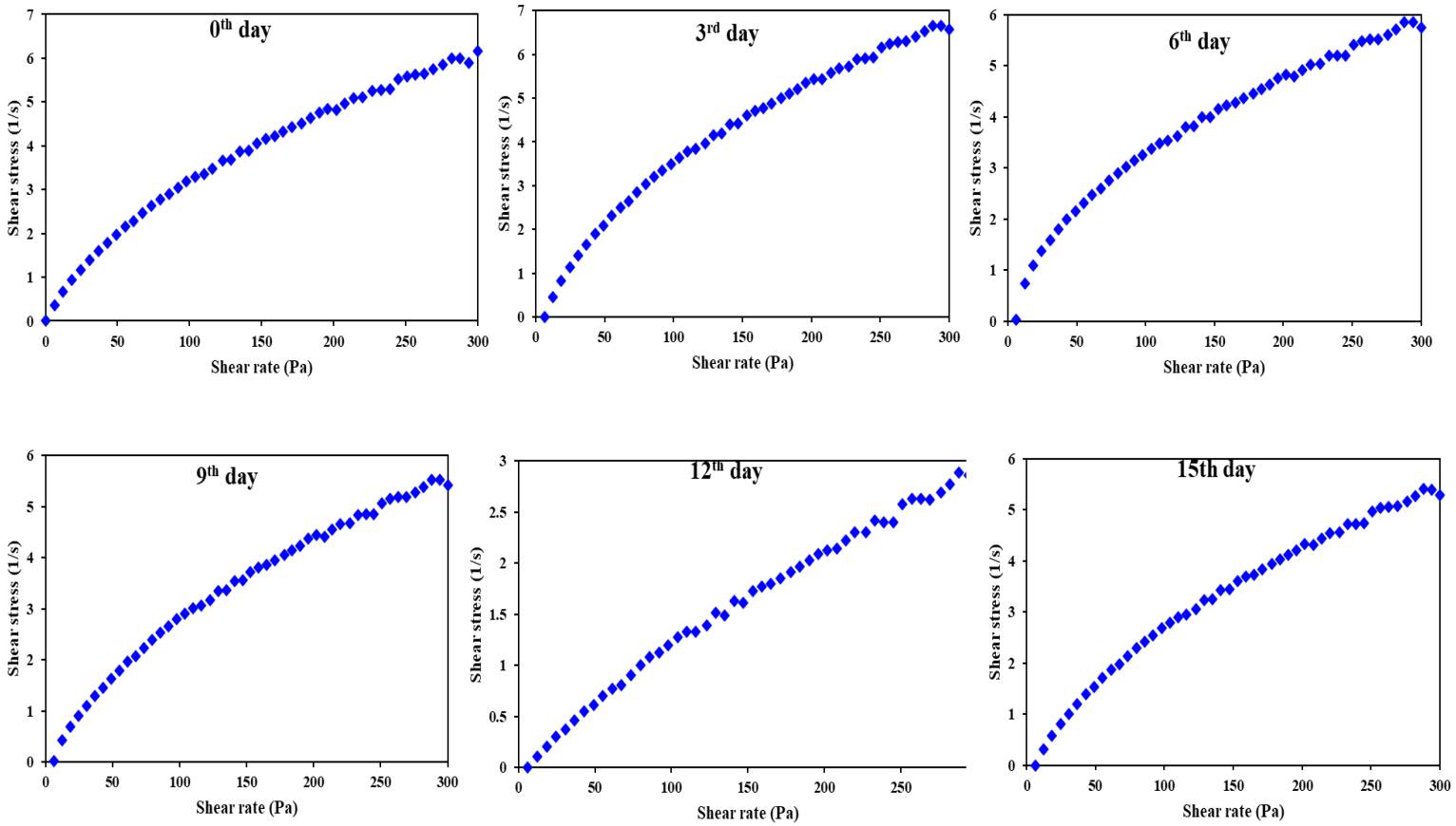


Fig. 4.7 Flow behavior of 20EU emulsions

Table 4.4 Apparent viscosity of 20EU emulsions

Day	Equation for power trendline	R^2 value of power trendline	Apparent viscosity @ 50 s-1
0^{th}	$y = 0.0662x^{0.82}$	0.983	0.033
3^{rd}	$y = 0.0143x^{1.14}$	0.938	0.030
6^{th}	$y = 0.0548x^{0.85}$	0.967	0.030
9^{th}	$y = 0.012x^{1.12}$	0.969	0.027
12^{th}	$y = 0.0046x^{1.17}$	0.977	0.013
15^{th}	$y = 0.0258x^{0.98}$	0.953	0.007

4.4.7 ATR-Fourier Transform Infrared Spectroscopy (FTIR)

The prepared emulsions were characterized by Fourier-transform infrared (FTIR) spectroscopy to elucidate potential chemical interactions. This was achieved by assessing the vibrational properties of various functional groups, as depicted in Figure 4.8. Specifically, certain key peaks were identified: at 704.19 cm^{-1} and 762.14 cm^{-1}, corresponding to aliphatic chloro compounds (C–Cl); 859.07 cm^-1, signifying peroxides (C–O–O– stretch); 928.89 cm^-1, representing aliphatic phosphates (P–O–C stretch); 1009.77 cm^-1, 1077.88 cm^-1, and 1149.10 cm^-1, indicative of aliphatic fluoro compounds (C–F); 1336.59 cm^-1, pointing to primary or secondary (OH in-plane bend group); 1638.92 cm^-1, relating to primary amines (NH bend); 2109.00 cm^-1, indicating cyanide ions, thiocyanate ions, and analogous ions; 2888.99 cm^-1, corresponding to methoxy (C–H stretch (CH3–O–); and 3276.54 cm^-1, representing ammonium ions, as documented by Punia et al. in 2021. The presence of water was unmistakably confirmed by absorption bands detected at 1647 cm^-1. Furthermore, the peak observed at 1341 cm^-1 could be attributed to the stretching vibration of the glycosidic bonds (1-4) within polysaccharides, in accordance with findings by Sandhu et al. in 2020. Regarding the components of roasted coffee oil, namely chlorogenic acid and caffeic acid, distinctive bands were identified at 3010 cm^-1 (>C=CH-) and 1710 cm^-1 (C=C stretching), as previously documented by Freiberger (2015). Notably, the FTIR analysis discerned no emergence of new peaks, affirming the effective encapsulation of roasted coffee oil within the starch particles.

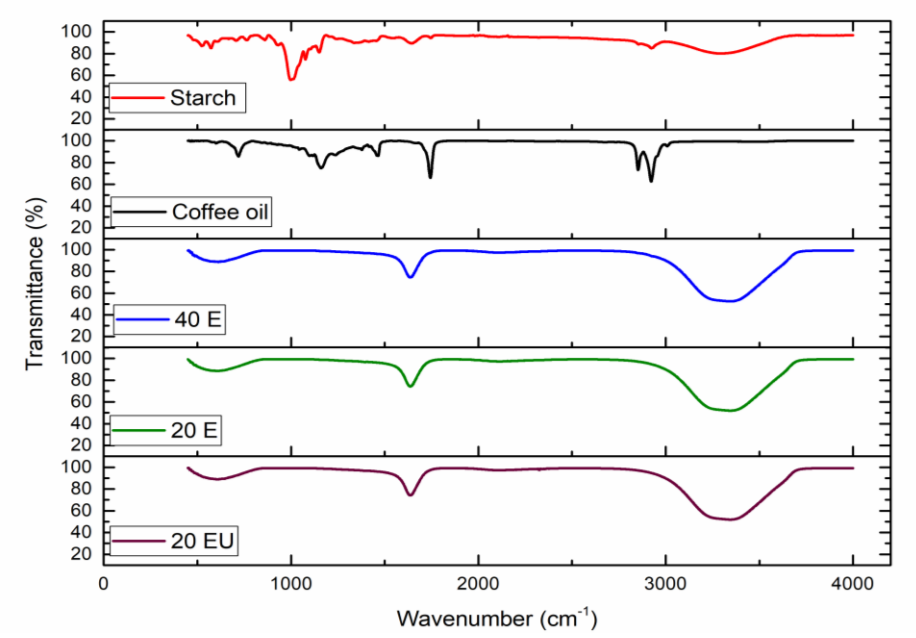
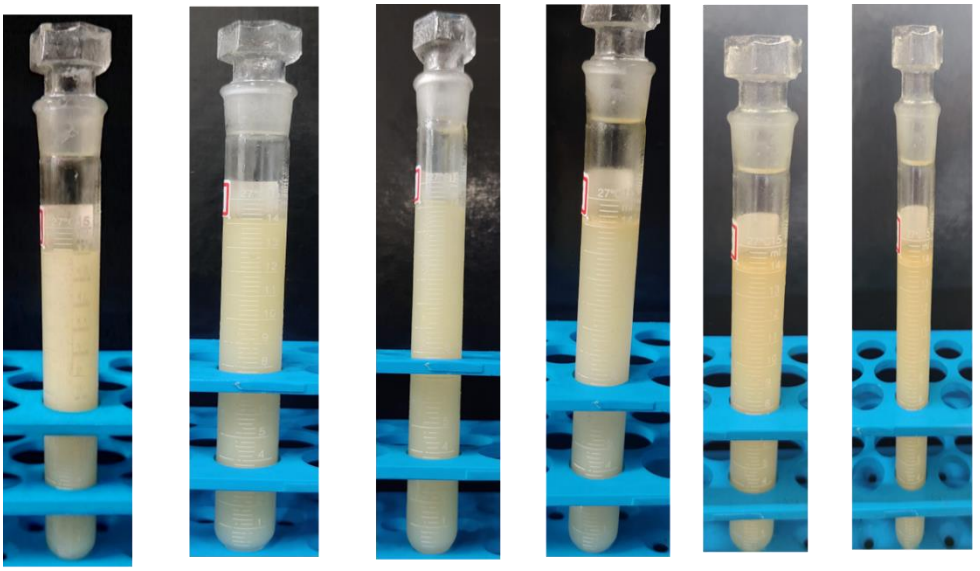


Fig 4.8 FTIR analysis of the prepared emulsions

4.4.8 Morphology observation of the prepared emulsions

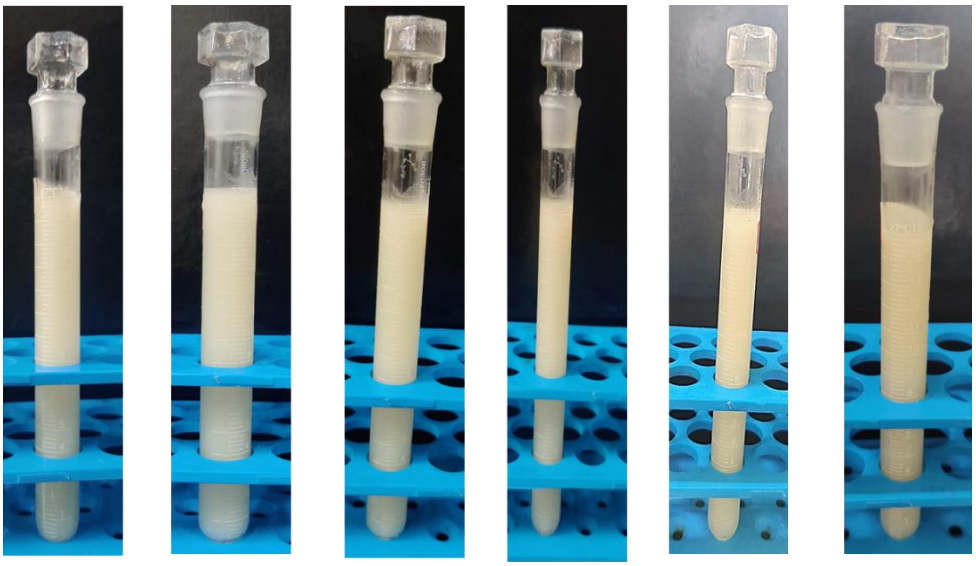
From the results of the storage study, it is evident that the prepared emulsions exhibit stability over a period of 15 days when stored under refrigerated conditions (4°C±1). Notably, an observable trend in the emulsions is the gradual loss in opacity as the storage duration progresses (from whitish to yellowish color due to the oil separation) (Fig 4.9). This color alteration corresponds to the changes observed in the emulsions following their storage.

40E



0th day | 3rd day | 6th day | 9th day | 12th day | 15th day

20E



0th day | 3rd day | 6th day | 9th day | 12th day | 15th day

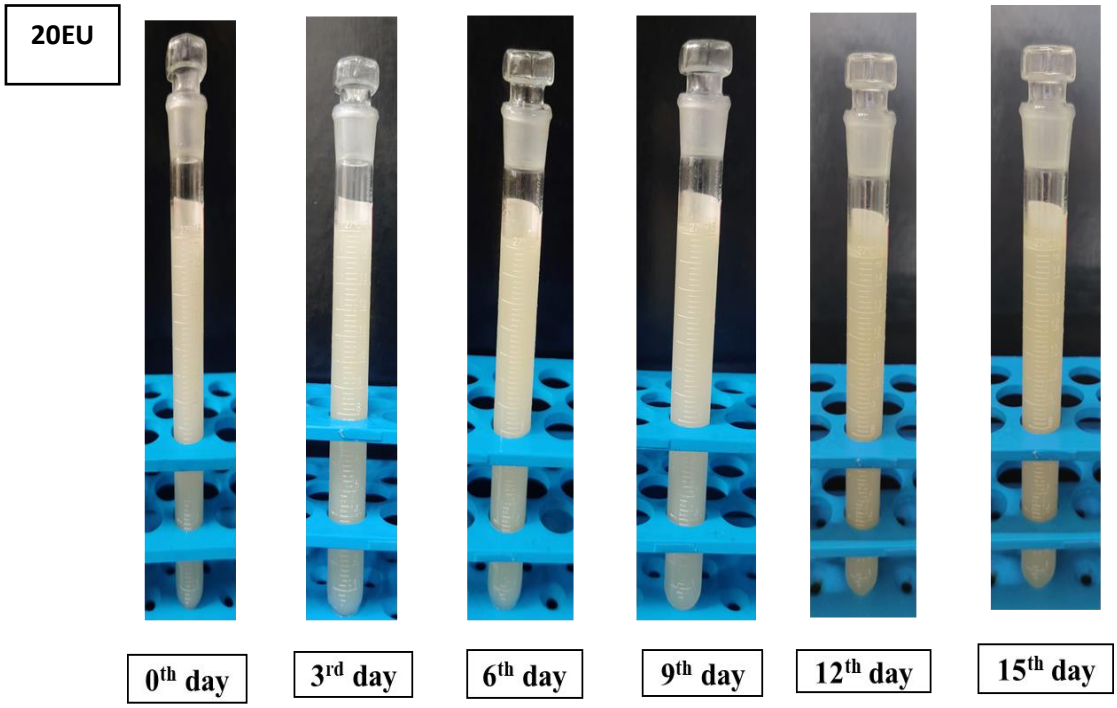


Fig 4.9 Storage study images of the prepared emulsions

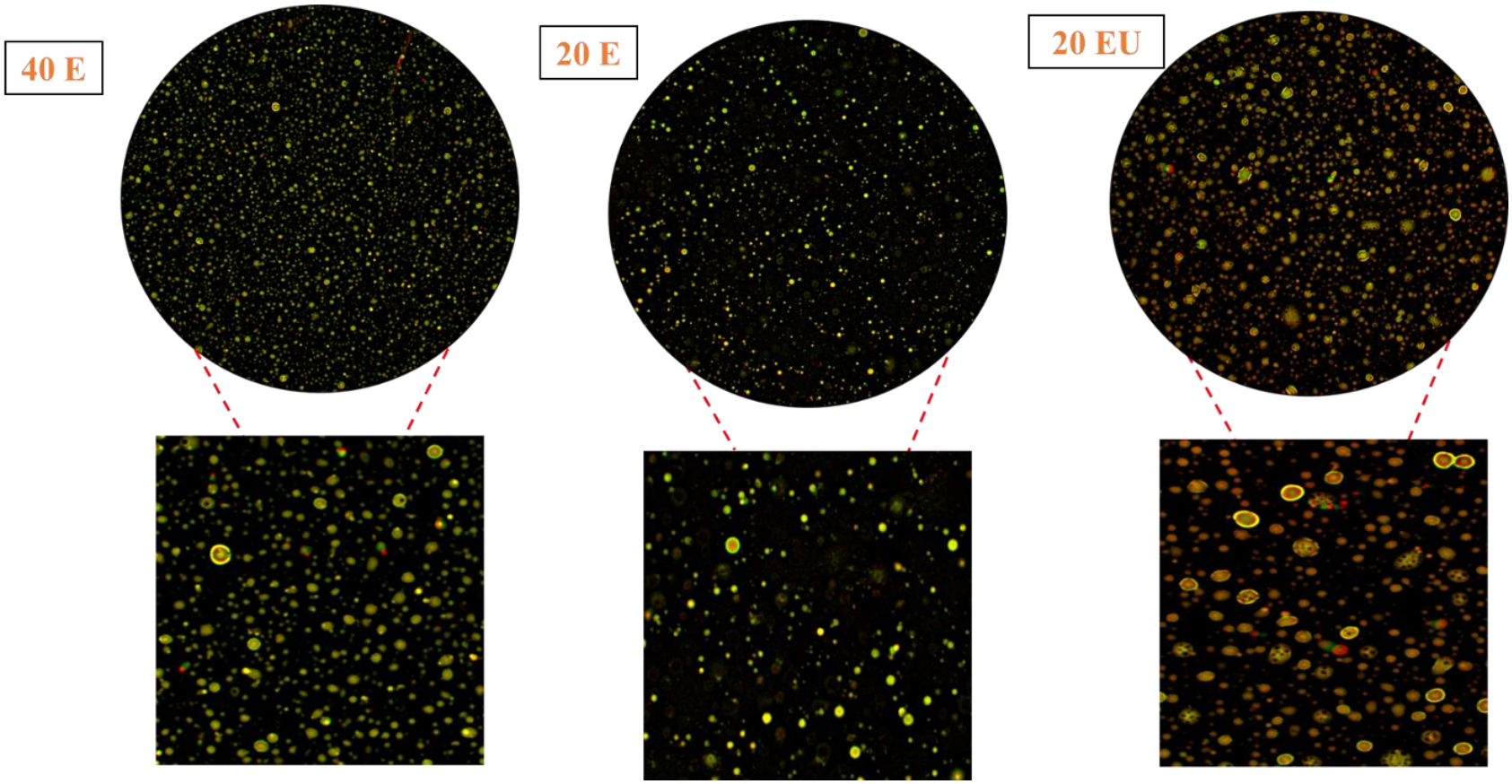


Fig. 4.10 Fluorescence microscopy image of fresh emulsion 40E, 20E and 20EU (Magnification: 40X).

For a comprehensive understanding of the distribution of oil droplets within the emulsions at different concentrations and to assess encapsulation efficiency, fluorescence microscopic imaging was employed. Superimposed images of emulsions stained with Nile red and safranin were constructed to illustrate the encapsulation efficiency (as shown in Fig. 4.10). These images provide a clear depiction of the encapsulation effectiveness of the prepared emulsions. Following imaging, the primary images were combined to create an image that elucidates the efficiency of emulsion entrapment. In this depiction, it is evident that the micronized pearl millet starch particles (in green) have completely encapsulated the oil (indicated by the red stain).

4.5 Conclusion

The utilization of micronized millet starch particles has demonstrated to be successful in the fabrication of oil-in-water (O/W) Pickering emulsions. To achieve stability, these emulsions were prepared by adjusting the starch concentration to values near the critical micelle concentration. Three formulations were employed: one comprising 2% micronized pearl millet starch and 40% coffee oil, and the other comprising 20% coffee oil with an additional 20% subjected to ultrasonication. Remarkably, these emulsions exhibited robust stability when stored under refrigerated conditions (4°C) for a duration of 15 days. Notably, the micronized pearl millet starch granules entirely enveloped the coffee oil within the fabricated Pickering emulsions. Moreover, Fourier-transform infrared (FTIR) analysis verified the absence of chemical alterations subsequent to the formulation of the Pickering emulsions. Consequently, the prepared Pickering emulsions, encapsulating coffee oil, hold promise for

utilization as flavor emulsions, with potential applications in food products, particularly in the preparation of flavored beverages.

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Chapter 5

Development of new product formulation (plant-based beverages) from sprouted finger millet, its sensory and shelf-life studies

5.1 Introduction

With the escalating awareness among consumers regarding the pivotal role of nutrition in maintaining good health, food products have transcended their conventional function of mere satiety providers. They are now being acknowledged as potent tools for the prevention and management of diseases. This evolving demand for food items with specific health-enhancing attributes has paved the way for a burgeoning market of functional foods, including a wide spectrum of processed beverages endowed with distinct health benefits (Ghoshal and Kansal, 2019; Nazir et al., 2019). While a universally accepted definition of functional foods and beverages remains elusive, there is a broad consensus that these products must, at the very least, be rich sources of bioactive compounds (Martirosyan and Singh, 2015; Nazir et al., 2019). Cereal milk, like its plant-based counterparts, constitutes a colloidal system characterized by the presence of sizeable particles encompassing starch granules, proteins, and fibres. If these particles attain excessive dimensions, it can lead to an undesirable sensory experience, characterized by a gritty, coarse, or chalky mouthfeel. Furthermore, during storage, the larger particles within the milk may succumb to gravitational forces, resulting in undesirable phenomena such as precipitation, sedimentation, or delamination, which can severely compromise product quality (Sethi, Tyagi, and Anurag, 2016).

Various manufacturing strategies have been devised to mitigate these challenges, with homogenization emerging as the most prevalent approach (Xiong et al., 2022). Homogenization serves to diminish particle size and ensure uniform dispersion of particles, thereby curbing sedimentation and enhancing beverage stability. Furthermore, the surging demand for plant-based milk alternatives has engendered novel opportunities for incorporating cereals into this domain. This trend augurs well

for the utilization of cereals as alternatives to traditional cow's milk. Nonetheless, the nutritional and technological challenges must aforementioned must be effectively addressed before cereal grain-based milk can establish itself as a viable substitute for cow's milk. In light of these considerations, millets emerge as a valuable resource for the development of dairy alternatives. Millets, characterized by their high protein content, lower starch content, and mild flavor profile, fill a critical void within the spectrum of plant-based milk sources. Their extensive cultivation, coupled with their ability to thrive under minimal maintenance conditions, positions millets as an ideal crop for utilization in dairy replacement through value-added processing. In India alone, millets are cultivated across 29 million hectares annually (Prakasha et al., 2018). Furthermore, millets represent an underutilized crop that warrants diversification through value-added applications, with millet milk being one such product.

Millet milk enjoys preference primarily due to its nutritional superiority when compared to alternative sources of plant-based milks. It boasts a high protein content while maintaining a low-calorie profile (Raajeswari and Nithya, 2016). This attribute renders it a desirable substitute for dairy, particularly in the present context where there is a growing inclination towards high-nutrition, low-calorie dietary choices. Notably, finger millet (Eleusine coracana), with its substantial calcium (348 ± 3.5 mg/100 g), zinc (36.6 ± 3.7 mg/100 g), and iron (4.27 ± 0.6 mg/100 g) content, presents significant potential for mineral enrichment in foods. It has the capacity to meet up to 50%, 300%, and 25% of the ICMR RDA for these minerals, respectively (Anitha and Sellamuthu, 2021; Kumar et al., 2020). This study introduces an innovative environmentally friendly method for creating emulsions suitable for food-grade applications, facilitating the delivery of active food ingredients.

The beverage matrix primarily comprises water, and thus, the inclusion of water-insoluble health-related compounds and flavor oils poses a significant challenge for researchers, necessitating an emulsification step. Furthermore, these functional compounds may be susceptible to degradation subsequent to their addition to food matrices, as they are subjected to various physicochemical stresses throughout food processing and storage. These functional compounds are particularly sensitive to environmental variations encompassing factors such as pH, temperature, and the presence of minerals, rendering their shelf-life contingent upon processing and storage conditions. Consequently, health-related compounds are susceptible to deteriorating properties due to oxidative reactions and other related processes. Similarly, flavor compounds undergo chemical transformations including oxidation, hydrolysis, and thermal degradation, potentially leading to the development of off-flavors. Hence, it becomes imperative to devise innovative strategies aimed at facilitating the dispersion of water-insoluble functional ingredients within aqueous food systems, while concurrently safeguarding them from instability mechanisms and degradation processes (Molet-Rodríguez et al., 2018). Beverage emulsions represent a solution in the form of concentrated emulsified systems tailored to effectively encapsulate and protect these water-insoluble ingredients, thereby enabling their seamless incorporation into beverages and drinks. Therefore, our chapter focussed on the incorporation of micronized pearl millet starch Pickering emulsions encapsulating coffee oil to sprouted finger millet for the preparation of coffee flavoured non-dairy beverage.

5.2 Objective

To develop coffee flavoured non-dairy beverage using sprouted finger millet milk and coffee oil encapsulated micronized pearl millet starch particles. The hypothesis of the chapter is as follows (Fig. 5.1 a):

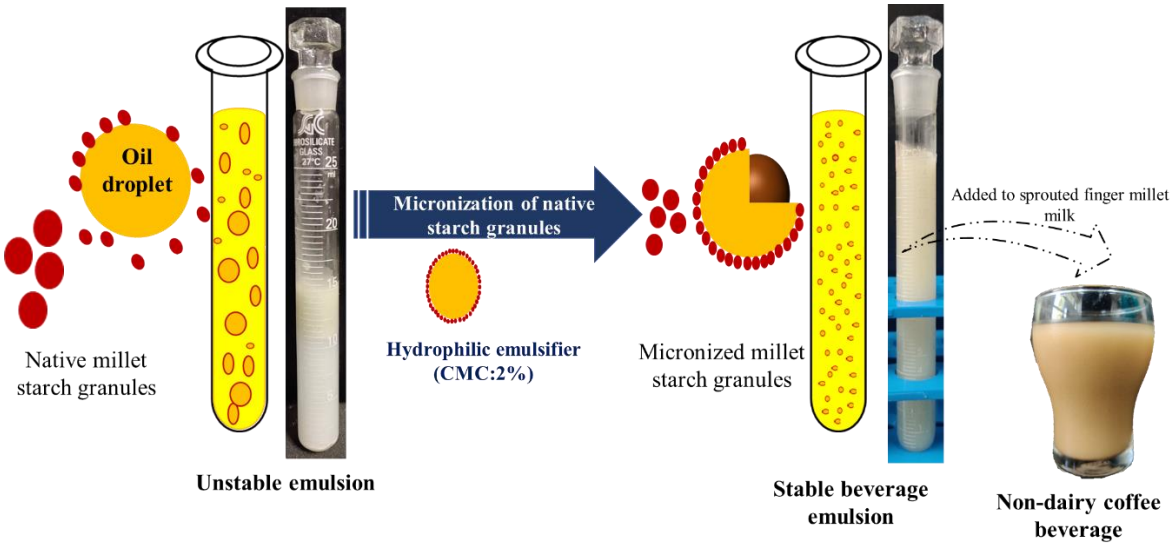

Fig 5.1a Hypothesis of the chapter

5.3 Materials and methods

5.3.1 Materials

Finger millet and pearl millet were received from Indian Council of Agriculture Research – Indian Institute of Millet Research, Hyderabad, India. Roasted coffee oil (Arabica) was purchased from Proderna Biotech Pvt Ltd., Zamrudpur, New Delhi, India. Gellan gum (food grade) was purchased from Urbanplatter, Maharashtra, India. Maltodextrin was purchased from Hi-Media (Mumbai, India). Sugar was purchased from the local market in Thiruvananthapuram, Kerala.

5.3.2 Experimental design

The outline of the study is depicted in the figure below:

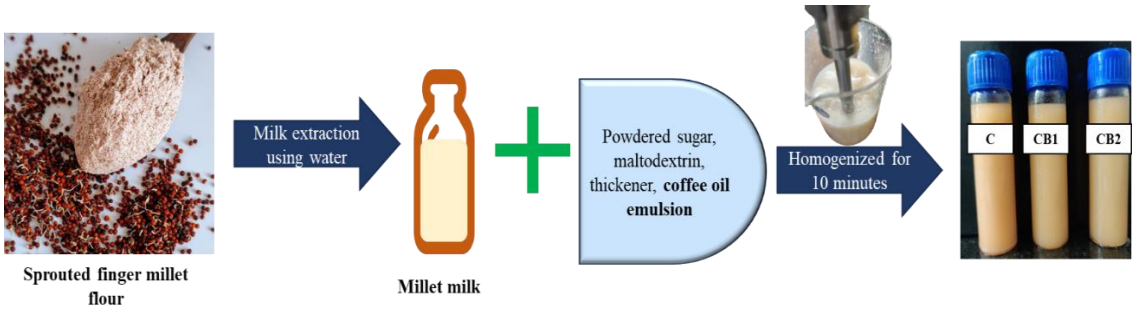

Fig 5.1b Outline of chapter 5

5.3.3 Preparation and optimization of plant-based dairy alternative from sprouted finger millet milk and coffee oil encapsulated pearl millet starch Pickering emulsion

Finger millet grains underwent a 24-h sprouting process aimed at enhancing their nutritional profile, as elucidated in our second chapter. The resulting sprouted finger millets were subsequently subjected to dehydration and pulverization using an electric mixer (MG 218; Zodiac Preethi, Chennai, India) to produce finger millet flour. This flour was then combined with water at a 1:2 ratio to initiate milk extraction, a process conducted twice to yield finger millet milk. The resulting milk was filtered using a muslin cloth.

The development of a plant-based (non-dairy) coffee beverage derived from sprouted finger millet milk involved extensive product development investigations. These investigations involved the incorporation of coffee oil encapsulated micronized pearl millet starch Pickering emulsions in various concentrations ranging from 5% to 30% v/v into the sprouted finger millet milk. Then the beverage was homogenized using a homogenizer (Omni International Tissue Master 125, United States) at 20000 rpm for 10 minutes. After numerous trials, the optimal

proportions of Pickering emulsions added to the sprouted finger millet milk were determined to be 15% emulsion (w/v), designated as CB-1, and 25% emulsion (w/v), designated as CB-2. The control sample that was used included only sprouted finger millet milk without the Pickering emulsion and was designated as C. The composition for the preparation of the three finalised beverages is presented in table 5.2.

5.3.4 Brix of the prepared plant-based beverages

The Brix (°) measurements of all three samples were assessed using an automated digital refractometer (Atago, RX-5000i, Japan). The instrument was calibrated with distilled water as the reference. Subsequently, the samples were carefully placed onto the dry prism surface for analysis.

5.3.5 Shelf life and characterisation studies of the prepared plant-based beverages

The prepared beverages were stored under refrigerated conditions (4 °C) and its shelf-life stability was analysed.

5.3.5.1 Bulk density

Bulk density, alternatively referred to as apparent density, is precisely defined as the quotient of the entire mass and the comprehensive volume of the material, encompassing both air and water content. Bulk density (g/mL) was determined by adding 5 mL of the beverage sample into an empty graduated 10 mL measuring cylinder. The volume of bulk density was calculated as the volume occupied by the mass of the sample added to the cylinder (Qiu et al., 2015).

5.3.5.2 Cloudiness

The absorbance at 600 nm on Shimadzu ultraviolet-visible 2600 (UV) spectrophotometer (Kyoto, Japan) at 32±1 °C using the method of Cao et al., (2012) with some slight modifications. The absorbance measured represented the cloudiness of the sample.

5.3.5.3 Color analysis

The color attributes, denoted as L*, a*, and b*, for the three emulsions were assessed utilizing a HunterLab Colorflex EZ spectrophotometer (Hunter Associates Laboratory Inc., Reston, USA). The spectrophotometer underwent calibration employing a standard white tile and a light trap to ensure accurate measurements. Subsequently, the samples were gently homogenized, and 5 mL of each sample were deposited into the cuvette, which was then positioned on a reflectance port. The L* value signifies the degree of lightness or darkness within a range from 0 (black) to 100 (white). The a* value reflects the intensity of redness (positive a*) or greenness (negative a*), while the b* value indicates the presence of yellowness (positive b*) or blueness (negative b*). The total color variation (ΔE) between the unprocessed and processed samples was computed using the following equation:

$$\Delta E = [(L_{\circ}^{*} - L^{*})^2 + (a_{\circ}^{*} - a^{*})^2 + (b_{\circ}^{*} - b^{*})^2]^{1/2}$$

Where,

$L_{\circ}^{*}$, $a_{\circ}^{*}$, and $b_{\circ}^{*}$ are the color values of the standard reference type;

L*, a* and b* are the color values of the test sample.

5.3.5.4 pH and total plate count

The pH of the beverage samples was measured using a pH meter, Oakton pH 700 (Benchtop meter Oakton Instruments, USA). Before commencing the pH measurements, the pH meter underwent calibration using a buffer solution with a pH value of 7.0 for accuracy.

Microbiological analysis for the control and both beverage samples was conducted on the first day of processing and subsequently repeated at seven-day intervals over a span of 28 days. The assessments encompassed the determination of the total plate count following ISO 4833:2003 guidelines, as well as yeast and mold count in accordance with ISO 6611:2004 protocols.

5.3.5.5 Sensory analysis of the prepared plant-based beverages

A hedonic scale test (9-point scale) was performed to determine the acceptability and any significant differences in the sensory attributes between the control and the test beverage samples. Care was taken to avoid interference from other sources. The samples were presented to twenty semi-trained panellists familiar with the techniques of sensory analysis. They were asked to score the product for its appearance/color, aroma/flavor, taste, mouthfeel, and overall acceptability with a scale representing quality grade description given in table 5.1.

Table 5.1 Hedonic scale grade description

Preference	Grade
Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6

Neither like nor dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike very much	2
Dislike extremely	1

5.4 Statistical analysis

All experiments were conducted at least in triplicate, with mean values and standard errors determined for these experiments. The means of all the parameters were examined for significance by analysis of variance (ANOVA) at a confidence level of 95%, using the Data Analysis ToolPak Add-in of Microsoft Office Excel, 365 Version 2204, USA. Duncan's multiple range tests were used to calculate the significance of differences between means.

5.5 Results and discussion

5.5.1 Preparation and optimization of plant-based dairy alternative from sprouted finger millet milk and coffee oil encapsulated pearl millet starch Pickering emulsions

Non-dairy beverages infused with coffee flavor were successfully formulated by utilizing sprouted finger millet milk in conjunction with the prepared micronized pearl millet starch Pickering emulsions. Finger millet, being a noteworthy source of essential nutrients including protein, calcium, zinc, and iron, also exhibits substantial vitamin content, polyphenolic compounds, amino acids, and robust antioxidant properties, as ascertained in our preceding Chapter 2 investigations.

Based on our prior analyses, it was determined that the protein content of finger millets decreased after 72 hours of germination. Consequently, a 24-hour germination period was selected for the preparation of these beverages. The resulting formulations contained approximately 7.7 grams of protein per 100 grams, a protein content akin to that of cow's milk, which contains 8 grams of protein per 100 grams. However, notably, the calcium content in the sprouted finger millet-based beverages was threefold greater than that observed in cow's milk. This underscores the potential of sprouted finger millet as a promising candidate for the development of plant-based milk products.

5.5.2 Brix

There was a significant increase ($p<0.05$) in the total soluble solids (°Brix) of the prepared beverage test samples when compared to the control. The control sample had a brix of 11.99% when compared to test samples; CB1-14.4%, and CB2-14.7%. hence our product is comparable to cow’s milk (12-13%) in terms of the total solids present in them. Germination results in the breakdown of starch to free sugars (Nirmala, Rao, & Muralikrishna, 2000) and hence increases the solubility of flour and content of total soluble solids, total solids, and total carbohydrates in the millet milk and finally in the beverage prepared. The higher the total soluble solids, the higher is the viscosity and consistency of the final product (Elechi et al., 2023).

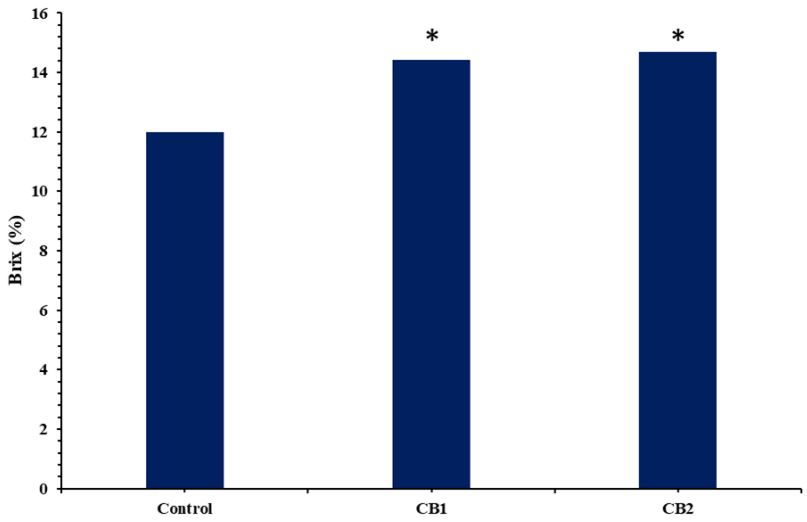

Fig 5.2 Brix (%) of the prepared beverages; CB1-15 % emulsion beverage, CB2-25% emulsion beverage. *$p \leq 0.05$ versus Control.

5.5.3 Shelf life and characterisation studies of the prepared plant-based beverages

The ingredient composition of the prepared control and test samples are presented in table 5.2. The prepared samples were characterized for different parameters such as bulk density, cloudiness, color, pH and total plate count as well as sensory analysis of the finalised products during a shelf-life period of 28 days.

Table 5.2 Ingredient composition of coffee flavoured plant-based beverages (100 ml)

Ingredients	**Control**	**CB-1**	**CB-2**
Sprouted finger millet milk	77.2 mL	62.2 mL	52.2 mL
Coffee oil encapsulated micronized pearl millet starch emulsion	Nil	15%	25%
Maltodextrin	7.5 g	7.5 g	7.5 g

Sugar	15 g	15 g	15 g
Gellan gum	0.15 g	0.15 g	0.15 g
Potassium sorbate	0.15 g	0.15 g	0.15 g

5.5.3.1 Bulk density

The bulk density measurements of both the control and test samples are presented graphically in Figure 5.3. Up to the 21st day of storage, no discernible differences in bulk density were observed between the control and test samples. However, on the 28th day, a significant discrepancy in the bulk density of CB2, when contrasted with the control sample, became evident. An increase in bulk density serves as an indicative marker of beverage instability, as heightened density levels can lead to the separation of the oil and water layers within the sample, thereby rendering it unstable. The composition of food matrices can be deconstructed into three discrete components: (i) the aqueous phase, (ii) the solid dry material, and (iii) the gaseous phase. During prolonged storage, changes in food volume may ensue as a result of shrinkage and/or collapse phenomena. It is plausible that the entirety of the displaced water volume may be supplanted by entrapped air, while the initial air content, denoted as the initial porosity, remains invariant. In such scenarios, neither shrinkage nor collapse phenomena manifest, thereby sustaining food volume at a constant equilibrium. Conversely, when shrinkage and/or collapse phenomena materialize, a discernible reduction in food volume is observed (Qiu et al., 2015).

In emulsions characterized by lower oil content, the droplets are relatively distant from one another, resulting in comparatively weaker inter-droplet interactions. Conversely, as the oil content increases, the droplets draw closer together, leading

to an augmented density of droplets. Consequently, the mean separation distance between the droplets decreases, thereby amplifying the dominance of London-van der Waals forces of attraction between the droplets. This intensified interaction leads to the packing of oil droplets and the escalation of inter-droplet collisions. As a consequence of this phenomenon, the flocculated droplets encapsulate a substantial portion of the continuous phase within themselves, which is subsequently released as the shear rate (or shear stress) is heightened. This release of the continuous phase engenders a reduction in the effective dispersed-phase concentration, resulting in a concurrent decrease in viscosity and the manifestation of a shear-thinning effect (Hebishy et al., 2017; Kundu et al., 2015).

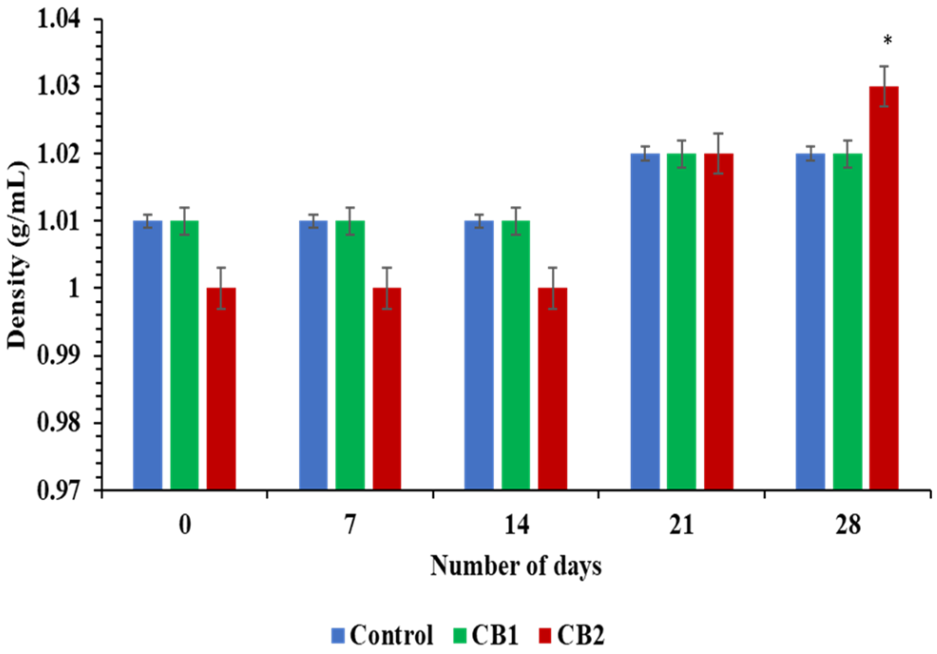

Fig 5.3 Bulk density of the beverage samples; CB1-15 % emulsion beverage, CB2-25% emulsion beverage. Data represented as mean ± SD, n = 3, *p ≤ 0.05 versus control.

5.5.3.2 Cloudiness

The assessment of cloudiness holds paramount significance within the realm of beverage research. In our prepared beverages, a notable cloudy appearance was evident. However, it is noteworthy that no significant disparities were observed in the cloudiness levels among the samples. Over the course of the storage study, a discernible reduction in cloudiness was documented. Initially, the beverages exhibited a cloudiness level in the range of 3.5, which subsequently diminished to less than 2.5 on the 28^{th} day of storage.

The manifestation of cloudiness arises from the suspension of particles, constituting a complex amalgamation of proteins, pectin, lipids, hemicellulose, cellulose, and various minor components. The diminishment in cloudiness can be attributed to several factors. Firstly, it can be attributed to a decrease in viscosity, concomitant with the diminishing stability (Cao et al., 2012). Additionally, alterations in average droplet size resulting from the aggregation of oil droplets, along with changes in the refractive index of both the oil and aqueous phases, can also be contributory factors influencing the observed variations in cloudiness among the samples (Mirhosseini et al., 2008).

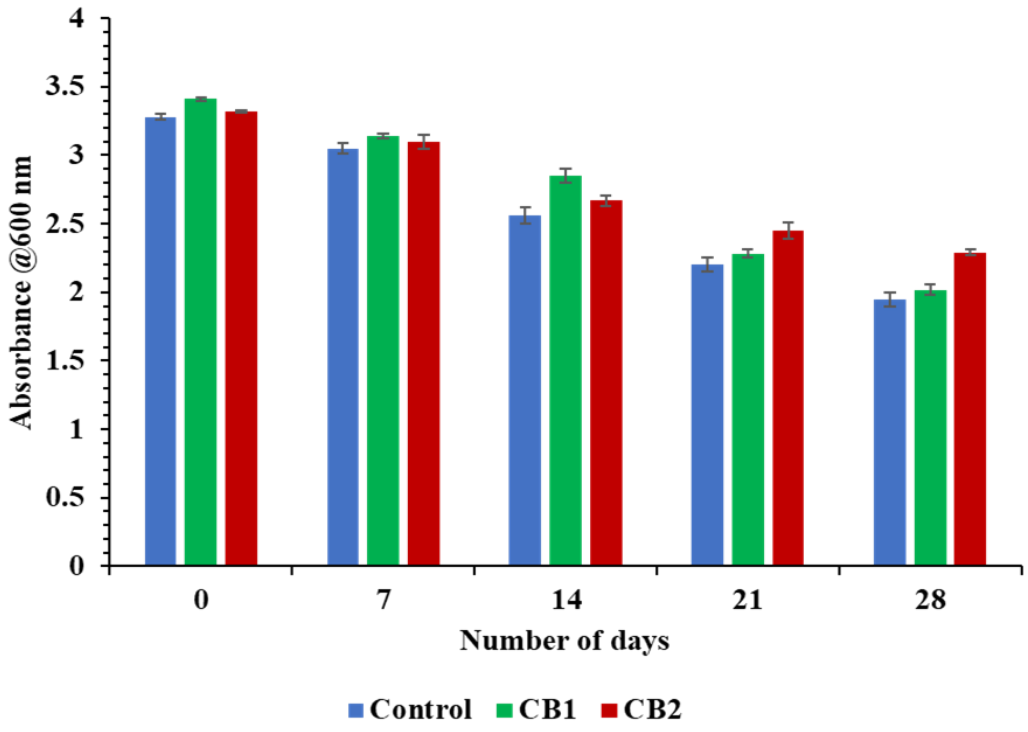

Fig 5.4 Cloudiness of the beverage samples; CB1-15 % emulsion beverage, CB2-25% emulsion beverage. Data represented as mean ± SD, n = 3.

5.5.3.3 Color analysis

It is imperative to underscore the critical significance of quantitative color assessment in the context of food quality evaluation. As per the guidelines provided by the American Meat Science Association (AMSA, 2012, pp. 1–136), visual assessments of food coloration bear direct relevance to consumer or sensory appraisals, thus establishing a foundation for comparative instrumental measurements, as undertaken in the present investigation.

In our study, a notable reduction in lightness was observed in the control samples when contrasted with the test samples. Interestingly, the incorporation of flavor emulsions into the test samples resulted in a discernible elevation in lightness values. Furthermore, a substantial alteration in the ΔE values was documented for

both the control (31.54±0.02) and test samples (CB1-6.53±0.02, CB2-4.83±0.01). This compellingly indicates that the introduction of coffee oil-flavored emulsions effectively preserved the color attributes of the test samples. Moreover, the a* and b* values collectively suggest that the beverages exhibited a yellowish-red coloration, a phenomenon corroborated by the visual representations from the storage study images of the beverages (Figure 5.5). These color variations primarily arise from disparities in the nature, concentrations, and sizes of colloidal particles present within the milk analogs, thereby yielding differential light scattering patterns. Additionally, the observed color distinctions can be attributed to variations in the types and concentrations of naturally occurring pigments, further contributing to divergent light absorption properties (Zheng et al., 2021).

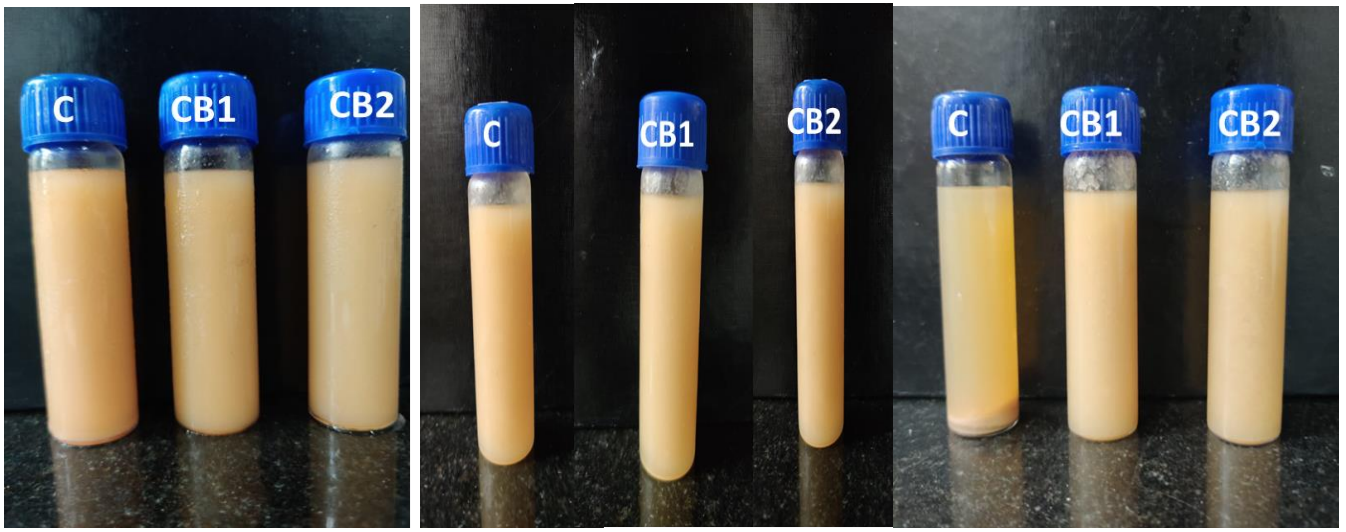

Fig 5.5 Storage study analysis of the beverage samples; CB1-15 % emulsion beverage, CB2-25% emulsion beverage.

Table 5.3 Color analysis of the beverage samples; CB1-15 % emulsion beverage, CB2-25% emulsion beverage, Data represented as mean ± SD, n = 3, p ≤ 0.05. (Letter 'a' represents least val

Days	**Control**			**CB1**			**CB2**		
	L*	a*	b*	L*	a*	b*	L*	a*	b*
0th	44.29±0.02	8.01±0.03	28.22±0.02	46.17±0.01	5.76±0.01	25.72±0.04	46.41±0.03	3.74±0.02	23.23±0.01
7th	43.45±0.02	7.78±0.05	27.55±0.03	47.25±0.02	5.70±0.02	25.64±0.03	46.65±0.02	3.75±0.04	23.20±0.04
14th	35.38±0.03	5.85±0.04	19.35±0.05	49.24±0.04	5.68±0.04	25.25±0.06	47.94±0.02	3.95±0.03	23.20±0.03
21st	27.74±0.03	4.75±0.03	10.82±0.04	50.5±0.03	5.26±0.03	24.97±0.03	48.95±0.02	4.25±0.05	23.17±0.02
28th	23.75±0.05	3.27±o.o2	4.75±0.03	52.55±0.05	5.17±0.02	24.43±0.02	50.85±0.06	4.61±0.03	22.20±0.05
ΔE		**31.54±0.02^{c}**			**6.53±0.02^{b}**			**4.63±0.01^{a}**	

5.5.3.4 pH and total plate count of the prepared plant-based beverages

The significance of monitoring variables such as pH cannot be overstated, given their pivotal role in influencing microbial proliferation within food matrices. In the present investigation, there was an absence of substantial pH fluctuations in the beverages up to the 21st day of storage, as depicted in Figure 5.6. During this period, the pH values remained within the range of 6.0. However, beyond this timeframe, a pronounced shift was observed as the pH values underwent a significant decrease, transitioning into a more acidic range at 5.7. In a study conducted by Makinen et al. (2016), the authors reported a pH value of 6.83 for a sample of cow's milk, which exhibited no significant difference when compared to the pH values observed in the formulated beverage. Also pH exerts a profound influence on the presence and distribution of microorganisms. Foods with a less acidic pH (pH > 4.5) are inherently more susceptible to the proliferation of pathogenic microorganisms, in addition to molds and yeasts (Jin & Kirk, 2018; Roberts & Greenwood, 2003).

In all three beverages, namely the Control, CB1, and CB2 formulations, discernable plate counts (as shown in Table 5.4) were initially observed on the 21st day of storage, with counts recorded at $3.45x10^5$, $3.60x10^5$, and $3.34x10^5$ CFU/mL, respectively. These counts exhibited a subsequent increase by the 28th day of storage. Conversely, yeast and mold counts for both the control and developed beverage samples remained undetectable. These findings can be attributed to the antimicrobial properties inherent to plant extracts. Secondary metabolites found in plant extracts have been well-documented to possess substantial inhibitory capabilities in response to microbial challenges. These secondary metabolites have exhibited antimicrobial activities against a broad spectrum of microorganisms, including bacteria, fungi, parasites, and viruses (El-Maati et al., 2016; Malu et al., 2009; Xie et al., 2015). The observed

microbial growth patterns align closely with the pH reduction observed in the beverage samples.

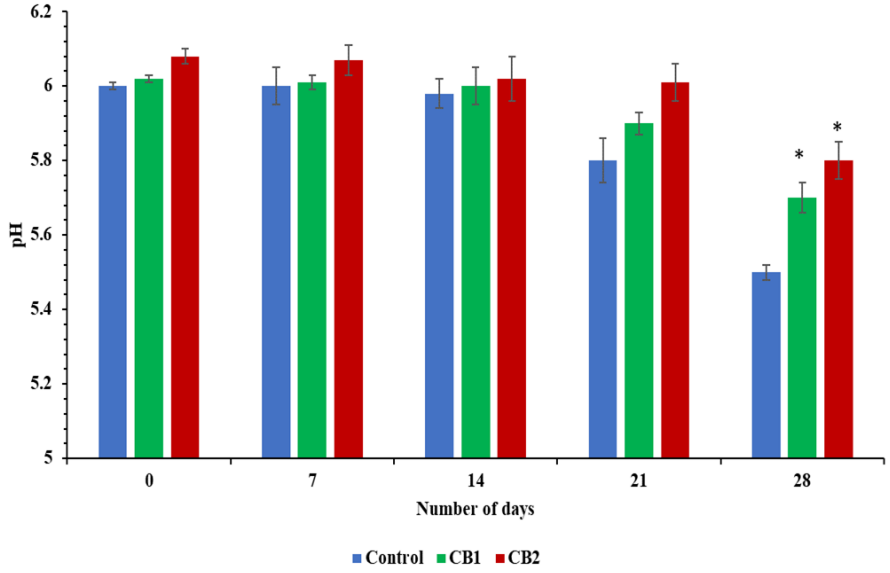

Fig 5.6 pH of the beverage samples; CB1-15 % emulsion beverage, CB2-25% emulsion beverage. Data represented as mean ± SD, n = 3, *p ≤ 0.05

Table 5.4 Total plate count for the beverage samples; CB1-15 % emulsion beverage, CB2-25% emulsion beverage.

Days	Control (CFU/mL)	CB1 (CFU/mL)	CB2 (CFU/mL)
0	Not detected	Not detected	Not detected
7	Not detected	Not detected	Not detected
14	Not detected	Not detected	Not detected
21	3.45×10^5	3.60×10^5	3.34×10^5
28	4.20×10^7	4.24×10^7	5.25×10^8

5.5.3.5 Sensory analysis of the prepared plant-based beverages

A hedonic assessment was conducted to ascertain the palatability and discern any noteworthy distinctions in sensory characteristics between the control and experimental beverage samples. Diligent measures were implemented to mitigate external interference. The samples were presented to a cohort of twenty panelists semi-trained panelists familiar with the techniques of sensory analysis.

Sensory evaluation constitutes a pivotal phase in elucidating consumer perceptions regarding the final product. Essential attributes, encompassing odour, color, texture, taste, and overall acceptance, were assessed for the plant-based milk samples within a rating scale ranging from 1 to 9. The beverage devoid of coffee oil encapsulated Pickering emulsion served as the control reference point. As depicted in Figure 5.7, discernible disparities manifested in the texture, taste, and overall evaluation of the milk samples. Notably, the inclusion of Pickering emulsions at a concentration of 15% induced a substantial increase in the texture score. This augmentation may be attributed to the enhanced consistency of the sample, which conferred a soft and pleasing taste profile (Rincon et al., 2020). Conversely, the sample enriched with 25% emulsions exhibited a diminished score in these attributes. This decline could be ascribed to the elevated viscosity of the beverage, resulting in an extended residence time within the oral cavity and subsequently imparting an unfavourable organoleptic impression. It is noteworthy that a majority of the panellists exhibited a preference for the beverage containing 15% emulsion over the 25% counterpart. With the elevation of emulsion concentration, a subtle aftertaste was observed in the beverage. Furthermore, it was discerned that the control sample possessed a notably thin consistency in comparison to the emulsion-enriched variants. The incorporation of emulsions

effectively contributed to enhancing both the consistency and palatability of the beverages.

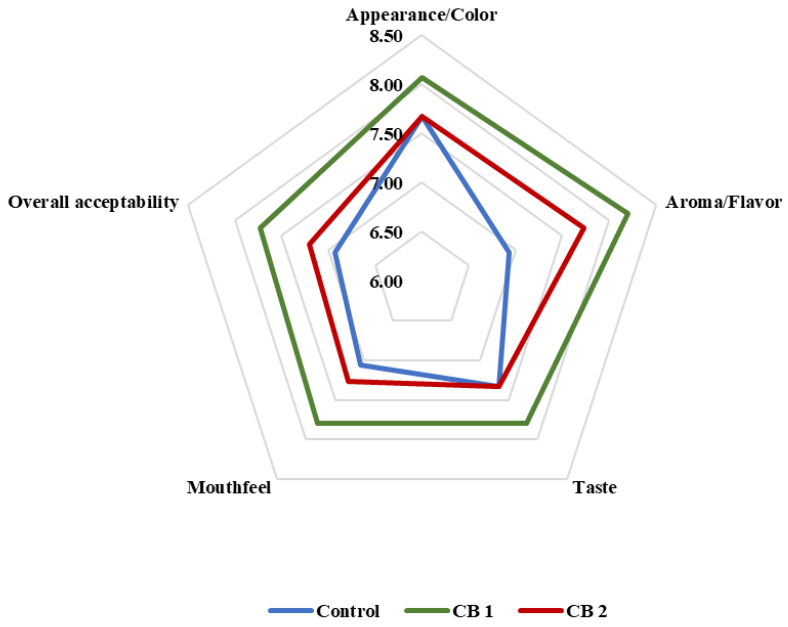

Fig. 5.7 Sensory analysis of the beverage samples; CB1-15 % emulsion beverage, CB2-25% emulsion beverage.

5.6 Conclusion

The search for dairy alternatives is actively pursued, primarily by individuals afflicted with lactose intolerance. Animal-derived milk and coconut milk are characterized by their high calorie and fat content. In this context, millet milk emerges as a more favourable option due to its elevated levels of protein, energy, ash, and carbohydrates, coupled with its reduced fat content, when compared with both coconut milk and dairy milk. The findings from the present investigation unequivocally demonstrate the acceptability of millet milk derived from sprouted finger millet milk and coffee oil encapsulated Pickering emulsions during sensory

evaluations. Additionally, these millet milk formulations exhibit a commendable shelf life of 21 days when stored under refrigerated conditions. This inherent stability renders them suitable for diverse applications as dairy milk substitutes. When considering the advantageous attributes of millet milk, including its cost-effectiveness as a raw material, ready availability, ease of processing, and cultivation, as well as its robust nutritional profile, it becomes evident that millet milk represents a superior alternative to cow's milk. Furthermore, it holds considerable benefits for farmers as a value-added product within their agricultural portfolio.

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Chapter 6

Summary and Conclusion

Millets, categorized as diminutive cereal grains, display notable resilience and thrive in arid regions, particularly across Africa and Asia. These hardy crops demand minimal maintenance yet yield seeds of exceptional quality, characterized by their rich energy content, elevated fiber levels, and significant concentrations of bioactive compounds and unsaturated fatty acids. Despite the existence of some studies concerning the nutritional aspects of millets, a comprehensive report encompassing micro and macro nutrients, dietary fiber, and bioactive compounds in both millets and germinated millets is conspicuously absent. It's worth noting that millets prominently feature starch as a major constituent, a versatile macromolecule applicable across both the food and non-food sectors. Starch, owing to its intrinsic attributes of cost-effectiveness, ubiquity, sustainability, and biodegradability, plays a pivotal role within the food industry. However, native millet starch exhibits limited functionality, constraining its utility within industrial contexts. Typically, both native and modified millet starches find extensive applications, serving as binding and thickening agents in baked goods, meat products, and snack seasonings, substituting for fats in ice creams, encapsulating flavors, stabilizing emulsions in juices and beverages, gelling agents in gums and gels, bolstering foam stability in marshmallows, and enhancing crispiness in fried snacks. Surprisingly, the untapped potential lies in exploring millet starch as Pickering particles.

Pickering emulsions refer to emulsions fortified with ultrafine solid particles. In contrast to conventional surfactants, these solid particles increase the energy levels for detachment from the oil-water interface upon successful adhesion. The irreversible adsorption of Pickering particles at the oil-water interface imparts remarkable stability, conferring resistance against coalescence and Ostwald Ripening. Empirical evidence underscores the potential of modified micron-sized and nano-sized solid

particles to establish robust physical barriers through selective adsorption at the oil-water interface, thereby augmenting emulsion stability. In alignment with the tenets of eco-friendliness, healthfulness, and sustainability, the research spotlight within Pickering emulsification has progressively shifted from inorganic agents such as titanium dioxide, silica, and clay to food-grade colloidal entities encompassing carbohydrates, proteins, and lipids.

Based on the accumulated information and identified research gaps, our study aims to address the following aspects: (1) Investigating the potential enhancement of millet's nutritional composition through germination processes. (2) Exploring physical modification techniques, specifically micronization, to enhance the Pickering properties of millet starch granules, rendering them suitable for stabilizing Pickering emulsions containing coffee oil. (3) Evaluating the applicability of coffee oil-encapsulated Pickering emulsions in food contexts, notably in the preparation of coffee-flavored plant-based beverages derived from germinated finger millet milk.

In the present study entitled "**Millet starch as food-grade Pickering particles in emulsion stabilization: Fabrication, characterization and application studies"** we aimed to transform native millet starch through the physical modification process of micronization, rendering it suitable for utilization as a reliable Pickering particle. These modified millet starch-based Pickering particles were employed to ensure the stability of coffee oil/water emulsions. Subsequently, the prepared emulsions, characterized by their stability, were integrated into sprouted finger millet milk to create a non-dairy coffee-flavoured beverage. The study also involved conducting investigations to assess the shelf-life stability of this newly formulated beverage.

Chapter 1 gives a general introduction and review of literature about millets, millet starch and its characteristics, Pickering emulsions, micronization as a method for physical modification of starch, relevance and applications of coffee oil emulsions and plant-based diary alternatives.

Chapter 2 entitled '**Determination of the nutritional, functional and phytochemical composition of nine millet varieties in their raw and germinated form'** presents an extensive investigation into the comprehensive composition of nine millet varieties, examining both their original and germinated states. The research found that germination significantly enhanced the nutritional and antioxidant characteristics of millet grains. These ancient grains exhibited a well-balanced amino acid profile, with particular richness in essential amino acids like methionine, valine, and tryptophan. Among the water-soluble vitamins analyzed, proso millet ranked highest, followed by amaranth, foxtail millet, and pearl millet. Notably, barnyard millet displayed the highest content of ascorbic acid, measuring at 98.39 ± 0.11 ng/g. In terms of minerals, finger millet emerged as the primary source of calcium, with a concentration of 303 ± 0.48 mg/100g, while foxtail millet exhibited substantial quantities of iron and zinc. Furthermore, germination significantly augmented the phytochemical content and antioxidant activity of millet grains. These preliminary findings laid the groundwork for exploring the potential applications of sprouted millet grains. Examination of the proximate composition revealed that carbohydrates were the predominant nutrients in millets, followed by protein and fat. The carbohydrate content in millets was primarily attributed to starch, comprising over 60% of the overall composition. Consequently, our study specifically concentrated on the isolation and characterization of starch extracted from millet grains.

Chapter 3 entitled '**Isolation of starch from millet grains, size reduction and its characterisation based on structural, functional, thermal and rheological properties'** deals with the isolation of starch fractions obtained from millet grains, followed by their subsequent reduction in particle size. Extensive characterization was performed to assess the structural, functional, and thermal properties of these starch fractions. The primary aim of this chapter is to establish and illustrate the feasibility of employing micronized millet starch particles, derived from various millet varieties, as Pickering particles for the purpose of emulsion stabilization.

In comparison to other millet varieties, micronized millet starch particles sourced from pearl millet (referred to as S1) and proso millet (referred to as S2) were identified as more suitable for the formulation of oil-in-water (O/W) emulsions based on multiple criteria, including their particle size (S1: 1.25 ± 0.21 μm, S2: 1.09 ± 0.14 μm), zeta potential (S1: -25.74 ± 0.05 mV, S2: -16.00 ± 0.14 mV), and hydrophilic properties (contact angle: S1: 55.4°; S2: 58.7°). The emulsions were prepared using a starch concentration below its critical micelle concentration (CMC) at 2.0%. The Pickering emulsions formed using pearl millet starch exhibited specific characteristics, including a droplet size of 1.01 ± 0.25 μm, zeta potential of -76.1 ± 0.02 mV, and turbidity of 81.23 ± 0.01%. Remarkably, micronized millet starch granules at a concentration of 0.4% (w/v) effectively stabilized 3.0% (w/v) O/W emulsions containing coffee oil. The results of this investigation unequivocally demonstrate the capacity of micronized starch granules derived from millet sources to serve as effective stabilizing agents for emulsions by acting as Pickering particles.

Chapter 4 entitled '**Optimisation and characterisation studies on Pickering emulsions of roasted coffee oil stabilised by physically modified pearl millet starch granules'** deals on the formulation of oil-in-water (O/W) Pickering emulsions

containing coffee oil, utilizing varying concentrations of pearl millet starch as the stabilizing Pickering particle. From multiple experimental runs, three distinct emulsion formulations were selected for in-depth analysis: a 40% coffee oil emulsion (designated as 40E), a 20% coffee oil emulsion (referred to as 20E), and a 20% ultrasonicated coffee oil emulsion (abbreviated as 20UE).

Subsequent to formulation, the emulsions underwent extensive characterization to assess critical parameters, encompassing droplet size, zeta potential, pH, density, color properties, Fourier-transform infrared (FTIR) spectroscopy analysis, and rheological behavior. The measured droplet sizes for the freshly prepared emulsions were as follows: 1.28 ± 0.20 μm (40E), 1.27 ± 0.36 μm (20E), and 1.86 ± 0.35 μm (20UE), respectively. Zeta potential values were recorded as follows: -34.4 ± 0.02 mV (40E), -41.4 ± 0.04 mV (20E), and -43.1 ± 0.02 mV (20UE). Furthermore, by analyzing the flow curve data derived from the emulsions, we computed the apparent viscosity, revealing that the emulsions exhibited non-Newtonian behavior. FTIR analysis corroborated the absence of any chemical interactions among the constituents, confirming the successful encapsulation of coffee oil within the emulsions. Importantly, fluorescence microscopy examination illustrated that the millet starch particles (stained green) effectively covered the oil phase (stained red), indicating their adsorption at the interface between the oil and water phases. This observation provides compelling evidence for the efficacy of millet starch as effective Pickering particles for emulsion stabilization.

Chapter 5 entitled '**Development of new product formulations (plant-based beverages) from sprouted finger millet, followed by its sensory and shelf-life studies'** delves into the formulation of a flavored plant-based dairy alternative beverage. This beverage was prepared using sprouted finger millet milk as the base

(referred to as Control - C), with the incorporation of Pickering emulsions at varying concentrations, specifically 15% (v/v) (designated as CB1) and 25% (v/v) (referred to as CB2). The stability of the formulated beverage was meticulously examined under refrigerated conditions over a 28-day period. Routine assessments, encompassing measurements of brix, pH, color attributes, and absorbance, were carried out at regular intervals of 7 days throughout the storage duration. The brix value for the control sample was determined to be 11.99%, while the prepared beverages exhibited brix values of 14.70% (CB1) and 14.41% (CB2). Notably, the pH of all samples remained constant at 6.00. To evaluate the sensory attributes and overall acceptability of the beverages, a sensory analysis was meticulously conducted. The results indicated that the beverage containing 15.0% emulsions (CB1) garnered higher acceptance scores in terms of color, taste, aroma/flavor, mouthfeel, and overall acceptability, surpassing both the control sample (C) and the one containing 25% emulsion (CB2) in these sensory aspects.

CONCLUSIONS

- The nutritional profile of nine millet grains in their raw and germinated form were assessed.
- Millets were found to be nutritionally superior in terms of all the macronutrients present in them.
- They had good amounts of amino acids, b vitamins and acsorbic acid.
- A thorough analysis of these grains unveiled the presence of a total of 23 distinct polyphenolic compounds, representing the first documented instance of their identification in this context.
- They also contain good amount of minerals when compared to the other staple cereals consumed.

- Physical treatments, such as size reduction or micronization, have been shown to induce modifications in millet starch. Micronization, in particular, results in the production of more uniform particles with enhanced functional, structural, and thermal stability.
- These modifications include alterations in particle size, zeta potential, hydrophobicity, and critical micelle concentration. As a result, these modified starch particles are well-suited for the formulation of oil-in-water (O/W) Pickering emulsions.
- The prepared emulsions exhibited stability for 15 days under refrigerated (4°C) conditions. They demonstrated a high degree of homogeneity, as evidenced by the polydispersity index values. Additionally, the emulsions displayed high zeta potential values exceeding ±30 mV, indicating their capacity to effectively stabilize emulsions formed using these particle dispersions.
- Plant-based coffee-flavored non-dairy beverages can be efficiently manufactured utilizing millet milk extracted from germinated finger millet and Pickering emulsions containing encapsulated coffee oil. The incorporation of such emulsions significantly improved both the consistency and palatability of the beverages, resulting in favorable sensory acceptability.

FUTURE ASPECTS

- The study's future prospects lie in investigating the connection between the heightened antioxidant activity resulting from germination and its potential health benefits for consumers.
- Identifying the Pickering functionality of millet starches other than pearl millet.
- More exploratory research in stabilizing concentrated emulsions.

- Modifying millet starch particles using other physical modification methods as well as enzymatic methods and identifying its capability to act as Pickering particles.
- Detailed investigative research on stability of Pickering emulsions in real food systems (interactions with the food product matrix) and understanding the de-structuring of emulsions in the gastrointestinal tract.
- Detailed study on the relationship between rheology and microstructure of the millet starch-based Pickering emulsions.

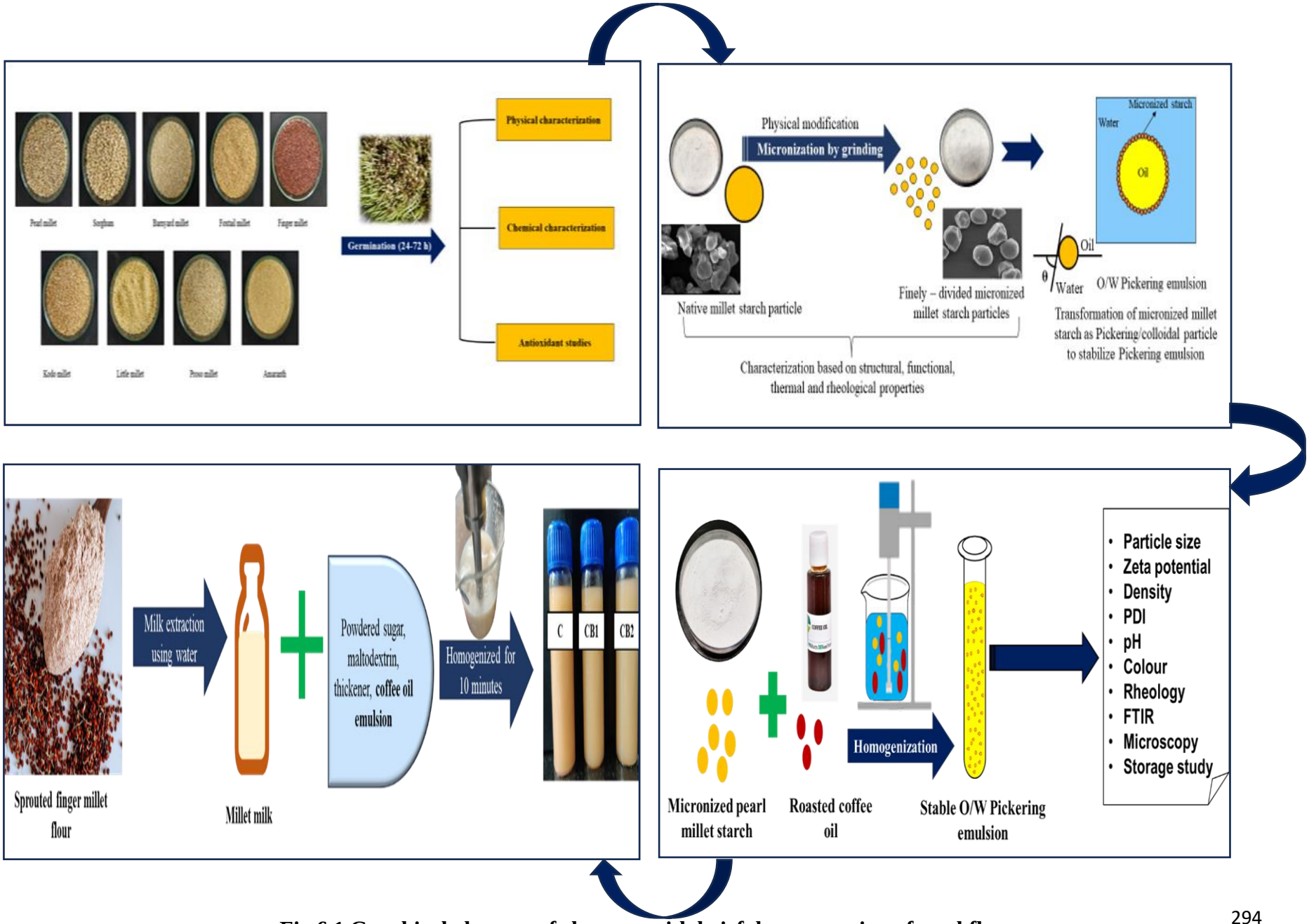

Fig 6.1 Graphical abstract of chapters with brief demonstration of workflow

APPENDIX

List of Instruments

Instruments	Manufacturer
Homogenizer	Omni International Tissue Master 125, United States
UV Spectrophotometer	Shimadzu UV-2600, Japan
Hot air oven	Globe Tex, Digital Laboratory Hot Air Oven, Ghaziabad, India
Overhead stirrer	Remi, RQ126/D, with 40V, Mumbai, India
FTIR-ATR Spectrophotometer	Perkin Elmer, USA
Malvern Zeta sizer	Zeta Nano-ZS; Malvern Instruments, UK
Scanning Electron Microscope	Carl Ziess EVO-18, Germany
HPLC	Shimadzu, Japan
LC-MS/MS	Shimadzu, Japan
Moisture analyzer	Mettler Toledo, India
Fluorescent Microscope	Olympus IX83, Olympus Corporation of the Americas, Center Valley, PA, USA
Conductivity meter	Labtronics, Labman, LT-51, Haryana
Oakton pH 700	Benchtop Meter, Oakton Instruments, USA
Color Spectrophotometer	Hunder Lab, ColorFlex, Virginia
XEUSS SAXS/WAXS	Xenocs, Grenoble, France
Refrigerated Centrifuge	Beckman Coulter, Pasadena, CA, USA
Rheometer	Anton Paar GmbH, Ostfildern-Scharnhausen, Germany
Drop shape analyser	KRUSS GmbH Hamburg, Germany
Multimode reader	Biotek Synergy 4, Winooski, VT, USA
ICP-MS	Thermo Scientific iCAP RQ, single quadrupole

ABSTRACT

Name of the student: Navami M M	**Registration No.: 10BB17A39023**
Faculty of study: Biological Science	**Year of submission: 2023**
ACSIR academic centre/CSIR Lab: CSIR-NIIST	**Name of supervisor: Dr P. Nisha**
Title of the thesis: 'Millet starch as food-grade Pickering particles in emulsion stabilization: Fabrication, characterization, and application studies.	

The increasing demand for alternative starch sources in the food industry has generated interest in exploring new sources. The applications of starch depend on its structural and functional properties. In the realm of both food and non-food sectors, native starches demonstrate limited suitability, necessitating the adoption of modification techniques to address their inherent limitations. Millet starch has emerged as one such potential alternative due to its unique characteristics and suitability for various applications. Chapter 1 gives a general introduction and review of literature about millet starch and its characteristics, Pickering emulsions, micronization, plant-based diary alternatives. In the first experimental chapter 2, nine varieties of millets were germinated till 72 hours and their nutritional, functional, phytochemical, and antioxidant composition were analyzed. Germination was found to improve the nutritional quality of the grains. Chapter 3 focuses on isolating and reducing the size of starch fractions from millet grains. These fractions are extensively characterized for structural, functional, and thermal properties. The primary goal was to demonstrate that micronized millet starch particles from various millet varieties can effectively act as Pickering particles for emulsion stabilization. The particle size of the starch granules reduced, and zeta potential values increased significantly upon micronization, and they were found to be hydrophilic. This makes them suitable for the preparation of o/w emulsions. Pearl millet starch showed the best Pickering particle property. Based on the understandings of chapter 3, the next chapter 4 focused on the preparation of micronized pearl millet starch Pickering emulsions encapsulating roasted coffee oil. Emulsions were prepared using 2% starch and varying oil concentrations. They were then analyzed based on droplet size, zeta potential, pH, density, PDI and microscopy analysis. The resultant emulsions were found to be stable under refrigerated conditions (4°C) for 14 days. In chapter 5 the prepared emulsions were added to sprouted finger millet milk to prepare non-dairy coffee flavored plant-based beverages. The prepared beverages with 15% emulsions were found to be more acceptable during sensory analysis. They also had good shelf-life stability till 21 days under refrigerated conditions. The study proves the efficacy of physically modified millet starch in stabilizing o/w emulsions, offering a non-chemical approach to particle modification. Micronization is simple, safe, and cost-effective, resulting in unique starch microparticles. These properties hold promise for various food and pharmaceutical formulations, expanding millet starch's commercial potential. Moreover, millets' nutritional attributes make them suitable dairy substitutes.

PUBLICATIONS

List of publications emanating from the thesis work

1. **Navami, M. M**., Billu Abraham., Archana Haridas., Nisha P. (2023). Nutritional profiling and quantitative analysis of amino acids and vitamins using LC-MS/MS in selected raw and germinated ancient grains, *JSFA Reports.* 2023;3(8):377–86. https://doi.org/10.1002/jsf2.141

List of publications not related to thesis work

1. Abraham, B., Reshmitha, T. R., **Navami, M. M**., George, L., Venugopalan, V. V., & Nisha, P. (2020). Phytochemical rich extract from the spent material generated from Industrial Dashamoola preparation (a medicinal Ayurvedic decoction) with antioxidant, antidiabetic and anti-inflammatory potential. *Industrial Crops and Products*, 151, 112451.
2. Heeba, S., **Navami, M. M**., Nayana, N., Nisha, P (2022). Influence of coating material and processing parameters on acrylamide formation in potato patties and mitigation strategies, International Journal of Food Engineering. *International Journal of Food Engineering*, 18(5), pp.399-409.

Book chapter

1. **Navami M.M**, Padma Ishwarya S, Nisha P. (2022). Quality standards for Millets in "The Handbook of Millets: Processing, Quality and Nutraceutical Potential edited by Dr. C. Anandharamakrishnan, Dr. Ashish Rawson, Dr C. K. Sunil.

NATIONAL/INTERNATIONAL CONFERENCES/SEMINARS PRESENTED

1. **Navami MM**, Nayana N, Nisha P. Valorization of spent *Emblica officinalis* generated from juice industries for functional foods and nutraceutical development, ICFOST 2017 at IICT, Hyderabad (Poster presentation)

2. Heeba S, **Navami MM**, Nisha P. Mitigation strategies of acrylamide formation in cutlet, iCRAFPT 2018 at IIFPT, Thanjavur (Poster presentation)

3. **Navami MM**, Nisha P. A comparative evaluation of dietary fibre content and prebiotic efficacy of traditional millet grains, IFCON 2018 at CFTRI, Mysore (Poster presentation)

4. **Navami MM**, Nisha P. Evaluation of the Prebiotic Efficacy of Dietary Fibre Isolated from Ancient Grains, CARBO-XXXIV: International conference on "Emerging frontiers in Carbohydrates Chemistry and Glycobiology, 2019, University of Lucknow (Poster presentation)

5. **Navami MM**, Nisha P. Effect of micronization on improving the capability of millet starch as Pickering particles for emulsion stabilization, ICFOST 2022, Trivandrum (Poster presentation).

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ERRATUM

JSFA Reports

Erratum to "Nutritional profiling and quantitative analysis of amino acids and vitamins using LC–MS/MS in selected raw and germinated ancient grains"

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In this article the affiliation of the first author and the corresponding author is incorrect.

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We apologize for this error.

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RESEARCH ARTICLE

JSFA Reports

Nutritional profiling and quantitative analysis of amino acids and vitamins using LC–MS/MS in selected raw and germinated ancient grains

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Abstract

Background: Ancient grains are grains that have remained unchanged over the past decades. Among them, millets and pseudocereals are highly nutritious underutilized grains that are gluten free. The present study compares the nutritional composition of nine important ancient grains in its raw and germinated forms.

Results: Among the grains studied, amaranth had the highest protein content (14.52%), followed by proso (13.04%) and foxtail millets (12.78%). Amino acid analysis was carried out using LC–MS/MS. Besides amaranth, barnyard, pearl, and proso millets contained considerable amount of essential amino acids. The lysine content of sorghum has increased four folds during germination. Valine, histidine, and glutamic acid contents increased marginally upon sprouting. These grains were also found to contain significant amount of B complex vitamins. The ascorbic acid content of the grains increased considerably upon germination especially in foxtail millet (78.03–30452.90 ng/g).

Conclusion: The present study is the first comparative study that has brought out the distinct nutritional superiority of the ancient grains in their raw and germinated forms. These gluten-free grains can be utilized to improve the nutritional demand and food security of the growing population.

KEYWORDS
amino acids, ancient grains, germinated grains, LC–MS/MS, millets, nutritional quality, vitamins

INTRODUCTION

Ancient grains can be defined as species or particular varieties of true cereals (sorghum, millets, teff, and wild rice), pseudocereals (amaranth, buckwheat, and quinoa), and pulses (cowpea and Marama bean). These are traditional staple foods cultivated since hundreds of years ago and consumed by communities outside the mainstream of technological development. Consequently, these grains have undergone relatively limited genetic improvement. Further, the ancient grains are gluten-free and can be consumed by celiac—patients, which is a major criterion that adds to their nutritive value.[1] Among ancient grains, millets and pseudocereals are important crops in the semiarid and tropical regions of Asia and Africa due to their resistance to diseases, pests, short growing season, and ability to thrive in less productive soils under heat and drought conditions.[2] Asian countries are the second most important block of millet producers that accounts for 38% of the global area, as well as 42% of worldwide production. As per the Food and Agriculture Organization Corporate Statistical Database (FAOSTAT) 2018, global millet production for the year 2016 was found to be 30.35 million tons. In India, millet production is ~10 million tons, out of which the production of small millet is about 467 thousand tons.[3] The Government of India has designated 2018 as the "National Year of Millets" and 2023 as the "International Year of Millets" by the United Nations in recognition of their resilience to climate change and their importance for nutritional, health, and food security.

Millets are small seeded cereals that belong to the family Poaceae, which are grown mainly in the semiarid regions of Asia and Africa. Sorghum and pearl millet are the major millet varieties. Other

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millet varieties comprise finger millet (*Eleusine coracana*), foxtail millet (*Setaria italica*), proso millet (*Panicum miliaceum*), kodo millet (*Paspalum scrobiculatum*), barnyard millet (*Echinochloa* spp.), and little millet (*Panicum sumatrense*).[3] A pseudocereal is a plant grown to produce starchy grain suitable for human food (excluding cereals, legumes, oilseeds, and nuts). The major pseudocereals are grain amaranth (*Amaranthus caudatus*; *Amaranthus cruentus*; *Amaranthus hypochondriacus*; family: Amaranthaceae), quinoa, and buckwheat.[4] The nutrient density of the staple grains can be increased by natural methods like soaking, sprouting, and fermentation. Germination or sprouting is a hydrothermal treatment of cereal grains under ambient environmental conditions, which leads to the synthesis of new compounds and elevates the nutritional quality of grains. The core purpose of germination is the increment of certain hydrolytic enzymes that are usually dormant in raw seeds.[5] The phytic acid and polyphenols that cohere to the enzymes in our digestive tract hinder the absorption of carbohydrates and proteins. These detrimental effects can be curbed by the process of germination.[6]

As the food industry is looking for alternative sources for developing non-glutinous products, there is an excellent scope for ancient grain-based health foods. Even though there are studies that suggest the nutritional superiority of ancient grains, there are no comprehensive reports on the influence of germination on the nutritive value of ancient grains. As there is an increase in demand for gluten-free products in the market, documentation of the nutritional profile of these ancient grains will add value to their food applications. Moreover, germination leads to a decrease in the anti-nutritional factors present in these grains. Therefore, in our present study, we aim to elucidate the complete nutritional profile of these ancient grains in their raw and germinated forms.

MATERIALS AND METHODS

Grain samples (pearl millet, sorghum, kodo millet, finger millet, little millet, barnyard millet, and foxtail millet) used in the study were received as gift samples from the Indian Council of Agriculture Research-Indian Institute of Millets Research, Hyderabad, India. Proso millet and amaranth were purchased from authenticated dealers in Trivandrum, Kerala, India.

Chemicals and consumables

All the chemicals used in the study were obtained from Sigma Aldrich unless otherwise stated. L-Amino acids standard reference kit was purchased from Sisco Research Laboratories, Maharashtra, India. Ammonium formate, formic acid (MS grade), and acetonitrile (MS grade) were purchased from Biosolve Ultra International, Bangalore, India. All solvents used were of MS grade. HPLC grade water was used throughout the study obtained from a water purification system (Millipore Milli-Q, Bangalore, India). Minigen Nylon syringe filters (0.2 µm diameter) were obtained from Genetix Biotech Asia Pvt. Ltd, New Delhi, India.

Sample preparation

Each raw grain sample (50 g) was ground to a powder (Mesh size 50) using a mixer. The samples were then stored in airtight containers for further analysis. For the preparation of germinated samples, 50 g of the grains were immersed in 1.25% sodium hypochlorite solution (seed: water ratio of 1:5, w/v) at room temperature for 30 min to disinfect them. Then it was washed thoroughly under tap water for 15–20 min for rinsing off the excess sodium hypochlorite. The grains were then covered with moist cotton cloth and left to sprout at room temperature (32 ± 3°C) for 0, 24, 48, and 72 h, respectively.[7] After germination, the grains were oven dried at 45°C and ground to powder form and stored in airtight containers for further analysis.

Proximate composition

The standard procedures of Association of Official Analytical Chemists (AOAC),[8] (method 930.15, 923.03, 920.39) were used for the determination of moisture, ash, crude fat, and protein contents of all the grain flours. All triplicate samples were oven-dried at 100°C, transferred to a desiccator, and allowed to cool at room temperature for moisture content. The sample weights were recorded before and after heat treatment in a muffle furnace (550°C for 12 h) for the ash content determination. Micro-Kjeldahl method was used for the protein estimation with nitrogen to a protein conversion factor of 6.25, and fat content was determined using Soxhlet extraction. The total carbohydrate content was calculated using the difference method (100-sum of protein, ash, fat, and moisture).

Sample preparation for LC–MS/MS analysis

The sample for LC–MS/MS analysis was prepared according to the procedure by Nimbalkar et al.[9] with some modifications. A homogeneous sample (1.0 g) was weighed into a sample container having 10 mL of Milli-Q water. The mixture was vortexed for 5 min, followed by centrifugation at 4°C at 10,000 rpm for 15 min. The supernatant was then passed through a 0.2 µm nylon membrane filter. Sample aliquot of 10 µL was injected for LC–MS/MS analysis.

Amino acid analysis using LC–MS/MS

Free amino acids (essential amino acids (E)—phenylalanine, leucine, methionine, threonine, valine, histidine, tryptophan, lysine, isoleucine, and nonessential amino acids (NE)—glutamic acid, glycine, proline, aspartic acid, tyrosine, hydroxyproline, alanine, serine, asparagine, cysteine, arginine, cysteine, and glutamine) were standardized using the LC–MS/MS system (Nexera with LCMS-8045, Shimadzu Corporation, Kyoto, Japan)—HPLC (Nexera LC-30AD) equipped with an autosampler (SIL-30AC), temperature-controlled column oven (CTO-20AC), and prominence diode array detector

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(SPD-M20A) coupled to triple quadrupole mass spectrometer (Nexera with LCMS-8045, Shimadzu Corporation, Kyoto, Japan). Working standards were prepared from the stock solution by dilution with Milli-Q water with a concentration ranging between 0.01 and 1 µg/mL. The quantification of all the amino acids was carried out using Shimadzu Shim-pack GISS C18 column (150 × 2.1 mm i.d., 1.9 µm) using a mobile phase of water/formic acid (100/0.1) for solvent A and 100% methanol for solvent B. Amino acids were eluted with a linear gradient system as follows: 0.5–4.9 min 5% of solvent B, 5.0–13 min 85% of solvent B, 13.1–15 min 5% of solvent B, a flow rate of 0.3 mL/min, and oven temperature of 40°C. The LC–MS/MS with electrospray ionization (ESI) was operated in multiple reaction monitoring (MRM) mode, both positive and negative. The injection volume was 10 µL, and ion spray voltage was 4 kV. The collision-induced dissociation (CID) gas was 230 kPa. Each calibration solution was analyzed in triplicate, and the average value of the results was used as the representative for each point. The results obtained are represented as amino acids µg/g with standard deviation (SD) ($n = 3$). The details of method validation are given in Supplementary Table 1a.

Analysis of water-soluble vitamins

Vitamins (thiamine, riboflavin, niacin, pyridoxine, folic acid, and ascorbic acid) were analyzed using the LC–MS/MS system (Nexera with LCMS-8045, Shimadzu Corporation, Kyoto, Japan). The quantification was carried out using a Shimadzu Shim-pack GISS C18 column (150 × 2.1 mm i.d., 1.9 µm). The mobile phase used was 10 mM ammonium formate in water and 0.1% formic acid for solvent A and 10 mM ammonium formate in methanol and 0.1% formic acid for solvent B. A linear gradient system was used as follows: 0.01–2 min 5% of solvent B, 2.0–5.0 min 30% of solvent B, 5.0–8.0 min 45% of solvent B, 8.0–12 min 90% of solvent B, 12–16 min 5% of solvent B, flow rate of 0.3 mL/min, and oven temperature of 33°C. The ESI was operated in both positive and negative MRM. The negative mode was used only for ascorbic acid. The injection volume was 10 µL. The ion spray voltage was 4 kV. The nebulizing gas flow was 3.0 L/min, and drying and heating gas flow was 10 L/min. Each calibration solution was analyzed in triplicate, and the average value of the results was used as the representative for each point. The results obtained are represented as vitamins ng/g with SD ($n = 3$). The method validation for the same is presented in Supplementary Table 1b.

Statistical analysis

All the experimental results were expressed as mean ± SD of triplicate measurements unless specified. The data were subjected to one-way analysis of variance (ANOVA), and the significance of differences between means was calculated by Duncan's multiple range tests using SPSS for Windows, SPSS Statistics 20 Software (IBM), and the significance was accepted at $p \leq .05$.

RESULTS AND DISCUSSION

Proximate composition

The proximate composition of the various millet grains is presented in Table 1. Results showed that carbohydrate is the primary nutrient present in millets followed by protein and fat. Amaranth was found to be the richest source of protein (14.52 ± 0.25%; dry basis [d.b]) followed by proso (13.04 ± 0.14%; d.b) and foxtail millets (12.78 ± 0.10%; d.b). It is reported to have the highest protein content (15.5%–16.10%) among the pseudocereals and wheat.[10] The protein content of ancient grains from the present study was within the ranges of the various reported studies.[12,13] The protein content of millet grains was found to be much higher (14.00%) when compared to the staple cereals, for example, maize (9.00%), wheat (12.00%), and rice (6.80%).[13] It is also reported that proso millet contained the highest amount of protein among all the millets followed by pearl millet.[14] Kodo and finger millet had significantly lower protein content when compared to the other small millets. Compared to the raw grains, the protein content decreased significantly ($p < .05$) for almost all the millet varieties upon germination, which may be due to the fact that the protease enzymes are activated during soaking and sprouting process, leading to degradation of protein content in the grains.[15]

The crude fat content indicated the following trend: amaranth (4.8 ± 0.14% d.b) > pearl millet (4.65 ± 0.07% d.b) > foxtail (4.30 ± 0.14% d.b), followed by little millet (3.53 ± 0.04% d.b) and barnyard millet (1.99 ± 0.01% d.b). The fat content of foxtail, proso, little, and kodo millets was reported to range between 2.3%–5.9%, 2.1%–5.2%, 3.10%–4.1%, and 1.1%–3.3%, respectively.[16] The values obtained in the present study fall within these reported ranges, except for proso. The lipid content of amaranth is reported to vary between 1.9% and 9.7% depending on the species and genotype.[17] The fat content of the germinated grains decreased significantly when compared to the raw samples, which is in accordance with the findings of Traoré et al.[18] This decrease could be due to the fact that lipids are used to produce the necessary energy for the biochemical and physiological modifications that occur in the seed during germination.[19]

Amaranth, barnyard, and proso millets were found to contain the highest ash content. There was not much significant change in the ash content of the grains upon germination. Total carbohydrate content was found to be higher in raw proso millet (67.04 ± 0.36% db) followed by sorghum (66.55 ± 0.06% db), finger millet (65.92 ± 0.55% db), and which are close to the reported values.[20] The carbohydrate content of germinated grains was seen to be gradually decreasing. This reduction in starch content can be attributed to the hydrolytic activity of the amylase enzyme degrading starch polymer chains. Another reason could be due to the use of starch as an energy source in the sprouting process.[21] Thus, the results suggest that sprouting modifies the proximate composition of these grains by enhancing the hydrolysis of complex insoluble organic compounds present in the seeds to form simpler water-soluble organic compounds.[15]

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TABLE 1 Proximate composition of raw and germinated ancient grains (percentage dry weight basis).

Parameters (% db)	Germination time (GT)	Pearl millet	Sorghum	Barnyard millet	Foxtail millet	Finger millet	Kodo millet	Little millet	Proso millet	Amaranth
Moisture	0th	8.92 ± 0.09^{f}	12.35 ± 0.07^{h}	6.55 ± 0.14^{b}	6.24 ± 0.06^{a}	10.77 ± 0.02^{g}	8.92 ± 0.09^{f}	6.61 ± 0.06^{c}	7.21 ± 0.13^{e}	7.08 ± 0.01^{d}
	24th	48.27 ± 0.06^{d}	46.15 ± 0.03^{c}	42.00 ± 0.03^{b}	38.39 ± 0.37^{a}	51.42 ± 0.05^{e}	42.24 ± 0.06^{b}	51.30 ± 0.04^{e}	38.05 ± 0.25^{a}	52.63 ± 0.08^{f}
	48th	45.04 ± 0.17^{g}	42.57 ± 0.04^{e}	39.63 ± 0.08^{d}	35.45 ± 0.05^{a}	44.55 ± 0.07^{f}	36.70 ± 0.03^{b}	36.45 ± 0.07^{b}	38.32 ± 0.45^{c}	61.47 ± 0.01^{h}
	72nd	48.69 ± 0.26^{h}	39.42 ± 0.06^{d}	37.20 ± 1.20^{c}	41.28 ± 0.06^{e}	46.29 ± 0.26^{g}	34.86 ± 0.06^{a}	42.59 ± 0.06^{f}	35.64 ± 0.56^{b}	53.33 ± 0.02^{i}
Total ash	0th	1.78 ± 0.04^{a}	1.43 ± 0.14^{a}	2.57 ± 0.09^{d}	2.36 ± 0.10^{c}	2.03 ± 0.01^{b}	1.99 ± 0.07^{b}	1.70 ± 0.09^{a}	2.60 ± 0.01^{c}	3.03 ± 0.12^{d}
	24th	2.19 ± 0.08^{a}	2.17 ± 0.25^{a}	2.17 ± 0.80^{a}	2.33 ± 0.56^{a}	2.07 ± 0.35^{a}	2.30 ± 0.03^{a}	2.54 ± 0.48^{a}	2.03 ± 0.25^{a}	2.04 ± 0.02^{a}
	48th	2.14 ± 0.35^{a}	2.29 ± 0.06^{a}	2.06 ± 2.46^{a}	2.13 ± 0.25^{a}	2.10 ± 0.05^{a}	2.24 ± 0.56^{a}	2.05 ± 0.65^{a}	2.06 ± 0.40^{a}	2.21 ± 0.06^{a}
	72nd	2.23 ± 0.50^{a}	2.13 ± 0.03^{a}	2.40 ± 0.04^{a}	2.19 ± 0.58^{a}	2.32 ± 0.09^{a}	2.03 ± 0.08^{a}	2.02 ± 0.08^{a}	2.50 ± 0.34^{a}	2.23 ± 0.45^{a}
Crude fat	0th	4.65 ± 0.07^{h}	1.30 ± 0.14^{b}	1.99 ± 0.01^{c}	4.30 ± 0.14^{g}	2.20 ± 0.07^{e}	2.09 ± 0.07^{d}	3.53 ± 0.04^{f}	1.13 ± 0.09^{a}	4.80 ± 0.14^{i}
	24th	2.76 ± 0.50^{b}	1.10 ± 0.72^{a}	3.50 ± 0.23^{c}	2.95 ± 0.04^{b}	2.30 ± 0.03^{b}	3.03 ± 0.05^{c}	2.69 ± 0.06^{b}	2.93 ± 0.26^{b}	2.40 ± 0.67^{b}
	48th	3.14 ± 0.04^{b}	2.87 ± 0.03^{a}	3.00 ± 0.78^{b}	3.68 ± 0.06^{c}	2.46 ± 0.60^{a}	3.55 ± 0.58^{c}	3.60 ± 0.07^{c}	2.94 ± 0.50^{a}	2.77 ± 0.57^{a}
	72nd	2.65 ± 0.06^{a}	2.75 ± 0.50^{a}	2.92 ± 0.79^{a}	3.23 ± 0.25^{b}	2.70 ± 0.47^{a}	2.89 ± 0.03^{a}	3.00 ± 0.97^{b}	2.77 ± 0.62^{a}	2.70 ± 0.36^{a}
Crude protein	0th	12.46 ± 0.25^{f}	10.89 ± 0.78^{e}	9.60 ± 0.14^{b}	12.78 ± 0.10^{g}	8.04 ± 0.28^{a}	9.55 ± 0.14^{b}	10.18 ± 0.06^{d}	13.04 ± 0.14^{h}	14.52 ± 0.25^{i}
	24th	5.08 ± 0.04^{a}	8.50 ± 0.06^{d}	8.00 ± 0.16^{d}	10.92 ± 0.45^{e}	5.50 ± 0.35^{a}	6.26 ± 0.05^{b}	6.96 ± 0.45^{b}	11.76 ± 0.40^{f}	7.19 ± 0.36^{c}
	48th	5.27 ± 0.08^{a}	7.77 ± 0.07^{c}	11.73 ± 0.02^{f}	9.55 ± 0.05^{e}	7.38 ± 0.05^{c}	6.48 ± 0.06^{b}	7.10 ± 0.26^{c}	12.65 ± 0.07^{g}	8.41 ± 0.25^{d}
	72nd	7.42 ± 0.15^{d}	6.87 ± 0.58^{c}	5.70 ± 0.04^{b}	7.58 ± 0.45^{d}	4.25 ± 0.34^{a}	9.70 ± 0.45^{e}	6.16 ± 0.59^{c}	12.70 ± 0.04^{f}	7.29 ± 0.58^{d}
Carbohydrate	0th	59.25 ± 0.06^{c}	66.55 ± 0.06^{h}	58.80 ± 0.11^{b}	60.68 ± 0.39^{d}	65.92 ± 0.55^{g}	63.72 ± 0.39^{e}	64.64 ± 0.39^{f}	67.04 ± 0.36^{i}	57.60 ± 0.21^{a}
	24th	41.7 ± 0.34^{d}	42.08 ± 0.04^{e}	44.33 ± 0.60^{f}	45.41 ± 0.06^{g}	38.71 ± 0.05^{c}	46.17 ± 0.65^{h}	36.51 ± 0.56^{b}	45.23 ± 0.25^{g}	35.74 ± 0.25^{a}
	48th	44.41 ± 0.40^{c}	44.5 ± 0.02^{c}	43.58 ± 0.57^{b}	49.19 ± 0.67^{d}	43.51 ± 0.36^{b}	51.03 ± 0.08^{f}	50.72 ± 0.56^{e}	44.03 ± 0.45^{c}	25.14 ± 0.05^{a}
	72nd	39.01 ± 0.46^{b}	48.83 ± 0.06	51.78 ± 0.32^{g}	45.72 ± 0.26^{d}	44.44 ± 0.24^{c}	50.52 ± 0.06^{f}	46.23 ± 0.68^{e}	46.39 ± 0.02^{e}	34.45 ± 0.09^{a}

Note: Values are means ± SD ($n = 3$), followed by different superscript letters are significantly different ($p \leq .05$). The letter "a" represents the least value.

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TABLE 2 Amino acid content of selected ancient grains (μg/g).

Sl. no	Amino acid standards	GT	Pearl millet	Sorghum	Barnyard millet	Foxtail millet	Finger millet	Kodo millet	Little millet	Proso millet	Amaranth
1	Phenylalanine[E]	0th	1.311 ± 0.23^{g}	0.011 ± 0.05^{b}	1.541 ± 0.24^{h}	0.008 ± 0.38^{a}	0.182 ± 0.07^{d}	0.168 ± 0.09^{c}	0.324 ± 0.10^{e}	1.048 ± 0.20^{f}	2.743 ± 0.08^{i}
		24th	2.467 ± 0.21^{e}	2.299 ± 0.18^{d}	<0.001	1.362 ± 0.07^{c}	0.185 ± 0.01^{a}	0.696 ± 0.03^{b}	0.654 ± 0.02^{b}	2.802 ± 0.16^{f}	1.346 ± 0.21^{c}
		48th	1.697 ± 0.21^{f}	2.529 ± 0.40^{g}	0.509 ± 0.04^{b}	0.854 ± 0.07^{d}	0.378 ± 0.04^{a}	0.613 ± 0.05^{c}	0.580 ± 0.16^{b}	1.581 ± 0.11^{e}	<0.001
		72nd	2.641 ± 0.31^{g}	2.603 ± 0.29^{g}	0.406 ± 0.07^{b}	0.610 ± 0.04^{c}	0.273 ± 0.01^{a}	1.036 ± 0.14^{e}	0.935 ± 0.04^{d}	1.276 ± 0.14^{e}	1.556 ± 0.07^{f}
2	Leucine[E]	0th	0.435 ± 0.12^{h}	0.422 ± 0.05^{g}	0.021 ± 0.41^{d}	0.097 ± 0.18^{f}	<0.001	0.030 ± 0.13^{e}	0.006 ± 0.07^{b}	0.002 ± 0.06^{a}	0.013 ± 1.27^{c}
		24th	1.953 ± 0.01^{f}	2.087 ± 0.03^{g}	<0.001	1.579 ± 0.03^{e}	0.755 ± 0.03^{b}	0.570 ± 0.08^{a}	1.295 ± 0.03^{d}	2.546 ± 0.19	0.970 ± 0.03^{c}
		48th	1.760 ± 0.10^{c}	1.978 ± 0.01^{e}	0.847 ± 0.01^{a}	1.168 ± 0.15^{b}	1.810 ± 0.02^{d}	0.874 ± 0.07^{a}	1.062 ± 0.02^{b}	1.819 ± 0.04^{d}	<0.001
		72nd	2.353 ± 0.08^{f}	1.955 ± 0.03^{e}	0.778 ± 0.08^{a}	1.341 ± 0.07^{b}	1.654 ± 0.10^{c}	0.758 ± 0.14^{a}	1.305 ± 0.06^{b}	1.772 ± 0.11^{d}	1.235 ± 0.05^{b}
3	Methionine[E]	0th	0.453 ± 0.05^{f}	0.049 ± 0.01	3.902 ± 0.11^{h}	0.200 ± 0.41^{e}	0.145 ± 0.07^{c}	0.069 ± 0.26^{b}	0.178 ± 0.02^{d}	1.48 ± 0.04^{g}	6.413 ± 0.02^{i}
		24th	2.622 ± 0.10^{d}	2.357 ± 0.36^{d}	<0.001	1.831 ± 0.05^{c}	0.478 ± 0.04^{a}	0.387 ± 0.05^{a}	0.881 ± 0.08^{b}	4.861 ± 0.06^{e}	0.849 ± 0.05^{b}
		48th	2.489 ± 0.31^{g}	2.271 ± 0.17^{f}	0.700 ± 0.04^{b}	1.082 ± 0.03^{d}	1.933 ± 0.05^{e}	0.577 ± 0.02^{a}	0.967 ± 0.02^{c}	2.578 ± 0.13^{g}	<0.001
		72nd	3.412 ± 0.13^{f}	1.271 ± 0.17^{c}	0.578 ± 0.06^{a}	1.092 ± 0.03^{b}	1.515 ± 0.04^{d}	0.566 ± 0.04^{a}	1.784 ± 0.11^{e}	1.661 ± 0.06^{d}	1.320 ± 0.25^{c}
4	Threonine[E]	0th	0.097 ± 0.04^{f}	0.094 ± 0.49^{e}	0.188 ± 0.10^{g}	0.019 ± 0.11^{a}	0.092 ± 0.01^{d}	0.061 ± 0.07	0.068 ± 0.06^{c}	0.499 ± 0.08^{h}	1.583 ± 0.01^{i}
		24th	2.594 ± 0.20^{f}	1.821 ± 0.10^{e}	<0.001	1.757 ± 0.06^{d}	0.616 ± 0.08^{c}	0.241 ± 0.02^{b}	0.076 ± 0.01^{a}	1.739 ± 0.13^{d}	0.382 ± 0.06^{b}
		48th	2.701 ± 0.23^{f}	2.557 ± 0.30^{e}	0.804 ± 0.05^{b}	0.523 ± 0.05^{a}	1.629 ± 0.08^{d}	0.778 ± 0.16^{b}	0.651 ± 0.06^{a}	1.565 ± 0.10^{c}	<0.001
		72nd	3.592 ± 0.19^{e}	2.576 ± 0.28^{e}	0.614 ± 0.07^{a}	1.241 ± 0.10^{c}	1.707 ± 0.10^{d}	0.677 ± 0.05^{a}	0.679 ± 0.03^{a}	1.705 ± 0.07^{d}	0.894 ± 0.06^{b}
5	Valine[E]	0th	2.801 ± 0.11^{f}	0.145 ± 0.08^{b}	5.739 ± 0.07^{i}	0.065 ± 0.06^{a}	0.524 ± 0.07^{d}	0.277 ± 0.11^{c}	0.633 ± 0.10^{e}	3.390 ± 0.06^{g}	5.583 ± 0.41^{h}
		24th	4.415 ± 0.18^{e}	4.639 ± 0.20^{e}	2.547 ± 0.14^{c}	3.401 ± 0.26^{d}	1.350 ± 0.07^{a}	1.310 ± 0.14^{a}	1.682 ± 0.17^{b}	5.739 ± 0.07^{f}	1.391 ± 0.26^{a}
		48th	3.74 ± 0.19^{c}	4.257 ± 0.08^{d}	1.207 ± 0.09^{a}	1.330 ± 0.11^{a}	2.711 ± 0.14^{b}	1.476 ± 0.16^{a}	1.359 ± 0.09^{a}	2.638 ± 0.18^{b}	<0.001
		72nd	5.705 ± 0.22^{e}	4.210 ± 0.15^{d}	1.294 ± 0.12^{a}	2.691 ± 0.04^{c}	2.800 ± 0.13^{c}	1.299 ± 0.18^{a}	1.522 ± 0.15^{b}	2.501 ± 0.23^{c}	1.585 ± 0.15^{b}
6	Lysine[E]	0th	0.492 ± 0.07^{f}	0.069 ± 0.15^{b}	6.177 ± 0.12^{i}	0.012 ± 0.16^{a}	0.142 ± 0.07^{d}	0.447 ± 0.06^{e}	0.761 ± 0.03^{g}	0.131 ± 0.11^{c}	3.082 ± 0.17^{h}
		24th	0.963 ± 0.04^{c}	2.618 ± 0.19^{e}	1.188 ± 0.10^{d}	1.290 ± 0.21^{d}	0.727 ± 0.10^{b}	0.265 ± 0.03^{a}	0.304 ± 0.03^{a}	0.911 ± 0.07^{c}	0.398 ± 0.04^{a}
		48th	0.816 ± 0.08^{b}	1.287 ± 0.14^{c}	1.647 ± 0.10^{e}	1.344 ± 0.11^{c}	0.430 ± 0.06^{a}	1.436 ± 0.10^{d}	1.640 ± 0.10^{e}	0.894 ± 0.08^{b}	<0.001
		72nd	0.210 ± 0.08^{a}	4.194 ± 0.17^{e}	0.466 ± 0.06^{b}	0.573 ± 0.05^{b}	<0.001	1.578 ± 0.17^{c}	2.912 ± 0.08^{d}	0.567 ± 0.03^{b}	1.639 ± 0.10^{c}
7	Isoleucine[E]	0th	1.004 ± 0.01^{h}	0.087 ± 0.02^{b}	0.844 ± 0.06^{f}	0.036 ± 0.05^{a}	0.304 ± 0.11^{e}	0.093 ± 0.11^{c}	0.186 ± 0.12^{d}	0.944 ± 0.06^{g}	2.102 ± 0.02^{i}
		24th	1.855 ± 0.08^{e}	2.196 ± 0.21^{f}	<0.001	1.463 ± 0.12^{d}	0.569 ± 0.04^{a}	0.558 ± 0.10^{a}	1.230 ± 0.09^{c}	2.459 ± 0.08^{g}	0.921 ± 0.05^{b}
		48th	1.791 ± 0.06^{c}	1.872 ± 0.03^{d}	0.814 ± 0.07^{b}	0.840 ± 0.14^{b}	1.779 ± 0.06^{c}	0.633 ± 0.05^{a}	0.882 ± 0.05^{b}	1.872 ± 0.05^{d}	<0.001
		72nd	2.391 ± 0.15^{e}	1.660 ± 0.26^{c}	0.609 ± 0.02^{a}	1.361 ± 0.15^{b}	1.551 ± 0.10^{c}	0.667 ± 0.45^{a}	1.337 ± 0.09^{b}	1.746 ± 0.10^{d}	1.321 ± 0.11^{b}
8	Tryptophan[E]	0th	8.220 ± 0.05^{i}	0.041 ± 0.20^{b}	6.050 ± 0.02^{g}	0.021 ± 0.20^{a}	0.150 ± 0.11^{c}	0.280 ± 0.05^{e}	0.170 ± 0.06^{d}	3.660 ± 0.04^{f}	8.160 ± 0.17^{h}
		24th	2.410 ± 0.26^{d}	3.173 ± 0.13^{e}	<0.001	1.636 ± 0.21^{c}	0.380 ± 0.02^{a}	0.215 ± 0.06^{a}	0.851 ± 0.06^{b}	3.489 ± 0.20	2.816 ± 0.18^{d}
		48th	1.340 ± 0.26^{d}	2.690 ± 0.22^{f}	<0.001	0.628 ± 0.06^{c}	1.458 ± 0.08^{d}	0.286 ± 0.03^{a}	0.464 ± 0.06^{b}	1.398 ± 0.12^{d}	1.818 ± 0.07^{e}
		72nd	0.310 ± 0.01^{b}	1.471 ± 0.12^{e}	<0.001	0.603 ± 0.03^{c}	0.843 ± 0.05^{d}	0.193 ± 0.38^{a}	0.507 ± 0.36^{c}	0.876 ± 0.09^{d}	<0.001

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TABLE 2 (Continued)

Sl. no	Amino acid standards	GT	Pearl millet	Sorghum	Barnyard millet	Foxtail millet	Finger millet	Kodo millet	Little millet	Proso millet	Amaranth
9	Histidine^{E}	0th	1.069 ± 0.10^{f}	0.037 ± 0.04^{a}	0.160 ± 0.20^{d}	0.039 ± 0.11^{a}	0.084 ± 0.14^{b}	0.125 ± 0.02^{c}	0.273 ± 0.76^{e}	1.268 ± 0.05^{g}	5.587 ± 0.01^{h}
		24th	4.472 ± 0.27^{d}	<0.001	4.733 ± 0.09^{d}	3.736 ± 0.17^{c}	<0.001	1.557 ± 0.28^{b}	3.464 ± 0.29^{c}	8.214 ± 0.14^{e}	0.331 ± 0.10^{a}
		48th	7.606 ± 0.26^{g}	<0.001	10.222 ± 0.11^{h}	1.279 ± 0.06^{b}	1.645 ± 0.13^{c}	2.288 ± 0.30^{d}	3.273 ± 0.31^{e}	4.675 ± 0.28^{f}	0.041 ± 0.02^{a}
		72nd	12.671 ± 0.11^{e}	1.283 ± 0.26^{a}	11.363 ± 0.30	1.673 ± 0.10^{b}	2.491 ± 0.27^{c}	1.464 ± 0.20^{a}	4.429 ± 0.28^{d}	1.454 ± 0.11^{a}	2.586 ± 0.18^{c}
10	Glutamic acid^{NE}	0th	0.543 ± 0.30^{c}	1.277 ± 0.06^{f}	4.394 ± 0.08^{i}	0.078 ± 0.06^{a}	0.468 ± 0.02^{b}	0.606 ± 0.13^{d}	1.905 ± 0.05^{g}	0.836 ± 0.10^{e}	3.027 ± 0.09^{h}
		24th	3.779 ± 0.11^{f}	2.674 ± 0.21^{d}	2.440 ± 0.25^{c}	2.842 ± 0.16^{e}	0.938 ± 0.05^{b}	0.546 ± 0.14^{a}	2.417 ± 0.19^{c}	5.703 ± 0.21^{g}	<0.001
		48th	4.666 ± 0.31^{e}	5.256 ± 0.17^{f}	1.646 ± 0.19^{b}	2.639 ± 0.33^{c}	3.378 ± 0.29^{d}	1.370 ± 0.10^{b}	2.553 ± 0.06^{c}	3.532 ± 0.11^{d}	0.551 ± 0.11^{a}
		72nd	5.845 ± 0.10^{e}	6.624 ± 0.17^{f}	1.498 ± 0.10^{b}	3.863 ± 0.10^{d}	3.721 ± 0.18^{d}	1.355 ± 0.08^{a}	2.390 ± 0.15	1.939 ± 0.05^{c}	1.404 ± 0.21^{b}
11	Glycine^{NE}	0th	0.034 ± 0.08^{c}	0.072 ± 0.06^{e}	0.177 ± 0.07^{g}	0.005 ± 0.07^{b}	0.048 ± 0.04^{d}	0.001 ± 0.11^{a}	0.129 ± 0.11^{f}	1.346 ± 0.06^{h}	1.485 ± 0.31^{i}
		24th	2.314 ± 0.16^{g}	1.913 ± 0.03^{f}	1.190 ± 0.07^{d}	1.427 ± 0.12^{e}	0.768 ± 0.11^{c}	0.575 ± 0.06^{b}	0.782 ± 0.07^{c}	2.455 ± 0.17^{g}	0.268 ± 0.06^{a}
		48th	2.420 ± 0.23^{f}	1.757 ± 0.17^{e}	0.613 ± 0.07^{c}	0.514 ± 0.07^{b}	1.308 ± 0.14^{d}	0.446 ± 0.07^{a}	0.523 ± 0.02^{b}	1.440 ± 0.27^{d}	<0.001
		72nd	2.542 ± 0.28^{e}	1.292 ± 0.23^{c}	0.628 ± 0.06^{a}	1.602 ± 0.06^{d}	1.675 ± 0.11^{d}	0.585 ± 0.05^{a}	1.209 ± 0.18^{c}	1.392 ± 0.07^{c}	0.887 ± 0.08^{b}
12	Proline^{NE}	0th	0.064 ± 0.05^{d}	0.038 ± 0.10^{a}	0.058 ± 0.02^{c}	0.041 ± 0.05^{b}	0.062 ± 0.07^{d}	0.105 ± 0.02^{e}	1.071 ± 0.12^{g}	0.146 ± 0.06^{f}	4.368 ± 0.09^{h}
		24th	2.776 ± 0.22^{d}	3.337 ± 0.11^{e}	<0.001	1.408 ± 0.06^{c}	0.535 ± 0.03^{a}	0.540 ± 0.11^{a}	1.199 ± 0.15^{b}	3.803 ± 0.16^{f}	0.595 ± 0.03^{a}
		48th	3.433 ± 0.17^{d}	4.278 ± 0.09^{d}	0.517 ± 0.07^{a}	0.544 ± 0.08^{a}	0.876 ± 0.37^{b}	0.580 ± 0.06^{a}	1.441 ± 0.09^{c}	1.405 ± 0.15^{c}	<0.001
		72nd	5.389 ± 0.45^{f}	4.786 ± 0.16^{e}	0.423 ± 0.06^{a}	1.620 ± 0.13^{d}	1.440 ± 0.12^{c}	0.405 ± 0.17^{a}	1.316 ± 0.15^{c}	0.760 ± 0.10^{b}	1.313 ± 0.17^{c}
13	Aspartic acid^{NE}	0th	0.299 ± 0.17^{h}	0.275 ± 0.02^{g}	0.166 ± 0.07^{d}	0.002 ± 0.02^{a}	0.038 ± 0.06^{b}	0.059 ± 0.07^{c}	0.839 ± 0.02^{i}	0.171 ± 0.02^{e}	0.199 ± 0.05^{f}
		24th	2.723 ± 0.10^{d}	3.328 ± 0.18^{e}	<0.001	1.549 ± 0.12^{c}	1.473 ± 0.11^{c}	0.062 ± 0.72^{a}	0.499 ± 0.06^{b}	0.375 ± 0.69^{b}	0.388 ± 0.04^{b}
		48th	3.664 ± 0.14^{e}	3.551 ± 0.12^{e}	1.690 ± 0.17^{d}	0.264 ± 0.14^{a}	1.590 ± 0.05^{d}	0.793 ± 0.21^{c}	0.409 ± 0.13^{b}	0.431 ± 0.11^{b}	0.565 ± 0.14^{b}
		72nd	4.784 ± 0.22^{f}	6.298 ± 0.11^{g}	1.575 ± 0.08^{e}	0.417 ± 0.08^{b}	1.278 ± 0.14^{d}	0.225 ± 0.10^{a}	0.831 ± 0.14^{c}	1.166 ± 0.12^{d}	0.511 ± 0.07^{b}
14	Tyrosine^{NE}	0th	0.824 ± 0.10^{f}	0.040 ± 0.04^{a}	1.588 ± 0.11^{g}	0.058 ± 0.20^{b}	0.444 ± 0.03^{e}	0.233 ± 0.05^{c}	0.436 ± 0.11^{d}	1.672 ± 0.09^{h}	8.877 ± 0.27^{i}
		24th	4.429 ± 0.10^{f}	3.537 ± 0.11^{f}	1.299 ± 0.05^{d}	2.373 ± 0.18^{e}	0.232 ± 0.02^{a}	0.619 ± 0.08^{b}	0.616 ± 0.05^{b}	2.733 ± 0.08^{e}	0.925 ± 0.05^{c}
		48th	3.420 ± 0.14^{f}	0.473 ± 0.06^{a}	0.813 ± 0.09^{b}	1.333 ± 0.17^{c}	2.660 ± 0.11^{e}	0.743 ± 0.04^{b}	0.289 ± 0.45^{a}	1.619 ± 0.24^{d}	<0.001
		72nd	5.606 ± 0.19^{c}	3.743 ± 0.13^{b}	0.477 ± 0.05^{a}	0.398 ± 0.03^{a}	1.720 ± 0.15	0.585 ± 0.01^{a}	0.497 ± 0.05^{a}	8.641 ± 0.20^{d}	0.496 ± 0.05^{a}
15	Hydroxyproline^{NE}	0th	0.951 ± 0.15^{h}	0.077 ± 0.03^{b}	0.783 ± 0.10^{f}	0.034 ± 0.09^{a}	0.271 ± 0.03^{e}	0.083 ± 0.06^{c}	0.171 ± 0.02^{d}	0.896 ± 0.05^{g}	2.148 ± 0.02^{i}
		24th	1.849 ± 0.10^{e}	2.170 ± 0.14^{f}	<0.001	1.651 ± 0.10^{c}	0.590 ± 0.07^{a}	0.483 ± 0.04^{a}	1.232 ± 0.19^{d}	2.578 ± 0.13^{f}	0.918 ± 0.02^{b}
		48th	1.781 ± 0.07^{b}	1.848 ± 0.08^{c}	<0.001	0.861 ± 0.10^{a}	1.758 ± 0.10^{b}	0.678 ± 0.02^{a}	0.896 ± 0.03^{a}	1.838 ± 0.11^{c}	<0.001
		72nd	2.350 ± 0.08^{e}	1.924 ± 0.08^{d}	0.425 ± 0.06^{a}	1.433 ± 0.10^{b}	1.501 ± 0.06^{c}	0.629 ± 0.05^{a}	1.350 ± 0.07^{b}	1.666 ± 0.08^{c}	1.286 ± 0.05^{b}
16	Alanine^{NE}	0th	0.110 ± 0.10^{f}	0.017 ± 0.10^{b}	0.023 ± 0.04^{c}	0.010 ± 0.15^{a}	0.058 ± 0.10^{e}	0.032 ± 0.10^{d}	0.372 ± 0.06^{g}	1.066 ± 0.17^{h}	1.708 ± 0.11^{i}
		24th	1.228 ± 0.09^{c}	2.260 ± 0.14^{e}	1.228 ± 0.16^{c}	1.450 ± 0.09^{d}	0.925 ± 0.06^{b}	0.748 ± 0.04^{a}	0.891 ± 0.05^{b}	3.855 ± 0.07^{f}	0.624 ± 0.10^{a}
		48th	2.859 ± 0.05^{f}	2.171 ± 0.10^{e}	0.703 ± 0.04^{a}	0.939 ± 0.01^{b}	1.733 ± 0.26^{d}	0.680 ± 0.15^{a}	1.200 ± 0.14^{c}	2.433 ± 0.10^{f}	<0.001
		72nd	3.169 ± 0.12^{e}	2.311 ± 0.20^{c}	0.620 ± 0.03^{a}	2.359 ± 0.07^{c}	2.517 ± 0.06^{d}	0.580 ± 0.07^{a}	1.532 ± 0.15^{b}	2.145 ± 0.09^{c}	1.266 ± 0.15^{b}

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TABLE 2 (Continued)

Sl. no	Amino acid standards	GT	Pearl millet	Sorghum	Barnyard millet	Foxtail millet	Finger millet	Kodo millet	Little millet	Proso millet	Amaranth
17	Serine^{NE}	0th	0.058 ± 0.16^{f}	0.018 ± 0.14^{b}	0.023 ± 0.08^{c}	<0.001	0.003 ± 0.06^{a}	0.047 ± 0.18^{d}	0.047 ± 0.11^{e}	0.071 ± 0.07^{g}	0.14 ± 0.20^{h}
		24th	1.251 ± 0.11^{c}	1.702 ± 0.14^{e}	0.942 ± 0.04^{b}	1.489 ± 0.08^{d}	0.482 ± 0.12^{a}	<0.001	0.577 ± 0.11^{a}	0.584 ± 0.14^{a}	0.542 ± 0.12^{a}
		48th	2.384 ± 0.25^{d}	2.415 ± 0.23^{d}	0.921 ± 0.03^{b}	0.474 ± 0.03^{a}	1.320 ± 0.08^{c}	0.922 ± 0.05^{b}	0.862 ± 0.25^{b}	1.385 ± 0.25^{c}	<0.001
		72nd	2.702 ± 0.14^{f}	3.330 ± 0.14^{g}	0.775 ± 0.07^{b}	0.378 ± 0.45^{a}	1.438 ± 0.06^{d}	0.764 ± 0.02^{b}	1.267 ± 0.08^{d}	1.844 ± 0.08^{e}	0.927 ± 0.06^{c}
18	Aspargine^{NE}	0th	0.254 ± 0.08^{e}	0.010 ± 0.06^{c}	0.001 ± 0.05^{a}	<0.001	<0.001	0.004 ± 0.07^{b}	0.374 ± 0.06^{f}	0.068 ± 0.10^{d}	0.814 ± 0.26^{g}
		24th	0.917 ± 0.03^{c}	1.845 ± 0.14^{c}	<0.001	0.458 ± 0.16^{b}	0.064 ± 0.18^{a}	0.032 ± 0.58^{a}	0.317 ± 0.09^{b}	1.872 ± 0.07^{c}	0.066 ± 0.01^{a}
		48th	0.881 ± 0.12^{c}	0.826 ± 0.06^{c}	1.203 ± 0.12^{d}	0.243 ± 0.05^{b}	0.845 ± 0.15^{c}	0.083 ± 0.02^{a}	0.432 ± 0.07^{b}	0.858 ± 0.09^{c}	<0.001
		72nd	1.442 ± 0.12^{e}	1.703 ± 0.16^{f}	0.728 ± 0.09^{c}	0.341 ± 0.05^{b}	0.084 ± 0.34^{a}	0.214 ± 0.05^{b}	0.640 ± 0.08^{c}	0.848 ± 0.06^{d}	0.383 ± 0.14^{b}
19	Cystine^{NE}	0th	0.051 ± 0.11^{a}	<0.001	0.279 ± 0.12^{b}	0.008 ± 0.05^{a}	<0.001	0.041 ± 0.12^{b}	<0.001	0.078 ± 0.04^{a}	0.435 ± 0.02^{c}
		24th	0.055 ± 0.03^{a}	0.047 ± 0.60^{a}	<0.001	0.079 ± 0.14^{a}	<0.001	<0.001	<0.001	0.247 ± 0.09^{b}	<0.001
		48th	0.379 ± 0.05^{b}	0.074 ± 0.47^{a}	0.022 ± 0.08^{a}	<0.001	0.041 ± 0.02^{a}	<0.001	0.031 ± 0.14^{a}	0.266 ± 0.10^{b}	<0.001
		72nd	0.716 ± 0.11^{c}	0.026 ± 0.01^{a}	0.042 ± 0.01^{b}	0.015 ± 0.02^{a}	0.064 ± 0.62^{b}	<0.001	0.072 ± 0.02^{b}	0.066 ± 0.02^{b}	0.045 ± 0.06^{b}
20	Arginine^{NE}	0th	0.679 ± 0.25^{f}	0.581 ± 0.17^{e}	0.837 ± 0.22^{g}	0.006 ± 0.10^{a}	0.237 ± 0.13^{c}	0.255 ± 0.09^{d}	0.146 ± 0.06^{b}	1.218 ± 0.02^{h}	1.427 ± 0.09^{i}
		24th	1.901 ± 0.02^{f}	0.255 ± 0.06^{b}	<0.001	0.816 ± 0.55^{d}	0.352 ± 0.18^{c}	0.055 ± 0.15^{a}	0.315 ± 0.04^{c}	1.213 ± 0.16^{e}	0.033 ± 0.01^{a}
		48th	0.441 ± 0.80^{a}	0.882 ± 0.06^{c}	<0.001	0.531 ± 0.09^{a}	0.909 ± 0.05^{d}	1.201 ± 0.16^{e}	0.660 ± 0.09^{b}	0.881 ± 0.06^{c}	<0.001
		72nd	0.231 ± 0.08^{b}	1.383 ± 0.13^{f}	0.157 ± 0.06^{a}	0.785 ± 0.01^{d}	0.255 ± 0.03^{b}	0.639 ± 0.05^{c}	0.918 ± 0.02^{e}	0.682 ± 0.05^{c}	0.147 ± 0.68^{a}
21	Cysteine^{NE}	0th	0.039 ± 0.01^{a}	0.091 ± 0.11^{d}	0.642 ± 0.20^{g}	0.056 ± 0.11^{b}	0.142 ± 0.01^{e}	0.078 ± 0.02^{c}	0.168 ± 0.03^{f}	0.167 ± 0.02^{f}	0.696 ± 0.11^{h}
		24th	0.766 ± 0.07^{d}	0.087 ± 0.01^{a}	0.254 ± 0.01^{b}	0.237 ± 0.06^{b}	0.047 ± 0.45^{a}	0.070 ± 0.04^{a}	0.085 ± 0.26^{a}	0.637 ± 0.09^{c}	0.042 ± 0.01^{a}
		48th	0.298 ± 0.06^{c}	0.139 ± 0.03^{b}	0.489 ± 0.04^{d}	0.088 ± 0.08^{a}	0.181 ± 0.08^{b}	0.076 ± 0.14^{a}	0.084 ± 0.34^{a}	2.886 ± 0.06^{c}	0.007 ± 0.04^{a}
		72nd	0.487 ± 0.06^{d}	0.138 ± 0.25^{b}	0.406 ± 0.02^{d}	0.265 ± 0.05^{c}	0.186 ± 0.04^{b}	0.780 ± 0.09^{e}	0.836 ± 0.04^{f}	0.287 ± 0.03^{c}	0.085 ± 0.02^{a}
22	Glutamine^{NE}	0th	0.509 ± 0.14^{f}	0.063 ± 0.06^{b}	6.873 ± 0.12^{i}	0.003 ± 0.06^{a}	0.136 ± 0.07^{d}	0.439 ± 0.12^{e}	0.992 ± 0.02^{g}	0.113 ± 0.07^{c}	3.112 ± 0.01^{h}
		24th	1.456 ± 0.07^{e}	2.876 ± 0.06^{g}	<0.001	1.355 ± 0.18^{f}	0.735 ± 0.09^{c}	0.293 ± 0.05^{a}	0.332 ± 0.10^{b}	1.580 ± 0.07^{f}	0.918 ± 0.09^{d}
		48th	1.184 ± 0.09^{c}	1.765 ± 0.16^{d}	2.389 ± 0.22^{e}	1.138 ± 0.08^{c}	0.667 ± 0.08^{a}	1.202 ± 0.13^{c}	1.350 ± 0.07^{c}	0.826 ± 0.08^{b}	<0.001
		72nd	0.230 ± 0.10^{a}	6.298 ± 0.16^{e}	0.598 ± 0.04^{b}	0.637 ± 0.12^{b}	<0.001	1.343 ± 0.10^{c}	2.401 ± 0.20	0.543 ± 0.11^{b}	1.868 ± 0.07^{d}

Note: Values are means ± SD ($n = 3$), followed by different superscript letters are significantly different ($p \leq .05$). The letter "a" represents the least value.
Abbreviations: E, essential amino acids; NE, nonessential amino acids.

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TABLE 3 Vitamin content of selected ancient grains (ng/g).

Vitamins		Pearl millet	Sorghum	Barnyard millet	Foxtail millet	Finger millet	Kodo millet	Little millet	Proso millet	Amaranth
Thiamine	0th	2.80 ± 0.16^{c}	1.55 ± 0.26^{b}	1.40 ± 0.04^{b}	11.57 ± 0.10^{g}	3.53 ± 0.13^{d}	3.50 ± 0.13^{d}	4.46 ± 0.07^{e}	5.17 ± 0.03^{f}	0.18 ± 1.04^{a}
	24th	3.40 ± 0.19^{a}	6.31 ± 0.30^{b}	6.29 ± 0.17^{b}	22.75 ± 0.09^{e}	<0.001	22.33 ± 0.26^{e}	10.54 ± 0.19^{d}	7.91 ± 0.06^{c}	<0.001
	48th	3.81 ± 0.18^{c}	4.63 ± 0.38^{d}	0.50 ± 0.07^{a}	26.66 ± 0.22^{g}	1.43 ± 0.12^{b}	16.46 ± 0.20^{f}	6.81 ± 0.07^{e}	4.80 ± 0.13^{d}	<0.001
	72nd	1.68 ± 0.27^{b}	6.73 ± 0.27^{d}	0.85 ± 0.07^{a}	12.42 ± 0.11^{e}	<0.001	5.69 ± 0.10^{c}	2.47 ± 0.15^{b}	<0.001	<0.001
Riboflavin	0th	4.24 ± 0.15^{d}	2.25 ± 0.09^{b}	3.71 ± 0.07^{c}	2.57 ± 0.40^{b}	1.63 ± 0.24^{a}	2.20 ± 0.10^{b}	2.15 ± 0.09^{b}	21.24 ± 0.07^{f}	9.60 ± 0.27^{e}
	24th	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	48th	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	72nd	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Niacin	0th	219.34 ± 0.19^{f}	390.67 ± 0.09^{i}	194.69 ± 0.16^{e}	277.34 ± 0.32^{g}	177.62 ± 0.15^{c}	136.81 ± 0.15^{a}	182.44 ± 0.06^{d}	283.94 ± 0.09^{h}	166.47 ± 0.24^{b}
	24th	165.13 ± 0.09^{f}	244.06 ± 1.86^{g}	127.70 ± 0.76^{e}	95.13 ± 0.80^{d}	64.97 ± 0.79^{a}	83.30 ± 0.54^{c}	167.24 ± 1.35^{f}	294.35 ± 1.47^{h}	70.92 ± 1.30^{b}
	48th	146.62 ± 1.26^{g}	296.10 ± 0.68^{i}	63.99 ± 0.79^{d}	51.90 ± 0.47^{b}	96.99 ± 0.60^{e}	29.30 ± 0.70^{a}	107.34 ± 0.93^{f}	205.49 ± 1.20^{h}	57.41 ± 0.53^{c}
	72nd	348.81 ± 1.16^{h}	181.70 ± 1.02^{g}	67.83 ± 0.62^{c}	57.36 ± 0.94^{b}	22.32 ± 2.11^{a}	51.67 ± 1.08^{b}	107.37 ± 1.30^{d}	167.69 ± 0.60^{f}	119.01 ± 0.47^{e}
Pyridoxine	0th	0.85 ± 0.06^{c}	2.12 ± 0.09^{d}	0.25 ± 0.05^{a}	0.74 ± 0.05^{c}	11.28 ± 0.08^{h}	2.75 ± 0.04^{e}	4.89 ± 0.06^{f}	6.50 ± 0.07^{g}	0.48 ± 0.05^{b}
	24th	4.20 ± 0.09^{c}	4.78 ± 0.10^{c}	5.71 ± 0.15^{d}	3.73 ± 0.17^{b}	6.36 ± 0.15^{e}	5.74 ± 0.12^{d}	14.17 ± 0.14^{f}	3.41 ± 0.06^{b}	2.14 ± 0.05^{a}
	48th	3.85 ± 0.09^{b}	5.20 ± 0.10^{c}	3.63 ± 0.06^{b}	2.79 ± 0.05^{a}	5.62 ± 0.15^{c}	6.80 ± 0.07^{d}	5.64 ± 0.08^{c}	2.22 ± 0.12^{a}	2.57 ± 0.07^{a}
	72nd	5.82 ± 0.06^{c}	1.24 ± 0.09^{a}	1.47 ± 0.19^{a}	<0.001	1.62 ± 0.14^{a}	7.50 ± 0.29^{d}	2.13 ± 0.08^{b}	2.51 ± 0.08^{b}	2.85 ± 0.03^{b}
Folic acid	0th	125.46 ± 0.12^{f}	84.50 ± 0.04^{b}	111.74 ± 0.13^{e}	189.79 ± 0.31^{i}	104.58 ± 0.60^{d}	91.80 ± 0.09^{c}	67.65 ± 0.06^{a}	136.52 ± 0.20^{g}	137.86 ± 0.11^{h}
	24th	134.14 ± 0.07^{f}	98.66 ± 0.57^{c}	6.58 ± 0.15^{a}	148.63 ± 0.62^{g}	25.20 ± 0.19^{b}	152.95 ± 0.23^{h}	106.68 ± 0.57^{d}	737.37 ± 3.91^{i}	123.20 ± 0.86^{e}
	48th	44.12 ± 0.07^{c}	234.61 ± 0.12^{h}	<0.001	83.27 ± 0.25^{f}	61.69 ± 0.64^{e}	1.33 ± 0.09^{a}	2.33 ± 0.12^{b}	171.70 ± 0.59^{g}	55.83 ± 0.09^{d}
	72nd	168.71 ± 0.52^{g}	118.71 ± 0.57^{d}	22.49 ± 0.05^{a}	126.91 ± 0.33^{e}	<0.001	29.47 ± 0.34^{b}	61.89 ± 0.12^{c}	162.88 ± 0.64^{f}	115.45 ± 0.76^{d}
Ascorbic acid	0th	55.34 ± 0.34^{b}	86.88 ± 0.38^{g}	98.39 ± 0.11^{i}	78.03 ± 0.36^{e}	46.60 ± 0.17^{a}	68.84 ± 0.30^{c}	73.21 ± 0.12^{d}	99.67 ± 0.16^{h}	83.24 ± 0.21^{f}
	24th	557.11 ± 0.66^{e}	901.85 ± 0.71^{g}	246.70 ± 1.05^{b}	244.66 ± 1.08^{b}	357.73 ± 0.77^{c}	505.46 ± 1.20^{d}	384.30 ± 0.62^{c}	807.51 ± 0.90^{f}	42.96 ± 0.68^{a}
	48th	670.72 ± 0.70^{c}	1812.84 ± 0.49^{j}	137.86 ± 0.58^{a}	1694.90 ± 0.72^{g}	374.78 ± 0.81^{b}	723.96 ± 0.74^{d}	1143.64 ± 1.08^{f}	1737.19 ± 1.61^{h}	1005.58 ± 0.99^{e}
	72nd	623.36 ± 0.80^{b}	827.47 ± 1.01^{d}	709.16 ± 1.32^{c}	30452.90 ± 2.02^{h}	703.46 ± 1.21^{c}	4556.93 ± 3.25^{g}	283.31 ± 1.11^{a}	3214.49 ± 2.39^{f}	1248.62 ± 1.19^{e}

Note: Values are means ± SD ($n = 3$), followed by different superscript letters are significantly different ($p \leq .05$). The letter "a" represents the least value.

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Amino acid composition of millets

In the present study, the quantification of amino acids in grains was carried out using LC–MS/MS (Table 2). The samples were analyzed, and the peaks were compared with those of reference compounds analyzed under the same conditions. The ancient grains were found to be fair sources of both essential and nonessential amino acids. Among the grains studied, raw amaranth was found to be the best source of amino acids (both essential and nonessential) as it contained the highest amount of almost all the amino acids, followed by barnyard, pearl, and proso millet. Amaranth was rich in phenylalanine (2.743 ± 0.08 μg/g), methionine (6.413 ± 0.02 μg/g), valine (5.583 ± 0.41 μg/g), and arginine (1.427 ± 0.09 μg/g). Amaranth grains are reported to be superior over other conventional cereals as it possesses a more balanced composition of essential amino acids.[2] The grain proteins of common wheat and durum wheat are rich in glutamic acid and proline, but low in essential amino acids, especially lysine and threonine, as well as tryptophan, methionine, and isoleucine.[22] Another study has reported that amaranth grains contained higher amounts of lysine, methionine, and arginine.[23] It is also reported that amaranth was a good source of amino acids especially phenylalanine (4.17 μg/g), methionine (4.09 μg/g), valine (3.78 μg/g), tryptophan (7.79 μg/g), lysine (3.33 μg/g), and isoleucine (03.20 μg/g), which are comparable to the values found in the present study.[9] In this study, cysteine was found to be present in meager quantities in millet grains, similar to that reported by Kamara et al.,[24] which means that S-S bonds were absent in them. The breakdown of protease resistant prolamins and the increase of essential amino acids upon germination have been reported.[18] An increase in the amount of phenylalanine and valine has been reported in sorghum varieties upon germination,[25] which is in line with our results as the content of phenylalanine increased from 0.011 μg/g in the raw form to 2.600 μg/g in germinated grain. The lysine content of sorghum increased four times upon germination from 0.069 to 4.194 μg/g. Besides, the nonessential amino acid content was found to decrease in the grains and glutamic acid increased in sorghum as observed by Afify et al..[25] It has been reported that the protein content of proso millet (11.6% of dry matter) is comparable to that of wheat, and its grain was also significantly more abundant in essential amino acids like leucine, isoleucine, and methionine than wheat protein as wheat is deficient in lysine and methionine.[25,26] The present study revealed a higher concentration of amino acids in the ancient grains. Thus, the traditional grains can be used as a substitute source for the conventional cereals, which are usually poor sources of lysine and methionine.

Vitamin composition of millets

Ancient grains were found to contain adequate amounts of B vitamins and ascorbic acid (Table 3). Proso followed by amaranth, foxtail, and pearl millet were found to be the richest source of most of the vitamins studied. Thiamine content was found to be highest in foxtail millet (11.57 ± 0.10 ng/g) followed by proso and pearl millet. Proso millet was found to be a good source of riboflavin (21.24 ± 0.07 ng/g). According to Kumar et al.,[27] foxtail millet (1.65 mg/100 g) and pearl millet (1.48 mg/100 g) contained reasonable amounts of riboflavin. The riboflavin content of the millets is found to be several folds higher than the staple cereals. It has also been observed that the riboflavin content was highest in proso millet (0.28 mg/100 g) and thiamine in foxtail millet (0.59 mg/100 g), which was in accordance with the results of the present study.[11] Niacin content was found in good amounts in almost all the grains. Saleh et al.[11] have stated that the niacin content of sorghum (jowar) is 4.3 mg/100 g, common millet (proso) is 4.5 mg/100 g, and barnyard millet is 4.2 mg/100 g. Pyridoxine was found to be the highest in finger millet (11.28 ± 0.08 ng/g). Foxtail millet (189.79 ± 0.31 ng/g) followed by amaranth (137.86 ± 0.11 ng/g) and proso millets (136.52 ± 0.20 ng/g) contained good amounts of folic acid. The vitamin B content in cereal grains generally increases due to sprouting and it also supports the seedling development and growth.[28] Riboflavin content of the grains diminished under germination. The reduction of some B vitamins during germination can be attributed to the fact that the de novo synthesis of vitamins is only initiated in later sprouting stages, and water-soluble vitamins can leach into the steeping water.[29] Variations in the vitamin contents of sprouted grains can be attributed to the type of grain and conditions of steeping and sprouting.[28]

Barnyard millet was found to be the most abundant source of ascorbic acid (98.39 ± 0.11 ng/g). There was a significant increase in the vitamin C content of the ancient grains upon germination. The increase in vitamin C during malting/germination is steered by the enzymatic hydrolysis of starch by amylases and diastases, which increase the availability of glucose for the biosynthesis of vitamin C.[30] This escalated content of glucose acts as a predecessor to the formation of vitamin C.[31]

CONCLUSIONS

From the study, it can thus be concluded that ancient grains are highly nutritious grains with a fair amount of protein, essential amino acids, and vitamins. They are nutritionally superior in terms of almost all the macronutrients present in them. These traditional grains contain a balanced amount of amino acids and are also rich in essential amino acids like methionine, valine, and tryptophan. They also contain good amounts of ascorbic acid and B vitamins. Thus, these ancient grains need to be exploited both in terms of their health as well as the nutritional benefits for alleviating the widespread nutritional deficiency around the globe, especially in tropical and subtropical countries. The regular incorporation of these gluten-free traditional grains in our daily diet can lead to significant health benefits and can also help in improving the overall health of people. Combination of millet grains can be used to improve its nutritional quality in our diet. Future trends can focus on the use of these grains for inclusion in gluten-free diets.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest in this work.

DATA AVAILABILITY STATEMENT

Data will be made available on request

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