



Effects of Microorganisms and Fermentation Time on the Characteristics of Flour from Whole Foxtail Millet (Hotong)

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Abstract. *Hotong (Setaria italica) has promising potential as a functional food ingredient, but its native flour requires technological enhancement for broader utilization. This study evaluated the effects of solid-state fermentation using Lactobacillus plantarum, Saccharomyces cerevisiae, and their combination for 24, 48, and 72 h on the physicochemical and functional properties of hotong flour. Fermentation significantly improved flour characteristics, with outcomes strongly influenced by microbial type and duration. L. plantarum at 72 h produced flour with the highest water-holding capacity and brightness, indicating suitability for products requiring high water-holding capacity and light color. In contrast, S. cerevisiae and mixed cultures enhanced antioxidant activity, while short-term L. plantarum fermentation better preserved phenolic content. These findings demonstrate that targeted fermentation conditions can be strategically selected to produce hotong flour with characteristics optimized for specific food applications, supporting its potential development as a versatile functional ingredient.*

Keywords: *Setaria italica; solid-state fermentation; whole grain; antioxidant activity; physicochemical properties.*

Type of the Paper: Regular Article.



1. Introduction

Setaria italica (commonly known as foxtail millet, or locally in Maluku hotong) is a nutrient-dense cereal with significant potential as a functional food source. It is rich in dietary fiber, protein, essential minerals, and bioactive compounds such as phenolics and flavonoids, which contribute to human health and play important roles in the prevention of degenerative diseases [1–3]. In Indonesia, particularly in the Maluku region, hotong has long been used in traditional food products. However, its use remains limited compared to that of more widely consumed cereals such as rice and wheat [4,5].

Despite its nutritional potential, the broader application of hotong in food systems faces several challenges. One of the primary limitations is the physicochemical characteristics of hotong flour, which is typically coarse in texture and exhibits poor water-binding capacity. These properties restrict its use in food products that require smooth texture and desirable softness [6]. Moreover, hotong contains anti-nutritional factors such as phytic acid and tannins, which inhibit the bioavailability of key nutrients, including minerals and proteins, thereby reducing the nutritional quality of the final product [7–10].

Fermentation has emerged as an effective bioprocessing technique to overcome these limitations. It has been widely demonstrated to improve the nutritional and functional qualities of various food substrates by enhancing bioavailability, reducing anti-nutrients, and enriching bioactive compound content [11]. For hotong, fermentation using microorganisms such as lactic acid bacteria (LAB) and *Saccharomyces cerevisiae* has shown promise in improving flour quality through mechanisms that include the degradation of anti-nutritional factors and enhancement of antioxidant activity [10,12–14]. Furthermore, fermentation may improve physicochemical attributes such as water absorption and textural properties, supporting the development of higher-quality functional food products [2].

However, existing studies have predominantly focused on the fermentation of foxtail millet in flour form. The potential benefits of fermenting whole foxtail millet grains prior to milling remain largely underexplored. Whole grain fermentation may allow more effective microbial penetration and breakdown of structural and nutritional components that are less accessible in flour form. Given the more complex physical structures and nutritional profile of whole grains, their fermentation could yield distinctive outcomes in terms of physicochemical properties, bioactive compound profiles, and antioxidant activity.

In addition to the form of the substrate, the type of microorganism and the duration of fermentation are critical variables influencing the success of the process. Yet, most research to date has utilized either LAB [3,15] or *S.cerevisiae* [16] individually, with limited investigation into their combined use. Synergistic fermentation using both microorganisms may enhance bioactive compound production, improve antioxidant properties, and more effectively reduce anti-nutritional factors [17]. Moreover, the optimization of fermentation duration is essential, as insufficient time may limit bio transformations, whereas excessive fermentation could compromise the structural integrity and quality of the flour.

Therefore, this study aims to address these research gaps by comparing the effects of fermenting whole hotong grains using different types of microorganisms (LAB, *S. cerevisiae*, and their combination) and fermentation durations. The outcome will be evaluated based on changes in physicochemical properties, bioactive compounds, and antioxidant activity. This study is expected to contribute new insights into optimal processing of hotong as a functional food ingredient with improved nutritional and technological properties

2. Materials and Methods

2.1. Materials

Foxtail millet (*Stetaria italica*), locally known as hotong, was sourced from Buru Island, Maluku Province, Indonesia (Fig. 1). The microorganism *Lactobacillus plantarum* was obtained

from Nanobio Laboratory (Indonesia). *Saccharomyces cerevisiae* was used in the form of commercial baker's yeast (Fermipan). All other chemicals used were of analytical grade.



Fig.1. Foxtail millet (hotong) grains.

2.2. Methods

2.2.1. Production of hotong flour

Hotong grains were washed under running water until the rinse water was clear, then soaked for 24 h. Fermentation was carried out as solid-state fermentation under anaerobic conditions in closed containers using three treatments: (1) *Lactobacillus plantarum* (60 mL culture in 450 mL water); (2) *Saccharomyces cerevisiae* (30 g yeast in 450 mL water); and (3) a combination of yeast (15 g) and *L.plantarum* (30 mL in 450 mL water, each per 300 g grains. Grains were thoroughly mixed with starter culture under sterile conditions and incubated at 37°C for 24, 48, or 72 h.

Fermented grains were oven-dried at 40°C until moisture content was below 12%, cooled to room temperature, and milled using food crusher (Krisbow, Indonesia). The flour was sieved through an 80 mesh screen to ensure uniform particle size and stored in airtight containers until analysis.

2.2.2. Color measurement

The color of the hotong flour samples was determined using a chromameter (Konica Minolta CR-400, Osaka, Japan). The instrument was calibrated with a standard white calibration plate provided by the manufacturer before measurements. Approximately 20 g of flour was placed in a transparent sample cup, and the surface was gently leveled to ensure uniform reflection and minimize light scattering. Measurements were recorded in the CIE L*, a*, b* color space, where L* represents lightness (0 = black, 100 = white), a* indicates the red-green axis (positive values = red, negative values = green), and b* indicates the yellow-blue axis (positive values = yellow, negative values = blue).

2.2.3. Whiteness index

The whiteness index (WI) based on CIE values was computed from the mean L*, a*, and b* using Eq. (1).

$$WI = 100 - \sqrt{(100 - L^*)^2 + (a^*)^2 + (b^*)^2} \quad (1)$$

2.2.4. Water holding capacity (WHC)

The water holding capacity of hotong flour was determined using a gravimetric method. Approximately 1.0 g of flour (as-is basis) was weighed into a 15 mL centrifuge tube, and 10 mL of deionized water was added (1:10, w/v). The mixture was vortexed for 15 s, allowed to equilibrate at 25°C for 30 min with intermittent vortexing every 5 min, and then centrifuged at 4000 × g for 15 min. The supernatant was carefully decanted, and the sediment was weighed. WHC (%wb) was expressed on a wet basis using Eq. (2).

$$WHC(\%) = \frac{m_{hydrated} - m_{dry}}{m_{hydrated}} \times 100 \quad (2)$$

2.2.5. Yield

The yield of hotong flour was calculated as the ratio of the weight of flour obtained after drying, milling, and sieving to the initial weight of raw millet grains, expressed as a percentage.

2.2.6. Proximate composition

The proximate composition of hotong flour, including moisture, ash, protein, fat, and crude fiber, was determined according to standard AOAC methods. Moisture content was measured by oven-drying at 105°C to constant weight, and ash content by incineration in a muffle furnace at 550°C. Protein was determined by the Kjeldahl method ($N \times 6.25$), and fat by the Soxhlet extraction using petroleum ether. Crude fiber was determined by sequential acid and alkali digestion.

2.2.7. Reducing sugar content

Reducing sugars were quantified by the dinitrosalicylic acid (DNS) colorimetric method with glucose as the standard. 1.0 g of flour was extracted with 10.0 mL of deionized water in a 50 mL tube by vortexing for 30 s and shaking for 30 min at 25°C, then centrifuged (4000 × g, 10 min), and the supernatant was collected. A glucose calibration curve (0.05-1.00 mg/mL) was prepared. For assay, 1.00 mL sample was mixed with 1.00 mL DNS reagent, heated in boiling water bath for 5 min, rapidly cooled to room temperature, and the absorbance was read at 540 nm against reagent blank with UV-Vis Spectrophotometer (Shimadzu, Japan). The glucoside equivalent concentration was obtained from calibration curve, and reducing sugars were calculated on a wet basis using Eq. (3).

$$\text{Reducing sugar (\%wb)} = \frac{C \times V_{tot} \times DF}{m_{sample}} \times 100 \quad (3)$$

Where:

- C : concentration of reducing sugars obtained from the calibration curve
- V_{tot} : total volume of extract
- Df : dilution factor

2.2.8. Total phenolic content

Total phenolic content was quantified by the Folin-Ciocalteu colorimetric assay using gallic acid as the standard. 1g of hotong flour was extracted with 10 mL 80% (v/v) methanol in a 50 mL tube (vortexed for 60 s, shaken for 60 min at 25°C, protected from light), then centrifuged at 4000 × g for 10 min, and the supernatant collected. The residue was re-extracted once with 10 mL of 80% methanol, the supernatants were pooled and brought to a known volume (V_{tot}). A gallic acid calibration curve (0-200 mg/L) was prepared fresh the same day. For the assay, 0.50 mL of sample or standard was mixed with 2.5 mL of 10% (v/v) Folin-Ciocalteu reagent. After 5 min, 2 mL of 75% (w/v) Na₂CO₃ was added, mixed, and incubated for 30 min at 25°C in the dark. Absorbance was read at 765 nm against a reagent blank. The gallic acid equivalent concentration (mg/L) was obtained from the calibration curve, and the total phenolic content was calculated as gallic acid equivalent (GAE) per 100 g sample using Eq. (4).

$$TPC (mg/100 g) = \frac{C \times V_{tot} \times DF}{m_{sample}} \times 100 \quad (4)$$

Where DF is the dilution factor, V_{tot} is the total extract volume (mL), and m_{sample} is the mass of sample (g).

2.2.9. Antioxidant activity DPPH (%RSA)

Antioxidant activity was measured using the DPPH assay and expressed as percent radical scavenging activity (%RSA). A 0.1 mM DPPH solution was prepared in methanol. Sample extracts were diluted to fall within the linear response range. For the assay, 0.1 mL of sample (or solvent for the control) was mixed with 3.90 mL of DPPH solution in a 1 cm pathlength cuvette, vortexed briefly, and incubated for 30 min at 25 °C in the dark. Absorbance was measured at 517 nm against methanol. To correct sample color/turbidity, a sample blank (0.1 mL sample + 3.390 mL methanol) was measured and its absorbance subtracted from the sample reading. % RSA was calculated using Eq. (5).

$$\%RSA = \left[1 - \frac{A_{sample} - A_{blank}}{A_{control}} \right] \times 100 \quad (5)$$

3. Results and Discussion

The hotong flour produced using different microbial starters and fermentation time is shown in Fig.2.

3.1. Physical properties of the flour

3.1.1. Water holding capacity (WHC)

Two-way ANOVA showed a significant interaction (p < 0.05) between microorganism type and fermentation time for the WHC of hotong flour. At 24 h, *L. plantarum* exhibited the lowest

WHC (97.71%, Table 1), while *S. cerevisiae* (121.24%) and the mixed culture (116.85%) were higher. After 48 h, the WHC of *L. plantarum* increased (113.51%), whereas that of both *S. cerevisiae* and combination decreased (114.05% and 117.08%, respectively). At 72 h, *L. plantarum* reached the highest WHC (128.94%), surpassing *S. cerevisiae* (115.43%) and the mixed culture (116.64%). This trend indicates that enzymatic starch degradation and granule restructuring during fermentation vary by microbial metabolism and duration, directly influencing water-binding capacity. Compared with other foxtail millet flours, the WHC of fermented hotong flour was relatively lower. Prior studies reported that reducing millet bran particle size increased WHC from 1.86 g/g to 3.16 g/g, while natural fermentation significantly modified WHC depending on conditions and cultivar [18,19].

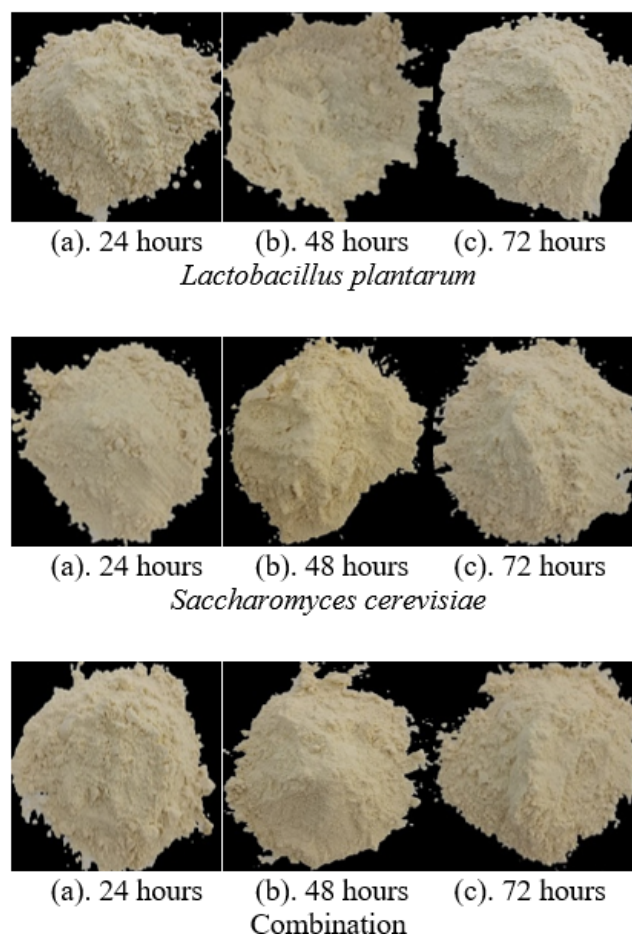


Fig. 2. Hotong flour with different microbial starters and fermentation time.

These discrepancies likely arise from compositional differences in the hotong matrix, particularly polysaccharides and cellulose, and from variations in fermentation technique. From an application perspective, long fermentation with *L. plantarum* (72 h) is advantageous for producing flour with high WHC ($\geq 120\%$), making it suitable for formulations requiring strong hydration properties. In contrast, short fermentation with *S. cerevisiae* is preferable where moderate WHC is desired.

Table 1. Physical properties of hotong flour

Physical Characteristics	Microorganism	Fermentation Time			Mean
		24 Hours	48 Hours	72 Hours	
Water Holding Capacity (% wb)	<i>Lactobacillus plantarum</i>	94.71 ± 0.07 ^g	113.51 ± 0.13 ^f	128.94 ± 0.16 ^a	112.39 ± 15.33 ^B
	<i>Saccharomyces cerevisiae</i>	121.24 ± 0.11 ^b	114.05 ± 0.13 ^e	115.43 ± 0.06 ^d	116.94 ± 3.47 ^A
	Combination	116.85 ± 0.06 ^c	117.08 ± 0.09 ^c	116.64 ± 0.23 ^c	116.86 ± 0.22 ^A
	Mean	110.97 ± 12.75 ^C	114.88 ± 1.72 ^B	120.34 ± 6.69 ^A	
Color L*	<i>Lactobacillus plantarum</i>	85.30 ± 0.02 ^d	85.81 ± 0.07 ^b	87.37 ± 0.04 ^a	86.16 ± 0.96 ^A
	<i>Saccharomyces cerevisiae</i>	84.83 ± 0.01 ^e	84.32 ± 0.09 ^f	85.62 ± 0.01 ^c	84.92 ± 0.59 ^B
	Combination	84.21 ± 0.01 ^f	84.87 ± 0.01 ^e	85.59 ± 0.05 ^c	84.89 ± 0.62 ^B
	Mean	84.78 ± 0.49 ^C	85.00 ± 0.69 ^B	86.19 ± 0.91 ^A	
Color a*	<i>Lactobacillus plantarum</i>	1.17 ± 0.01 ^e	1.14 ± 0.03 ^e	0.98 ± 0.04 ^f	1.10 ± 0.09 ^B
	<i>Saccharomyces cerevisiae</i>	1.64 ± 0.01 ^{abc}	1.69 ± 0.00 ^{ab}	1.43 ± 0.07 ^d	1.59 ± 0.13 ^A
	Combination	1.71 ± 0.00 ^a	1.58 ± 0.00 ^{bc}	1.56 ± 0.02 ^c	1.62 ± 0.08 ^A
	Mean	1.51 ± 0.26 ^A	1.47 ± 0.26 ^A	1.32 ± 0.27 ^B	
Color b*	<i>Lactobacillus plantarum</i>	17.65 ± 0.02 ^d	16.61 ± 0.11 ^f	17.07 ± 0.04 ^e	17.11 ± 0.47 ^B
	<i>Saccharomyces cerevisiae</i>	18.73 ± 0.04 ^b	19.52 ± 0.12 ^a	17.57 ± 0.03 ^d	18.61 ± 0.88 ^A
	Combination	18.95 ± 0.01 ^b	18.66 ± 0.19 ^b	18.02 ± 0.04 ^c	18.54 ± 0.43 ^A
	Mean	18.44 ± 0.62 ^A	18.26 ± 1.34 ^B	17.55 ± 0.43 ^C	
Whiteness Index (WI)	<i>Lactobacillus plantarum</i>	77.00 ± 0.00 ^{cd}	78.13 ± 0.13 ^b	78.74 ± 0.01 ^a	77.96 ± 0.79 ^A
	<i>Saccharomyces cerevisiae</i>	75.84 ± 0.04 ^e	74.91 ± 0.04 ^g	77.25 ± 0.03 ^c	76.00 ± 1.06 ^B
	Combination	75.28 ± 0.00 ^f	75.93 ± 0.15 ^e	76.87 ± 0.00 ^d	76.03 ± 0.72 ^B
	Mean	76.04 ± 0.79 ^C	76.32 ± 1.48 ^B	77.62 ± 0.89 ^A	

Notes: Values with different lowercase letters in the same row differ significantly due to interaction effects (Tukey α 0.05). Values with different uppercase letters in the same column differ significantly for single factors. Values without letters are not significantly different

3.1.2. Color

Fermentation time and microorganism type jointly influenced ($p < 0.05$) the L* value of hotong flour. At 24 h, *L. plantarum* exhibited the highest lightness (85.30), followed by *S. cerevisiae* (84.83) and the mixed culture (84.21). After 48 h, L* in *L. plantarum* increased (85.81), while that of *S. cerevisiae* decreased (84.32) and that of the mixed culture slightly rose (84.87). The highest brightness was achieved at 72 h with *L. plantarum* (87.37), surpassing both *S. cerevisiae* (85.62) and the mixed culture (85.59). The increasing L* values in *L. plantarum* suggest

degradation of phenolic pigments and suppression of polyphenol oxidation, leading to enhanced brightness. In contrast, *S. cerevisiae* likely produced pigmented metabolites such as melanoidins, reducing lightness at earlier stages. The mixed culture yielded relatively stable L^* values but did not match the brightening effect of *L. plantarum*. Compared with millet flour, which typically shows an L^* value of 80-85 under fermentation [20,21], hotong flour exhibited similar or slightly higher brightness. This may reflect differences in pigment and phenolic composition, as well as fermentation conditions. Practically, 72 h fermentation with *L. plantarum* is recommended for applications such as bread and noodles where a bright appearance is desired.

The interaction between microorganism type and fermentation time significantly affected the a^* value ($p < 0.05$), which represents the red-green color component of hotong flour. At 24 h, the mixed culture produced the strongest reddish hue (1.71), followed by *S. cerevisiae* (1.64), while *L. plantarum* remained the lowest (1.17). After 48 h, *S. cerevisiae* rose slightly (1.69), the mixed culture declined (1.58), and *L. plantarum* stayed low (1.14). By 72 h, all treatments decreased, with *L. plantarum* reaching the lowest intensity (0.98), suggesting a marked reduction in red-brown coloration. The consistent suppression of a^* by *L. plantarum* points to its role in minimizing browning, likely through pigment degradation and inhibition of non-enzymatic reactions. In contrast, the mixed culture at early fermentation enhanced redness, probably due to accumulation of oxidized phenolics or early Maillard products, while *S. cerevisiae* showed a transitional pattern. Compared with millet flour, where fermented samples generally exhibited a^* values between 1.10 and 2.00 [21], hotong flour tended to be within a similar range. From an application perspective, extended fermentation with *L. plantarum* is suitable for achieving a lighter, more neutral flour, while short-term mixed culture fermentation provides a reddish-golden tone desirable in baked goods such as cookies or crackers.

The b^* value (yellow-blue component) of hotong flour was significantly influenced by the interaction between microorganism type and fermentation time ($p < 0.05$), reflecting dynamic shifts in color during fermentation. At 24 h, the mixed culture yielded the highest b^* value (18.95), followed by *S. cerevisiae* (18.73), while *L. plantarum* showed the lowest (17.65). By 48 h, *S. cerevisiae* reached the peak value (19.52), *L. plantarum* dropped further to 16.61, and the mixed culture remained high (18.66). After 72 h, all treatments decreased, with *S. cerevisiae* at 17.57, the mixed culture at 18.02, and *L. plantarum* recovering slightly to 17.07 compared with 48 h. These fluctuations suggest that yellowness intensity depends on both the microorganism and fermentation stage. Mixed cultures and *S. cerevisiae* enhanced b^* values during the first 24-48 h, likely due to partial breakdown of darker phenolics and accumulation of yellow-reflecting compounds such as oxidized flavonoids. In contrast *L. plantarum*, consistently reduced b^* values, possibly through oxidative enzymatic activity that degrades yellow pigments. The general decline

in b^* after 72 h may also reflect the onset of non-enzymatic browning or further degradation of pigments formed earlier. Compared with millet flour, where fermented samples typically range between 12.30 and 14.27 depending on the fermentation and drying conditions [21], hotong flour showed slightly higher b^* values (16-18) during early fermentation. This suggests a stronger inherent yellow tone in hotong, likely due to higher precursor pigments such as xanthophylls or flavonols [22,23]. From an application perspective, mixed culture fermentation for 24–48h appears optimal for producing flour with bright yellow intensity, desirable in bakery products that emphasize natural color appeal.

3.1.3. Whiteness index

The whiteness index of hotong flour was significantly affected by the interaction between microorganism type and fermentation duration ($p < 0.05$), with distinct differences across treatments. After 24 h of fermentation, the highest WI was observed in *L. plantarum* (77.00), followed by *S. cerevisiae* (75.84), while the mixed culture produced the lowest value. At 48 h, *L. plantarum* maintained the highest WI (78.13), whereas *S. cerevisiae* and the mixed culture declined to 74.91 and 75.93, respectively. The maximum WI was achieved at 72 h with *L. plantarum* (78.74), showing a consistent increase over time. In contrast, *S. cerevisiae* and the mixed culture only reached 77.25 and 76.87 at 72 h. The progressive rise in WI under *L. plantarum* fermentation suggests its crucial role in suppressing browning reactions or degrading native color pigments in hotong. Conversely, *S. cerevisiae* and the mixed culture yielded lower WI values, likely due to pigment formation such as melanoidins from early Maillard reactions. The enhanced brightness observed in prolonged *L. plantarum* fermentation may be attributed to its oxidative or hydrolytic enzyme activity, which favors acid production over other byproducts that thereby promoting pigment breakdown and flour whitening [24]. When compared with millet flour, hotong displayed relatively higher WI values following fermentation. Given that hotong naturally possesses a lighter base color, it exhibits a greater potential for brightness improvement during fermentation. From an application standpoint, fermentation with *L. plantarum* for 72 h is particularly recommended to produce flour with maximum whiteness, which is advantageous for food products such as noodles, bread, and cookies where a bright or white appearance is desirable.

3.2. Chemical characteristics of the flour

3.2.1. Proximate composition

The proximate composition of hotong flour such as, moisture, ash, protein, fat, and crude fiber content is shown in Table 2.

Table 2. Chemical properties of hotong flour

Chemical Characteristics	Microorganisms	Fermentation Time			Mean
		24 Hours	48 Hours	72 Hours	
Moisture Content (%)	<i>Lactobacillus plantarum</i>	10.20 ± 0.04 _b	10.17 ± 0.01 _b	8.45 ± 0.04 _f	9.61 ± 0.90 _B
	<i>Saccharomyces cerevisiae</i>	10.94 ± 0.03 _a	8.74 ± 0.04 _e	9.43 ± 0.06 _d	9.70 ± 1.01 _A
	Combination	9.91 ± 0.04 _c	9.49 ± 0.06 _d	9.53 ± 0.02 _d	9.64 ± 0.21 _{AB}
	Mean	10.35 ± 0.47 _A	9.47 ± 0.61 _B	9.14 ± 0.53 _C	
Ash Content (%)	<i>Lactobacillus plantarum</i>	1.36 ± 0.01	1.54 ± 0.01	1.60 ± 0.00	1.50 ± 0.11 _A
	<i>Saccharomyces cerevisiae</i>	1.32 ± 0.02	1.50 ± 0.00	1.54 ± 0.03	1.45 ± 0.12 _B
	Combination	1.25 ± 0.03	1.44 ± 0.04	1.49 ± 0.04	1.39 ± 0.12 _C
	Mean	1.31 ± 0.05 _C	1.49 ± 0.05 _B	1.54 ± 0.05 _A	
Protein Content (%)	<i>Lactobacillus plantarum</i>	11.78 ± 0.03 _a	11.67 ± 0.01 _b	11.26 ± 0.03 _d	11.57 ± 0.26 _A
	<i>Saccharomyces cerevisiae</i>	11.67 ± 0.04 _b	11.45 ± 0.01 _c	11.25 ± 0.00 _d	11.45 ± 0.19 _B
	Combination	11.75 ± 0.02 _{ab}	11.27 ± 0.00 _d	11.19 ± 0.04 _d	11.40 ± 0.27 _C
	Mean	11.73 ± 0.06 _A	11.46 ± 0.18 _B	11.23 ± 0.04 _C	
Fat Content (%)	<i>Lactobacillus plantarum</i>	1.40 ± 0.01 _a	1.37 ± 0.01 _a	1.36 ± 0.04 _a	1.37 ± 0.02 _A
	<i>Saccharomyces cerevisiae</i>	1.41 ± 0.01 _a	1.35 ± 0.01 _{ab}	1.35 ± 0.00 _{ab}	1.37 ± 0.03 _A
	Combination	1.41 ± 0.01 _a	1.37 ± 0.04 _a	1.28 ± 0.02 _b	1.35 ± 0.06 _A
	Mean	1.40 ± 0.01 _A	1.36 ± 0.02 _B	1.33 ± 0.04 _C	
Crude Fiber Content (%)	<i>Lactobacillus plantarum</i>	9.56 ± 0.04 _c	9.57 ± 0.00 _c	9.27 ± 0.02 _e	9.46 ± 0.15 _A
	<i>Saccharomyces cerevisiae</i>	9.76 ± 0.03 _a	9.41 ± 0.03 _d	9.16 ± 0.03 _f	9.44 ± 0.27 _A
	Combination	9.66 ± 0.03 _b	9.26 ± 0.01 _e	9.22 ± 0.00 _{ef}	9.38 ± 0.21 _B
	Mean	9.66 ± 0.09 _A	9.41 ± 0.14 _B	9.22 ± 0.05 _C	
Reducing Sugar Content (%)	<i>Lactobacillus plantarum</i>	0.36 ± 0.01 _a	0.27 ± 0.01 _b	0.15 ± 0.01 _e	0.27 ± 0.09 _A
	<i>Saccharomyces cerevisiae</i>	0.21 ± 0.01 _d	0.38 ± 0.01 _a	0.22 ± 0.01 _{cd}	0.26 ± 0.09 _A
	Combination	0.24 ± 0.01 _c	0.23 ± 0.01 _{cd}	0.21 ± 0.01 _d	0.22 ± 0.01 _B
	Mean	0.29 ± 0.07 _A	0.27 ± 0.07 _B	0.19 ± 0.03 _C	

Notes: Values with different lowercase letters in the same row differ significantly due to interaction effects (Tukey α 0.05). Values with different uppercase letters in the same column differ significantly for single factors. Values without letters are not significantly different

The interaction between microorganism type and fermentation duration significantly affected the moisture content of hotong flour. The highest value was recorded after 24 h of fermentation with *S. cerevisiae* (10.94%), whereas the lowest was obtained after 72 h with *L. plantarum* (8.45%). This pattern indicates that microbial metabolic dynamics during fermentation influence the final moisture level. The marked reduction in moisture content observed with *L.*

plantarum suggests more intensive enzymatic activity, leading to substrate degradation and enhanced water evaporation or structural modification of the flour matrix. In contrast, *S. cerevisiae* showed more variable moisture fluctuations, likely due to its fermentative traits that generate metabolites with differing water-binding capacities. These findings align with previous studies reporting that fermentation can alter moisture levels through hydrolysis and volatile compound formation, which facilitate water reduction [25–27]. Lower final moisture is beneficial for prolonging shelf life, reducing water activity (A_w), and minimizing microbial spoilage during storage. Thus, the combined effects of microorganism type and fermentation time are critical for controlling moisture content and determining the functional quality of fermented hotong flour.

The two-way ANOVA revealed that the interaction between microorganism type and fermentation duration had no significant effect on hotong flour ($p > 0.05$); therefore the discussion focuses on the main effects. The highest mean ash content was obtained with *L. plantarum* (1.50%), followed by *S. cerevisiae* (1.45%), while the lowest was observed in the mixed culture (1.39%). Extending fermentation time from 24 h (1.31%) to 48 h (1.49%) and 72 h (1.54%) significantly increased ash levels. These findings suggest that *L. plantarum* possesses more effective enzymatic activities, such as phosphatases and oxidoreductases, for mobilizing mineral ions from the flour matrix, whereas longer fermentation promotes microbial proteolytic and carbohydrate-hydrolytic degradation of organic components, thereby concentrating the mineral fraction [28]. The rise in ash content reflects a potential increase in micronutrients such as calcium, magnesium, and phosphorus, supporting its value for functional food applications. Nevertheless, excessive ash may affect sensory attributes, particularly taste and texture, indicating that an optimal fermentation duration (e.g., 48–72h) should be carefully selected based on the desired product quality.

The results showed that both microorganism type and fermentation duration had a significant effect on the protein content of hotong flour ($p < 0.05$), with a clear interaction between the two factors. At 24 h, the highest protein content was obtained with *L. plantarum* (11.78%), followed closely by the mixed culture (11.75%) and *S. cerevisiae* (11.67%). By 48 h, however, the protein level began to decline, most noticeably in the mixed culture (11.27%), while *L. plantarum* (11.67%) and *S. cerevisiae* (11.45%) still retained relatively higher values. After 72 h, the mixed culture dropped further to the lowest value (11.19%), whereas *L. plantarum* (11.26%) and *S. cerevisiae* (11.25%) remained almost the same. The reduction in protein content over time can be explained by the proteolytic activity of the microorganisms, which break down proteins into smaller peptides and amino acids for their own growth [29]. This effect becomes more pronounced during longer fermentation. In the mixed culture, competition for substrates likely accelerated this breakdown, resulting in a sharper decrease compared with single strains [30,31]. Similar patterns

have also been reported in other studies on legumes and cereals, where microbial protease activity gradually reduced crude protein levels [32,33]. From an application perspective, the findings suggest that choosing the right microorganism and fermentation is crucial. If the goal is to preserve protein content, *L. plantarum* with 24 h of fermentation seems the most favorable option. On the other hand, longer fermentation or mixed cultures might be useful if the target is to enhance other properties, such as flavor development or antioxidant activity, even though this comes with some reduction in protein content.

Variance analysis revealed a significant interaction ($p < 0.05$) between microorganism type and fermentation duration for the fat content of hotong flour, directing discussion toward this interaction pattern. After 24 h of fermentation, the treatments with *L. plantarum*, *S. cerevisiae*, and their combination showed comparable fat levels of 1.40%, 1.41%, and 1.41%, respectively. At 48 h, the most pronounced decrease was observed in the *S. cerevisiae* treatment (1.35%), while *L. plantarum* and the combined culture maintained levels around 1.37%. By 72 h, the combination treatment exhibited the sharpest decline, reaching 1.28%, whereas *L. plantarum* and *S. cerevisiae* remained at approximately 1.36% and 1.35%, respectively. This pattern indicates that lipid degradation capacity varies depending on the interaction between microbial species and fermentation period. The reduction in fat content, particularly under combined culture after extended fermentation, is likely attributable to synergistic lipolytic activity, where lipases from each microorganism collaboratively hydrolyze triglycerides into free fatty acids that either dissolve or disrupt the flour matrix [34,35]. At the early stage (24 h), lipid reserves remained relatively stable as lipase activity had not yet reached its peak. Over time, the accumulation of enzymes and microbial metabolites accelerated lipid hydrolysis, especially in the mixed culture that provided a broader enzymatic spectrum. These findings support the view that multi-microbial fermentation can enhance fat degradation and modify lipid composition in the final product, emphasizing the need for process designers to tailor fermentation duration and microbial composition according to desired nutritional and sensory outcomes.

3.2.2. Crude fiber content

A significant interaction ($p < 0.05$) between microorganism type and fermentation duration affected the crude fiber content of hotong flour (Table 2). At 24 h, *S. cerevisiae* produced the highest crude fiber content (9.76%), followed by the mixed culture (9.66%) and *L. plantarum* (9.56%). After 48 h, the largest decrease occurred in the mixed culture (9.26%) compared with *L. plantarum* (9.57%) and *S. cerevisiae* (9.41%). By 72 h, the sharpest decline was observed with *S. cerevisiae*, yielding the lowest value (9.16%), while *L. plantarum* and the mixed culture measured 9.27% and 9.22%, respectively. The interaction pattern suggests that microbial enzymatic activities, particularly cellulase and hemicellulases, vary not only among species but also with

fermentation time. *S. cerevisiae* tends to degrade fiber more rapidly in the early phase, whereas *L. plantarum* maintains fiber relatively steadily up to 48 h before moderate degradation. The mixed culture shows a strong early synergistic effect, followed by a slightly slower decline than *S. cerevisiae* alone. These findings align with previous reports that multi-microbial fermentation can modify cell wall polysaccharide structure and shift crude fiber proportions in the substrate [36]. Particularly, controlling the microorganism and fermentation time allows adjustment of fiber content in hotong flour for functional food applications, for example short fermentation with *S. cerevisiae* for a higher fiber content, or intermediate fermentation with *L. plantarum* to balance texture and fiber content.

3.2.3. Reducing sugar content

The interaction between microorganism type and fermentation duration had a significant effect ($p < 0.05$) on the reducing sugar content of hotong flour. At 24 h, *L. plantarum* produced the highest reducing sugar level (0.36%), whereas *S. cerevisiae* and the mixed culture showed lower values of 0.21% and 0.24%, respectively. By 48 h, the trend reversed, *S. cerevisiae* increased sharply to 0.38%, while *L. plantarum* and the mixed culture decreased to 0.27% and 0.23%, respectively. At 72 h, the lowest reducing sugar was observed with *L. plantarum* (0.15%), followed by *S. cerevisiae* (0.22%) and the mixed culture (0.21%). The pronounced decline in reducing sugar under *L. plantarum* treatment indicates more intensive carbohydrate metabolism, where enzymes such as glucosidases and isomerases break down disaccharides and oligosaccharides into non-reducing products or channel them into secondary polysaccharide synthesis [37]. In contrast, the mid-stage increase in reducing sugar with *S. cerevisiae* (48 h) likely reflects accumulation of glycolytic intermediates before their utilization for cell growth, consistent with the observation in cassava and sorghum flour fermentation, where *S. cerevisiae* generates higher reducing sugars during the logarithmic growth phase [38]. The mixed culture displayed a moderate pattern, showing synergistic effects but less pronounced than single cultures in modifying sugar content. These findings align with studies on cassava and maize flour fermentations, which highlight the critical role of microbial selection and fermentation time in controlling reducing sugar levels to match the desired functional properties of the final product [39,40]. From an application standpoint, fermentation with *L. plantarum* for 72 h is suitable for producing low sugar hotong flour, supporting processing stability (e.g. in noodles or biscuits) and reducing the risk of caramelization during baking, whereas short fermentation with *S. cerevisiae* may be preferable when a naturally sweeter flavor profile is desired.

3.3. Functional properties of hotong flour

3.3.1. Total phenolic content

The interaction between microorganism type and fermentation duration had a significant effect ($p < 0.05$) on the reducing sugar content of hotong flour (Table 2). At 24 h, *L. plantarum* produced the highest reducing sugar level (0.36%), whereas *S. cerevisiae* and the mixed culture showed lower values of 0.21% and 0.24%, respectively. By 48 h, the trend reversed: *S. cerevisiae* increased sharply to 0.38%, while *L. plantarum* and the mixed culture decreased to 0.27% and 0.23%, respectively. At 72 h, the lowest reducing sugar was observed with *L. plantarum* (0.15%), followed by *S. cerevisiae* (0.22%) and the mixed culture (0.21%). The pronounced decline in reducing sugar under *L. plantarum* treatment indicates more intensive carbohydrate metabolism, where enzymes such as glucosidases and isomerases break down disaccharides and oligosaccharides either into non-reducing products or into secondary polysaccharide synthesis. In contrast, the mid-stage increase in reducing sugar with *S. cerevisiae* (48 h) likely reflects accumulation of glycolytic intermediates before their utilization for cell growth, consistent with the observation in cassava and sorghum flour fermentation, where *S. cerevisiae* generates higher reducing sugars during the logarithmic growth phase. The mixed culture displayed a moderate pattern, showing synergistic effects but less pronounced than single cultures in modifying sugar content. These findings align with studies on cassava and maize flour fermentations, which highlight the critical role of microbial selection and fermentation time in controlling reducing sugar levels to match the desired functional properties of the final product [41,42]. From an application standpoint, fermentation with *L. plantarum* for 72 h is suitable for producing low sugar hotong flour, supporting processing stability (e.g., in noodles or biscuits) and reducing the risk of caramelization during baking, whereas short fermentation with *S. cerevisiae* may be preferable when a naturally sweeter flavor profile is desired.

3.3.2. Antioxidant activity

A significant interaction ($p < 0.05$) between microorganism type and fermentation duration was observed in relation to the DPPH radical scavenging activity (%RSA) of hotong flour. At 24 h, *Saccharomyces cerevisiae* and the mixed culture both reached RSA values of 19.51%, notably higher than *Lactobacillus plantarum* (14.84%) (Table 3). After 48 h, the highest RSA to *S. cerevisiae* (20.26%), followed by the mixed culture (19.51%) and *L. plantarum* (17.55%). By 72 h, the mixed culture exhibited the strongest synergistic effect, achieving the highest RSA (20.26%), while *S. cerevisiae* (20.11%) and *L. plantarum* (19.36%) followed closely. These dynamics highlight the complementary roles of microbial antioxidant enzymes, such as phenol oxidase from *L. plantarum* and β -glucosidase from *S. cerevisiae*, which act differently across

fermentation stages to regulate both release and degradation of radical scavenging compounds [17,24,43].

Table 3. Functional properties of hotong flour.

Functional Characteristics	Microorganisms	Fermentation Time			Mean
		24 Hours	48 Hours	72 Hours	
Total Phenolic Content (mg/100g)	<i>Lactobacillus plantarum</i>	1.50 ± 0.00 ^a	0.92 ± 0.01 ^b	0.50 ± 0.01 ^c	0.97 ± 0.45 ^A
	<i>Saccharomyces cerevisiae</i>	0.59 ± 0.01 ^d	0.66 ± 0.01 ^c	0.89 ± 0.01 ^b	0.71 ± 0.14 ^B
	Combination	0.53 ± 0.01 ^e	0.42 ± 0.01 ^f	0.38 ± 0.01 ^g	0.44 ± 0.07 ^C
	Mean	0.87 ± 0.49 ^A	0.66 ± 0.22 ^B	0.59 ± 0.24 ^C	
Antioxidant Activity (DPPH (%RSA))	<i>Lactobacillus plantarum</i>	14.84 ± 0.11 ^d	17.55 ± 0.11 ^c	19.36 ± 0.11 ^b	17.25 ± 2.04 ^C
	<i>Saccharomyces cerevisiae</i>	19.51 ± 0.11 ^b	20.26 ± 0.11 ^a	20.11 ± 0.11 ^a	19.96 ± 0.36 ^A
	Combination	19.51 ± 0.11 ^b	19.51 ± 0.11 ^b	20.26 ± 0.11 ^a	19.76 ± 0.62 ^B
	Mean	17.95 ± 2.41 ^C	19.10 ± 1.25 ^B	19.91 ± 0.44 ^A	

Notes: Values with different lowercase letters in the same row differ significantly due to interaction effects (Tukey α 0.05). Values with different uppercase letters in the same column differ significantly for single factors. Values without letters are not significantly different

From an application standpoint, 72 h fermentation with *S. cerevisiae* or the mixed culture offers the most favorable balance between antioxidant release and matrix stability, although the absolute RSA remains below solvent-extracted values. To further enhance antioxidant capacity, integrating multistrain fermentation with post-fermentation strategies such as standardized solvent extraction or enzymatic hydrolysis could be effective, as demonstrated in earlier studies on *S. italica*.

4. Conclusions

This study demonstrates that solid-state fermentation of whole foxtail millet (hotong) grains is an effective strategy to enhance both physicochemical and functional properties of the resulting flour, directly addressing the objective of evaluating the effects of microorganism type and fermentation duration. The findings confirm that these two variables significantly influence product characteristics. Fermentation with *Lactobacillus plantarum* for 72 h produced flour with the highest water-holding capacity (128.94% wb) and brightness ($L^* = 87.37$), indicating its suitability for food products requiring high hydration and light color. In contrast, *Saccharomyces cerevisiae* and mixed cultures achieved the highest antioxidant activity (20.26% RSA), while shorter fermentation with *L. plantarum* (24 h) retained the highest total phenolic content (1.50 mg/100g). The novelty of this study lies in the application of fermentation to whole grains rather than flour, demonstrating distinct improvements in functional quality and highlighting the importance of microbial selection and process duration. These findings provide a basis for tailoring fermentation conditions to develop hotong flour with specific functional attributes for diverse food

applications. Future research may focus on process optimization at industrial scale and exploration of additional microbial consortia to further enhance nutritional and functional properties.

Abbreviations

AOAC	Association of Official Analytical Chemists
ANOVA	Analysis of Variance
Aw	Water activity
CIE	Commission Internationale de l'Éclairage
DPPH	2,2-diphenyl-1-picrylhydrazyl
DF	Dilution factor
DNS	Dinitrosalicylic acid
GAE	Gallic Acid Equivalent
% RSA	Percent Radical Scavenging Activity
UV–VIS	Ultraviolet–Visible spectrophotometer
WI	Whiteness Index
WHC	Water Holding Capacity

Data availability statement

Data will be made available on request at <https://bit.ly/Datahotong>

CRedit authorship contribution statement

Belsy V. Erubun: Conceptualization; Investigation; Data curation; Formal analysis; Methodology; Visualization; Writing – original draft. **Helen C. D. Tuhumury:** Conceptualization; Methodology; Supervision; Validation; Project administration; Resources; Writing – review and editing. **Gillian Tetelepta:** Investigation; Data curation; Resources; Validation; Writing – review and editing.

Declaration of Competing Interest

The authors declare no competing interests.

Declaration of Use of AI in the Writing Process

The authors used Scite AI during the preparation of this manuscript to support literature review process and Grammarly to improve grammar, paraphrase text, enhance clarity. After using the tool/service, the authors carefully reviewed and edited the content and take full responsibility for the content of the publication.

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