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Impact of fermentation time on the *in vitro* enzymatic digestibility of traditionally extracted pearl millet (*Pennisetum glaucum*) starch

Manhal Gobara Hamid [a,b], Khitma A. Sir Elkhatim [a,b], Yousif M.A. Idris [b], Mohammed Elsafy [c], Mahbubjon Rahmatov [c], Tilal Abdelhalim [a], Faraz Muneer [c,*]

[a] Biotechnology and Biosafety Research Center, Agricultural Research Corporation, P.O. Box 126, Shambat, Khartoum North, Sudan
[b] College of Agricultural Studies, Sudan University of Science and Technology, Shambat, Khartoum North, Sudan
[c] Department of Plant Breeding, Swedish University of Agricultural Sciences (SLU), P.O. Box 190, 234 22, Lomma, Sweden

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ABSTRACT

In this study, we used a traditional Sudanese fermentation method to extract starch from pearl millet (*Pennisetum glaucum* L.) varieties and investigated the impact of fermentation time (1 and 7 days) on *in vitro* starch digestibility. From fermented grains two distinct layers of starch were obtained i.e. the upper layer (*Jir Khadim),* and the lower layer (*Jir Hur*). The *in vitro* digestibility analysis of all the starch samples showed that both starch layers exhibited rapid hydrolysis rates within the first 60 min of enzymatic digestion, although the 7-day fermented starches exhibited significantly higher hydrolysis rates after 120 min than 1-day fermented samples. Scanning electron microscopy have shown that the longer fermentation time impacted starch granule morphology with surface perforations, indentations, and granule degradation that might have contributed to higher starch digestibility. Pearl millet varieties with higher amylose content i.e. Ashana and Bioda, showed slower hydrolysis rates, higher resistant starch (RS) levels, and reduced readily digestible starch (RDS). These findings demonstrate that fermentation time significantly affects starch digestibility and fraction distribution, with amylose content and fermentation-induced structural modifications playing key roles. This study underlines the potential for standardizing indigenous food-processing methods for developing modern food products with tailored digestibility profiles.

1. Introduction

Starch is an important storage carbohydrate that provides food with nutritional and functional properties. It contributes to texture, viscosity, water retention, and mouthfeel in directly consumed and processed food products (Punia et al., 2021). Beyond its nutritional role, predicting and controlling starch digestibility is vital because of the high consumption of starchy foods and the associated health concerns across different population groups (Chi et al., 2022; Taylor et al., 2015). The occurrence of increasing obesity and diet-related chronic diseases (cardiovascular diseases and type II diabetes) is a major concern which are linked to high consumption of starch-rich food products and their digestibility (Korompokis et al., 2021). Starch and starchy food products exhibit varying degrees of enzymatic digestibility, which leads to different glycemic responses upon consumption (Jenkins et al., 1987). Different individuals, including children, athletes, middle-aged and older individuals, have varying requirements for starch digestion rates and extents (Chi et al., 2022). Therefore, the understanding of structural features that govern the required food properties and starch hydrolysis during digestion are pre-requisite to mitigate any negative health implications of starch based diets. This includes assessing rate of starch enzymatic digestion and the glycemic index of starchy foods, which are influenced by several factors, such as pre-processing methods, cooking techniques (Bravo et al., 1998), and pre-treatments like grain dehulling (Alonso et al., 2000).

Starch functionality is influenced by the susceptibility to enzymatic breakdown and it is largely affected by the proportions of rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) present in various types of starch sources (Wang et al., 2015). The human gastrointestinal system utilizes two types of enzymes for starch digestion: salivary and pancreatic α-amylases and intestinal brush border enzymes, including glucoamylase, maltase-glucoamylase, and sucrase-isomaltase. These enzymes primarily hydrolyze and metabolize RDS and SDS in the small intestine, whereas RS remains undigested and

* Corresponding author.
E-mail address: faraz.muneer@slu.se (F. Muneer).

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passes through the large intestine (Zhang et al., 2020). Uncooked starch is less susceptible to enzymatic hydrolysis (Du et al., 2014), whereas gelatinized starch undergoes structural disorganization, which increases its accessibility to digestive enzymes and facilitates hydrolysis (Chung, Liu, Peter Pauls, et al., 2008). Starch digestibility among species varies owing to factors such as the source of starch, surface granule organization (e.g., pores, size, and architecture), amylose-to-amylopectin ratio, degree of crystallinity, and type of crystalline polymorphic form (Lindeboom et al., 2004; Sandhu & Lim, 2008; Sandhu & Siroha, 2017; Stevnebø et al., 2006). In addition, starch's physicochemical properties, which can influence digestibility, are affected by different isolation methods, processing and storage conditions (Du et al., 2014).

Among starch isolation methods, spontaneous fermentation has been historically used as a pre-processing method in many traditional food recipes (Saeed et al., 2022). During fermentation, microbial communities, primarily comprising bacteria (mainly lactic acid bacteria) and fungi (yeast), break down starch through enzymatic activity and the production of organic acids. This process enhances the functional properties of starch, making it more suitable for the preparation and improvement of various starch-based foods (Bian et al., 2022; Usha et al., 1996). In addition, spontaneous fermentation of grains is also known to reduce anti-nutritional factors such as tannins and phytates, thereby improving the overall digestibility profile of pearl millet starches (Gupta & Gaur, 2024). Previous studies have shown that conventional isolation methods induce changes in the thermal, textural, and pasting properties of starch from various crop sources (Correia et al., 2012, Correia et al., 2013; Estrada-León et al., 2016; Zhang et al., 2020). However, conventional methods for starch extraction and modification often rely on chemicals, which can have adverse environmental and health impacts. In contrast, environmentally friendly approaches like fermentation offer a green and sustainable alternative. Thus, understanding starch digestibility from unconventional sources such as pearl millet and exploring the effects of traditional pre-processing of grains (e.g. fermentation) for starch extraction can significantly expand their use in food products (Punia et al., 2021). Examining how these pre-treatments influence starch breakdown and overall structure-digestibility relationship offer opportunities for targeted starch modification during food production and improvement of nutritional value of starchy food. Additionally, leveraging traditional methods that are not very well documented can help preserve cultural heritage while meeting modern dietary needs and innovation.

Pearl millet is a staple cereal crop in Sudan. Grains are often fermented using indigenous methods to isolate starch fractions, known as *Jir*. These starches produce a pure white product for "*Jiryiia*" porridge preparation. The indigenous pre-processing of pearl millet grains to produce *Jir* involves complex fermentation steps that significantly influence the final product quality. However, limited information is available on how these indigenous extraction methods affect pearl millet starch's *in vitro* digestibility. Further research is needed to understand the impact of these indigenous methods on pearl millet starch digestibility and hydrolysis rate. This study aimed to investigate the impact of fermentation time through 1) assessing two fermentation durations (1 and 7 days) on starch isolation from five pearl millet varieties; 2) evaluating how the fermentation and isolation processes affected the hydrolysis rate of the extracted starch layers, free glucose and total starch contents and 3) measuring the *in vitro* digestibility of the different starch fractions.

2. Materials and methods

2.1. Materials

This study examined four Sudanese pearl millet genotypes along with one released cultivar. The cultivar Ashana is well known for its resistance to downy mildew, early maturation, and high yield. Other genotypes include Bioda, Dembi-green, Dembi-red, and Mayoa. Bioda, a white-seeded pearl millet favoured by farmers and commonly consumed as a porridge with milk. In contrast, Dembi-green, Dembi-red, and Mayoa are traditional genotypes that are highly valued in Western Sudan for their drought tolerance and yellow seeds. All genotypes were obtained from the Nayala Research Station of the Agricultural Research Corporation (ARC) in Sudan.

2.2. Isolation of starch (Jir) from pearl millet grains

Starch from pearl millet grains was extracted using a traditional method practiced by Sudanese women in Darfur, Sudan, as described by Dirar (1993, p. 41) with minor modifications. The extraction process began with dehulling, sun-drying, and winnowing of grains. The dehulled grains were then placed in a plastic bucket with water (approximately 3:1 water-to-grain ratio) for spontaneous fermentation, lasting either 1 day or 7 days. During fermentation, evaporated water was replenished, and surface water was periodically replaced with fresh water to maintain optimal conditions. The traditional two-step (1 and 7 days) spontaneous fermentation process of different millets (pearl and finger millets) for starch production involves both lactic acid bacteria (LAB) and yeast fermentation. In the first fermentation step, LAB dominate, with their count increasing as the yeast count decreases. This step is marked by a drop in pH (around 4) and an increase in titratable acidity. During the second fermentation step, the dynamics shift: the LAB count decreases while the yeast count rises. *Pediococcus* species are predominant during the first step, while *Lactobacillus acidophilus,* and *Lactobacillus jensenii* dominate in the second step. *Saccharomyces sinensis* emerges as the dominant yeast after both fermentation steps. During the whole process, LAB contribute to the formation of lactic acid and yeast produces succinic acid resulting in acidification of starch slurry (Zhao et al., 2019, Gupta et al., 2024).

After fermentation, the grains were sieved, sun-dried in a dust-protected area, and pounded in a wooden mortar to obtain fine flour. The fine flour was suspended in a large quantity of water and strained through a fine cloth to separate starch. This process was repeated with the residue remaining in the strainer until all starch-containing materials were extracted. The suspension was then covered and left undisturbed overnight to allow starch to settle. The surface water was carefully skimmed. The settled starch was separated into two distinct layers: an upper layer (*Jir Khadim*), characterized by a firmer texture, and a glittery lower layer (*Jir Hur*). Each layer was carefully collected, sun-dried in aluminium trays, and stored in zip-polythene bags at 4 °C for further analysis.

2.3. Scanning electron microscopy

The starch samples were mounted on sticky tape attached to metal stubs and sputter-coated with gold particles using a Cressington 108 auto (Cressington, Watford, UK) at 20 mA. The microstructure of the isolated starch samples was examined using a Hitachi SU3500 scanning electron microscope (Hitachi, Tokyo, Japan) at an acceleration voltage of 5 kV.

2.4. Analysis of free glucose (FG) content

The glucose concentration of the *Jir* samples was determined using a 3,5-dinitroalisalicylic acid (DNS) assay (Miller, 1959). Starch samples (50 mg) were extracted with 5 ml of 80% ethanol at 85 °C. Aliquots (100 μl) of the extracts were mixed with 200 μl DNS reagent and heated at 98 °C for 10 min. The absorbance was measured at 540 nm using a Thermo Scientific Multiskan Go microplate spectrophotometer (Thermo Scientific, MA, USA). A standard glucose curve was used to calculate the glucose concentration.

2.5. Analysis of total starch content

Total starch content of the *Jir* samples was determined following Miller (1959), modified according to McCleary and Monaghan (2002). Starch samples (50 mg) were extracted with 80% ethanol, solubilized with 2M potassium hydroxide (KOH), and hydrolyzed with 0.1 ml of α-amylase (8300 U) and 0.1 ml of amyloglucosidase (3300 U). The resulting glucose concentration was measured using the DNS assay, and starch content was calculated using the following formula:

$$\text{Starch concentration} = \frac{\text{V x MW}}{\text{Ɛx d x v}} \text{ x } \frac{162}{180} \; x\, \Delta\text{AD} - \text{Glucose [g / L]}$$

Where.

V = final volume [mL[
MW = molecular weight of D-glucose 180.156 [g/mol]
Ɛ = extinction coefficient of DNS at 540 nm 13000 [l x mol-1 x cm-1]
d = light path [cm]
v = sample volume [mL]
$\frac{162}{180}$ = Adjustment from free D-glucose to anhydrous D-glucose.

$$\text{Starch (g / 100g)} = \frac{\text{starch concentration g\L}}{\textit{sample weight}} \; x\, 10$$

2.6. In vitro enzymatic digestibility

The *in vitro* digestibility of *Jir khadim and Jir hur* starches was determined using a method described by Wang et al. (2014) with minor modifications. The starch samples (100 mg) were gelatinized at 95 °C for 20 min, cooled to 37 °C, and incubated with an enzyme suspension (6 ml) containing amyloglucosidase and porcine pancreatic α-amylase. Samples were collected at intervals (0, 5, 10, 20, 30, 60, 90, 120, and 180 min), mixed with 80% ethanol to deactivate enzymes, and glucose release was quantified using the DNS assay. Hydrolysis rates (%) were calculated and plotted against reaction time using the following formula:

$$\text{Hydrolysis rate (\%)} = \frac{\text{Content of hydrolyzed glucose x 0.9}}{\text{Weight of starch sample } x \text{ Percentage of TS}} \text{ x 100\%}$$

Hydrolysis curves plotted of the reaction time and hydrolysis rate of different starches.

2.7. Determination of rapidly digestible starch, slowly digestible starch and resistant starch content

Starch fractions were classified as rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) based on their hydrolysis rate in the gastrointestinal tract (Englyst & Hudson, 1996). RDS represents starch digested within the first 20 min, SDS refers to starch digested between 20 and 120 min, and RS consists of undigested after 120 min. The content of each fraction was calculated based on the hydrolysis data using the following equations.

$$\text{RDS (\%)} = \frac{(\text{Glucose}_{20\ \text{min}} - \text{FG})\text{x } 0.9}{\text{Weight of starch sample}}$$

$$\text{SDS (\%)} = \frac{(\text{Glucose}_{120\ \text{min}} - \text{Glucose}_{20\ \text{min}} - \text{FG})\text{x } 0.9}{\text{Weight of starch sample}}$$

$$\text{RS (\%)} = \frac{(\text{TS} - \text{Glucose}_{120\ \text{min}} - \text{FG})\text{x } 0.9}{\text{Weight of starch sample}}$$

$\text{Glucose}_{20\ \text{min}}$ and $\text{Glucose}_{120\ \text{min}}$

$\text{Glucose}_{20\ \text{min}}$ and $\text{Glucose}_{120\ \text{min}}$ represents the glucose released within 20 min and 120 min, respectively.

2.8. Statistical analysis

The hydrolysis data were analyzed using a mixed-effects model in R with the Linear Mixed-Effects Models (*lme4*) package in R. Cultivars, fermentation layers, and time were included as fixed effects with random intercepts specified for each cultivar. Post-hoc pairwise comparisons were performed using Tukey's Honest significant difference (HSD) test (Estimated Marginal Means (*Emmeans*) package in R), with significance set at $p < 0.05$. A three-way ANOVA, using the *car* package with function (*Anova*) in R, was conducted to assess the effects of cultivar, fermentation duration, and starch layer on hydrolysis rates and starch properties.

3. Results and discussion

3.1. In vitro hydrolysis of pearl millet starches

The fermentation time significantly influenced the mean hydrolysis (%) of *Jir*, with notable variation observed among pearl millet varieties and extracted starch layers (*Jir khadim and Jir hur*) during *in-vitro* digestion (Fig. 1). The digestion profiles generally showed rapid hydrolysis during the initial 0–60 min, followed by a gradual increase from to 60–180 min (Fig. 1). Overall, the 7-days fermented starches showed relatively higher hydrolysis rates at 120 min compared to 1-day fermented starches. Among the analyzed varieties, Ashana displayed a gradual increase in hydrolysis during the initial 0–30 min for both starch layers (Fig. 1a). However, the 1-day fermentation samples plateaued at 74% hydrolysis after 120 min, whereas the 7-days fermented samples reached 90% hydrolysis rate (Fig. 1a). The higher hydrolysis rate after the 7-days of fermented Ashana starch is likely due to morphological changes in the starch granules. Specifically, the upper and lower layers exhibited surface perforations (porous structure, solid white arrows), indentations (dotted red arrows), and granule degradation (solid red arrow), as observed in the SEM micrographs (Fig. 2a–d).

For Dembi-green, the 1-day fermented upper layer showed a relatively higher hydrolysis rate (60%) during the initial 0–30 min compared to the other samples with an average of approximately 40% (Fig. 1c). In contrast, the 7-days fermented upper layer of starch from Bioda exhibited a slower hydrolysis rate (50%) during the initial 0–30 min (Fig. 1b). This slower hydrolysis rate may be due to the presence of non-starch components (e.g. proteins, fiber and lipids) (Fig. S1, supplementary information), which can hinder enzymatic degradation (Fig. 2e and f, solid arrows). Both Dembi-red and Mayoa showed very high hydrolysis rates (60–85%) for both fermentation times during the initial 0–30 min compared to other cultivars (Fig. 1d–f). The SEM micrographs showed significant structural feature alterations in these samples, including surface porosity, granule degradation, and distinct surface indentations especially after 7-days of fermentation (Fig. 3a–d, solid arrows; Fig. S2, Supplementary information). In addition, the relatively small granule sizes in Dembi-red and Mayoa likely contributed to their rapid hydrolysis rates. The amylose content was another critical factor that affected the hydrolysis rates. Ashana (18%) and Bioda (17.9%) demonstrated significantly higher amylose contents compared to Dembi-red (16.9%) and Mayoa (15.7%), correlating with slower hydrolysis rates. These findings align with previous studies demonstrating that higher amylose content is associated with decreased hydrolysis rates (Sandhu et al., 2017; Stevnebø et al., 2006).

Structural changes in fermented starch also enhanced its enzymatic accessibility, leading to higher hydrolysis rate. In contrast, the slower hydrolysis observed in certain starches (for example, Bioda) may result from proteins, lipids, non-starchy polysaccharides, and polyphenols, which form physical barriers that impede enzyme binding and activity (Annor et al., 2017; Chi et al., 2022; Ren et al., 2016).

Previous study have shown that starch granule size and shape influence starch hydrolysis rates (Sandhu et al., 2017). In this study, most millet starch granules were polygonal in shape, while some exhibited

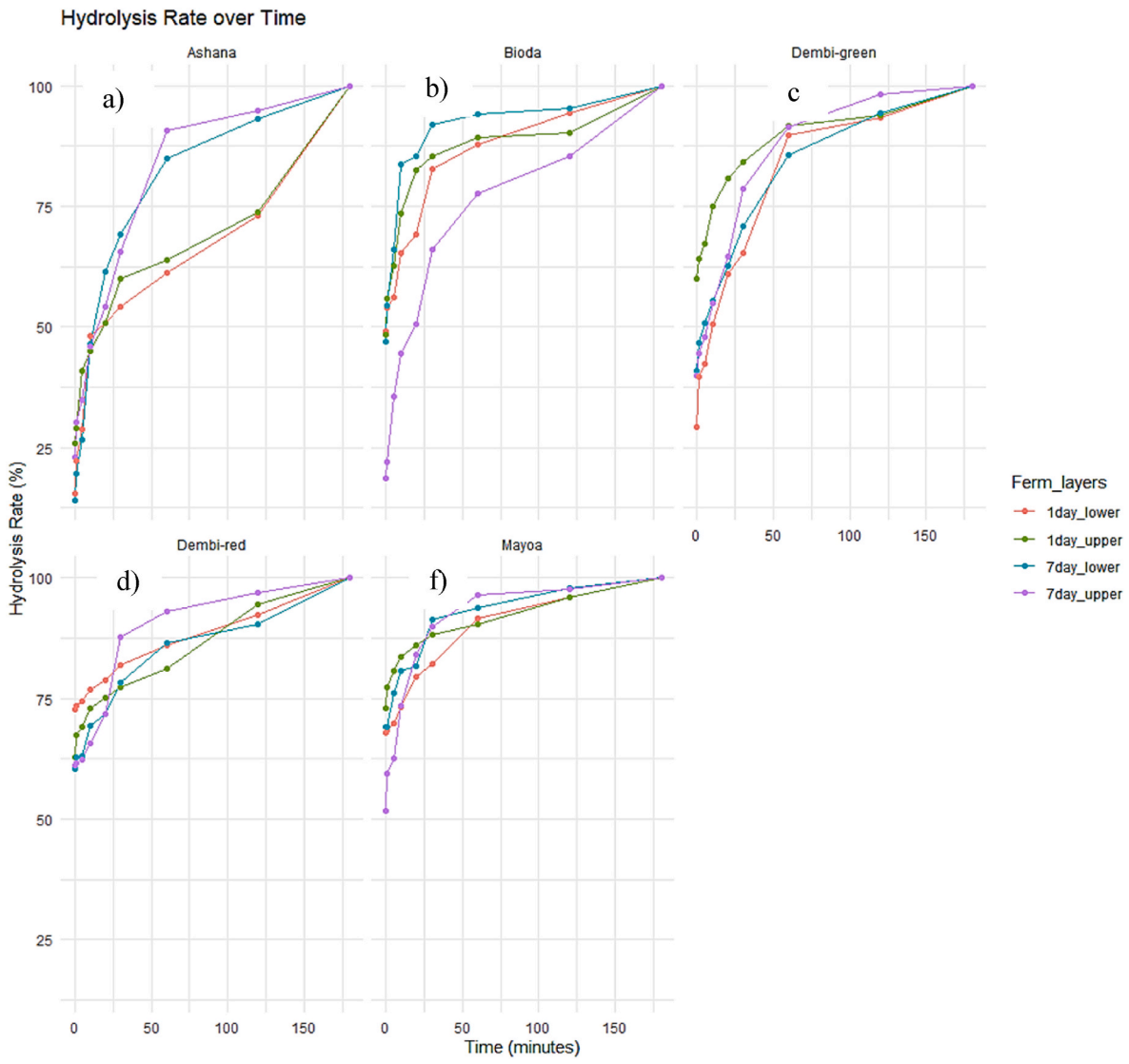

Fig. 1. Impact of fermentation-mediated traditional starch extraction on the hydrolysis of starch from five verities of pearl millet. Two starch layers; *Jir khadim* = *upper layer, Jir hur* = *lower layer*, fermentation time; 1 and 7-days.

indentations and perforations, particularly in larger granules, which is consistent with previous reports on hydrolyzed starches (Annor et al., 2017). Pores, channels, and cavities increase the internal surface area, facilitating enzyme binding, as shown in studies on maize and potato starches hydrolysis (Dhital et al., 2010). Granule morphology also affects amylase accessibility and hydrolysis efficiency (B. Zhang, Dhital, & Gidley, 2015). Digestion often begins at the granule pores and proceeds inward (Zhang et al., 2006). However, reduced starch granule integrity may affect the digestion rate, although it does not necessarily influence the extent of digestion (Chi et al., 2022). Granule size alone may not be the primary factor determining enzymatic hydrolysis patterns.

3.2. Maximum hydrolysis rate and reaction speed

To better understand the dynamic and nonlinear processes of starch hydrolysis, the digestion profiles were fitted to a first-order kinetic equation (Table 1). Variations were observed in the maximum hydrolysis rates ($C\infty$) and reaction speed after 180 min among all samples. Both Ashana and Bioda exhibited higher $C\infty$ values after 7 days of fermentation compared to 1 day (Table 1), revealing that extended fermentation of high-amylose starches facilitates enzymatic hydrolysis, albeit with a relatively slower reaction speed compared to other samples.

For Dembi-green, Dembi-red, and Mayoa, smaller variations in $C\infty$ and reaction speed were observed across fermentation times and starch layers (Table 1). The highest k values, indicating faster reaction speeds, were found in the Dembi green, 1-day lower layer (2.022) and Dembi-red 1-day upper layer (2.038). Conversely, the lowest k values were observed in the Ashana 7-day lower layer (0.053) and Bioda 7-day lower layer (0.058), reflecting slower reaction speeds.

These findings align with those of previous studies on pearl millet starch, where the hydrolysis rates ranged between 74.845% and 94.585% (Chung, Liu, Peter Pauls, et al., 2008). A comparison of *Jir* starch with starches from other crops, including pea, lentil, chickpea, wheat, and mung bean, revealed distinct differences in $C\infty$ values. The $C\infty$ values for pulse starches were 76.8% for pea, 76.9% for lentil, and 75.4% for chickpea. Zhang et al. (2018) reported $C\infty$ values of 75.0% for corn, 102% for wheat, and 78.8% for mung bean starch.

3.3. Impact of in vitro digestibility on various starch fractions

In pearl millet cultivars, fermentation times, starch layers, and their interactions showed significant ($P < 0.05$) variation across all the evaluated starch fractions (Table 2). During fermentation periods, *Jir*

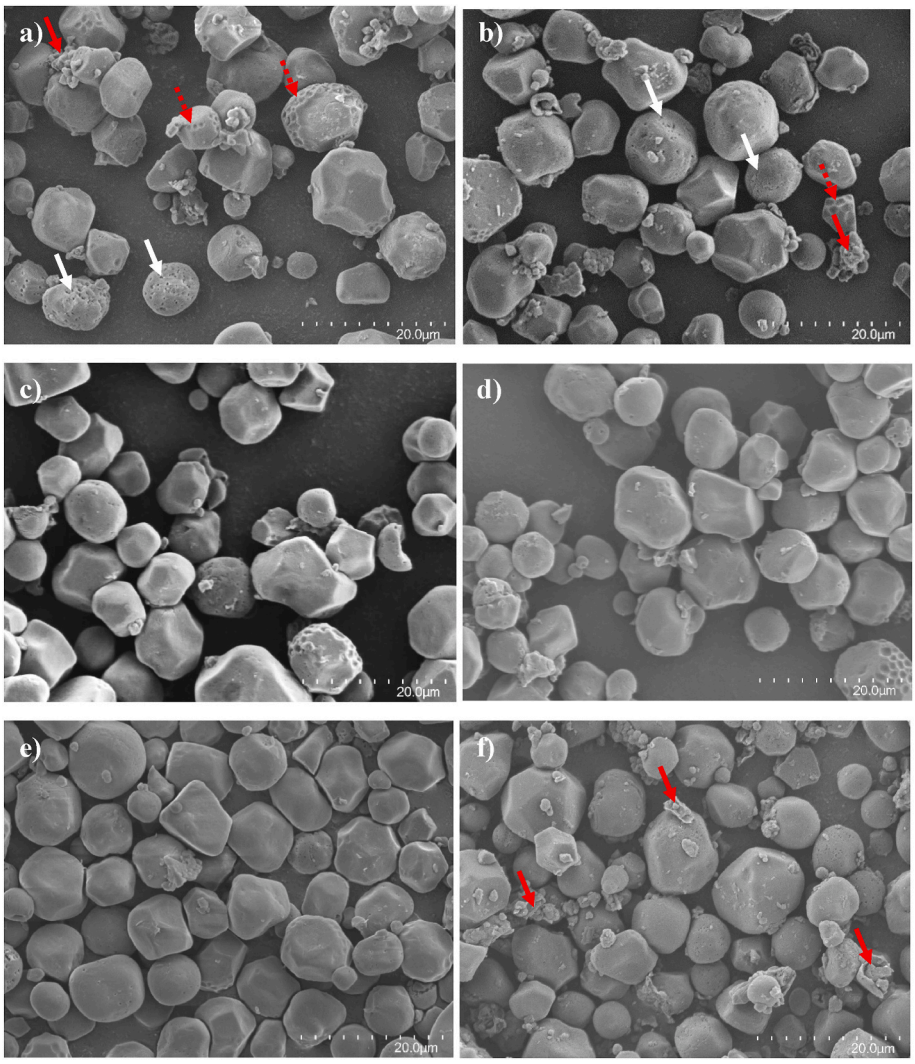

Fig. 2. SEM micrographs of upper and lower layer of fermented starch (*Jir*) after 1 and 7 days: a) Ashana: 7 days, lower layer, b) Ashana:7 days, upper layer, c) Ashana:1 day, lower layer and d) Ashana:1 day, upper layer, and e) Bioda: 7 days, lower layer, f) Bioda: 7-days upper layer.

produced with 7-day fermentation exhibited significantly higher ($P < 0.05$) levels of slowly digestible starch (SDS) compared to the 1-day fermentation, whereas the opposite trend was observed for rapidly digestible starch (RDS) and resistant starch (RS) (Table 2). Significant differences were also noted between the extraction layers, with the upper layer containing a higher RDS and significantly lower SDS fractions. However, the RS content did not vary significantly ($P < 0.05$) with the fermentation periods, extraction layers, or their three-way interaction (Table 2).

Among the cultivars, significant differences were observed across all the starch fractions. Ashana had the lowest RDS and the highest RS content. In contrast, Dembi-green showed significantly higher RDS and the lowest SDS levels, whereas Mayoa had the highest SDS content among the cultivars. The RDS and SDS values of pearl millet starches were substantially higher than those reported for cereal starches such as maize, waxy maize, wheat, and rice (Zhang et al., 2006). A similar trend was observed when pearl millet starches were compared to legume starches from yellow pea, lentil, and chickpea (Chung, Liu, Donner, et al., 2008). Notably, only the RS values of the Ashana cultivar were comparable with those of maize, rice, and wheat.

This study suggests that fermentation induces structural changes in pearl millet starches, thereby enhancing their susceptibility to enzymatic digestion. These effects may be partly attributed to the partial degradation of the granule's outer shell during fermentation, which facilitates enzyme attachment and penetration into the granule's internal structure. Additionally, the relatively low amylose content in pearl millet compared to cereals and legumes likely contributed to the higher RDS levels observed in these samples (Sandhu et al., 2008). Previous studies on the spontaneous fermentation of pearl millet and finger millet starches have shown a significant decrease in RS content, suggesting that fermentation enhances starch digestibility. This increase in RS values is primarily attributed to the enzymatic activity of microbes that break down amylose (amorphous) and amylopectin molecules (branched and crystalline fraction) typically inaccessible to digestive enzymes. Fermentation facilitates this process by breaking down the external starch granule structures that otherwise hinder access to the inner starch components, thereby improving *in vitro* digestibility (Bian et al., 2022; Gupta et al., 2024; Zhao et al., 2019). This study also showed physical changes in the starch granule surface (Figs. 2 and 3, Supplementary information, Fig. S2), likely contributing to the degradation of its internal molecular organization and leading to changes in the ratios of amylose and amylopectin, thereby contributing to changes in overall *in vitro* digestibility profile of studied starches.

Reducing the rate of starch digestion through physical, chemical, and physiological approaches is a key strategy for converting RDS into SDS and RS, potentially improving the balance between digestion rate and resistance to complete digestibility (G. Zhang & Hamaker, 2009). Hydrothermal modification has been shown to increase the RS content; for

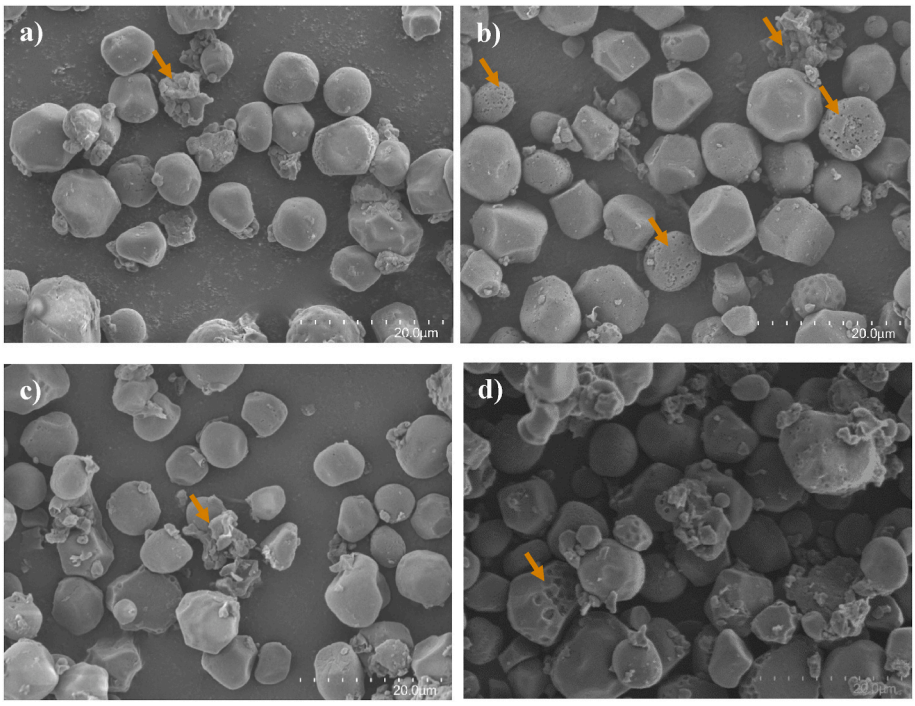

Fig. 3. SEM micrographs of upper layer starches fermented for 1 and 7 days; a) Dembi-red: 1 day, b) Dembi-red: 7 days, c) Mayoa: 1 day, and d) Mayoa: 7 days.

Table 1
Impact of fermentation days on First-order kinetic equation parameters, (C∞) maximum hydrolysis extent; (k) kinetic constant of different starches.

Variety	Fermentation	Layer	C∞ (%)	K (min^{-1})
Ashana	1 Day	lower	74.845	0.093
Ashana	1 Day	upper	76.183	0.069
Ashana	7 days	lower	94.585	0.053
Ashana	7 days	upper	92.752	0.057
Bioda	1 Day	lower	83.602	1.047
Bioda	1 Day	upper	79.561	1.078
Bioda	7 days	lower	87.359	0.058
Bioda	7 days	upper	88.590	0.859
Dembi-green	1 Day	lower	89.226	2.022
Dembi-green	1 Day	upper	84.608	1.638
Dembi-green	7 days	lower	86.458	1.109
Dembi-green	7 days	upper	88.802	1.491
Dembi-red	1 Day	lower	81.436	1.749
Dembi-red	1 Day	upper	84.349	2.038
Dembi-red	7 days	lower	82.566	1.351
Dembi-red	7 days	upper	79.932	1.528
Mayoa	1 Day	lower	84.749	1.400
Mayoa	1 Day	upper	90.288	0.076
Mayoa	7 days	lower	90.977	0.106
Mayoa	7 days	upper	83.281	0.163

instance, annealing and hydrothermal treatments enhance both RDS and RS levels in native starch granules from corn, pea, and lentil by increasing starch chain interactions (H. J. Chung, Liu, & Hoover, 2009). Similarly, Ashwar et al. (2016) demonstrated that dual autoclaving significantly increased the RS content by promoting hydrogen bond strengthening, intermolecular re-approximation, and recrystallization. Thus, modifying the viscosity of starchy foods through various treatments offers an effective means of controlling starch digestion rate (Chi et al., 2022). The results of this study indicate that fermentation of pearl millet starches contributes to increased levels of RDS. However, the extent of this effect is influenced by the amylose content and fermentation time, as demonstrated by the Ashana cultivar, which exhibits high amylose levels and distinct starch digestibility. Whereas Mayoa showed the highest SDS and lowest RDS contents besides having lower amylose content.

Table 2
Effects of fermentation periods, extract layers and their interactions on rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistance starch (RS) contents in different pearl millet cultivars.

Varieties	RDS%	SDS%	RS%
Ashana	53.8E	29.2B	17.0A
Bioda	71.0C	19.3C	9.8B
Dembi-green	82.9A	14.0D	3.1C
Dembi-red	75.0B	19.3C	5.7C
Mayoa	62.9D	32.9A	4.2C
Fermentation			
1 Day	71.4A	18.2B	10.4A
7 days	66.9B	27.7A	5.5B
Layers			
Lower	68.2B	24.1A	7.7A
Upper	70.0A	21.8B	8.2A
3-way ANOVA			
Cultivars, V	386.3***	179.5***	72.2***
Fermentation, F	78.0***	331.0***	70.5***
Layer, L	13.1***	20.4***	0.8^{NS}
V*F	60.7***	41.7***	44.8***
V*L	23.0***	12.7***	8.7***
F*L	322.8***	348.6***	1.0^{NS}
V*F*L	106.7***	75.9***	1.8^{NS}

3.4. Free glucose and total starch

Pearl millet cultivars, fermentation times, starch layers, and their potential interactions exhibited significant ($P < 0.05$) variations in FG content and total starch (TS) (Table 3). Among the pearl millet cultivars, Bioda and Dembi green showed significantly higher FG levels compared to the other three analyzed cultivars. In contrast, Ashana exhibited the highest TS content (64.7%), followed by Bioda (51%), which was significantly higher compared to TS levels of other cultivars. Regarding fermentation periods, the 7-day fermentation significantly ($P < 0.05$)

Table 3
Effects of pearl millet cultivars, fermentation periods, extract layers and their interactions on free glucose (FG) and total starch (TS(.

Varieties	FG (%)	TS (%)
Ashana	22.7C	64.7A
Bioda	30.7A	51.0B
Dembi-green	29.8A	35.6D
Dembi-red	27.0B	35.1D
Mayoa	23.6C	39.8C
Fermentation		
1 Day	26.4B	41.2B
7 days	27.1A	49.2A
Layers		
Jir hur	30.4A	49.4A
Jir Khadim	23.1B	41.1B
3-way ANOVA		
Varieties, V	239.5***	3647.2***
Fermentation, F	12.8***	1831.4***
Layer, L	1226.6***	1957.6***
V*F	9.7***	565.4***
V*L	19.7***	382.0***
F*L	144.4***	1019.5***
V*F*L	49.8***	274.1***

increased both FG and TS compared to the 1-day fermentation. Among the extraction layers, *Jir hur* contained significantly higher FG and TS content than *Jir Khadim* (Table 3).

Compared with wheat starch, traditionally extracted pearl millet starch has lower FG levels (Zhang et al., 2018). This reduction may be further intensified during fermentation because glucose, with its simple chemical structure, is readily consumed by microorganisms. In particular, Lactic acid bacteria metabolize glucose through glycolysis, utilizing it as a primary energy source during fermentation, thereby reducing FG levels. The highest TS content observed in pearl millet starch was comparable to that of corn and mung bean starch but remained lower than wheat starch (Zhang et al., 2018). The TS levels in isolated beans, for example, have been reported to range from 94.5% to 100% (Chung, Liu, Peter Pauls, et al., 2008). The relatively low TS content in extracted pearl millet starch likely reflects reduced purity. Fermentation introduces by-products that alter the starch composition, diminishing both purity and overall TS content.

4. Conclusions

Traditional isolation and fermentation methods significantly influence the physicochemical properties of starch, thereby impacting its *in vitro* digestibility. Starch isolated after 7 days of fermentation displayed significant structural alterations, including a porous surface morphology, physical indentations, and partial degradation of granules. These structural changes were closely linked to increased hydrolysis rates during *in vitro* digestion, particularly within the first 60 min, where hydrolysis rate reached up to 70% in most samples. The study demonstrated that impact of fermentation on starch fractions distribution, with rapidly digestible starch (RDS) being the predominant fraction, followed by slowly digestible starch (SDS) and resistant starch (RS). Amylose content also played a critical role in hydrolysis rates, with higher amylose content like in Ashana showed lower RDS and higher RS levels than low-amylose varieties. Notably, Ashana, despite its higher amylose content, demonstrated a higher maximum hydrolysis rate in samples fermented for 7 days, underscoring the complex interplay between starch composition and fermentation-induced modification. These findings underscore the potential of traditional fermentation techniques to enhance the functional properties of starch, tailoring its digestibility and suitability for various food application.

CRediT authorship contribution statement

Manhal Gobara Hamid: Writing – original draft, Methodology, Data curation. **Khitma A. Sir Elkhatim:** Methodology, Data curation. **Yousif M.A. Idris:** Writing – review & editing. **Mohammed Elsafy:** Writing – review & editing, Visualization, Formal analysis. **Mahbubjon Rahmatov:** Writing – review & editing, Visualization, Funding acquisition, Conceptualization. **Tilal Abdelhalim:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Faraz Muneer:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2025.117523.

Abbreviations

ARC	Agricultural Research Corporation
DNS	3,5-Dinitrosalicylic Acid
EMMEANS	Estimated Marginal Means (package in R)
FG	Free Glucose
HSD	Honest Significant Difference
JIR	Starch extracted from pearl millet grains (with specific layers, *Jir Khadim* (upper) and *Jir Hur* (lower))
KOH	Potassium Hydroxide
LME4	Linear Mixed-Effects Models (package in R)
MW	Molecular Weight
RDS	Rapidly Digestible Starch
RS	Resistant Starch
SDS	Slowly Digestible Starch
SEM	Scanning Electron Microscopy
TS	Total Starch
U	Units (enzyme activity)
(C∞)	Maximum hydrolysis extent
(k)	Kinetic constant

Data availability

Data will be made available on request.

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