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Improvement of Resistant Starch Content in Millet (*Panicum miliaceum* L.) Flour by Fermentation and Heat Moisture Treatment

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Abstract: Resistant starch is considered a valuable prebiotic source, and its content is associated not only with dietary fiber but also closely related to the amylose fraction of starch. Millet is a cereal grain rich in dietary fiber. Compared with staple cereals such as rice, wheat, and corn, millet contains higher levels of dietary fiber and antioxidants. The primary component of millet is starch, which accounts for approximately 70% of the grain, consisting of amylose and amylopectin. The research objective – to determine the best modification method to produce millet flour with the highest RS3 content. This study uses a completely randomized design with five treatment variations, repeated three times, applying different fermentation types followed by High Moisture Treatment (HMT). The analysis included the contents of starch, amylopectin, and resistant starch. The results show that significant differences in resistant starch levels were observed between treatments without HMT and those subjected to HMT. Fermentation followed by HMT effectively enhanced the resistant starch content of millet flour, highlighting the importance of combining biological and physical modifications. Among the treatments, fermentation with *Lactobacillus rhamnosus* SKG 34 followed by the HMT process was identified as the most effective approach, resulting in the highest resistant starch content of 3.85%. These findings demonstrate the potential of this combined modification strategy for improving the functional properties of millet flour.

Keywords: Fermentation; High Moisture Treatment; Millet Flour; Resistant Starch.

Introduction

Millet is one of the cereal grains widely recognized for its high dietary fiber content, which contributes to a greater satiety effect. The dietary fiber content of millet flour ranges from 12.55% to 15.26%, consisting of 1.98–2.08% soluble fiber and 10.57–13.18% insoluble fiber [1]. In general, millet is rich in vitamins (e.g., B-complex vitamins), dietary fiber, protein, starch, and minerals (such as calcium and iron), and its overall nutritional composition is superior to that of common cereals such as rice, wheat, and corn [2]. The primary component of millet is starch, accounting for approximately 51-79% on a dry basis [3]. Starch from different millet varieties is known to exhibit diverse compositions and properties, including amylose contents ranging from 3% to 38.6% [4]. The relatively high starch and dietary fiber contents of millet indicate its potential as an alternative source for resistant starch (RS) production.

Resistant starch (RS) is classified as an insoluble fiber; however, despite being measured as insoluble fiber, RS exhibits physiological functions similar to those of soluble fiber [5]. Resistant starch content of unmodified millet flour ranged from 1.26% to 1.95% [6]. Resistant starch is a highly promising prebiotic source, in addition to oligosaccharides. RS is defined as starch that is resistant to hydrolysis by gastric acid and cannot be digested by pancreatic enzymes. Consequently, it is not absorbed in the small intestine but can be fermented by microorganisms in the large intestine [7]. Other advantages of RS include its ability to prevent constipation even when consumed in large amounts, reduce cholesterol levels, and lower the glycemic index.

The RS content of starch can be increased through physical, chemical, and enzymatic modification techniques, commonly referred to as resistant starch type III (RS3). One of the most effective alternative approaches to enhance RS content is the use of lactic acid bacteria (LAB) fermentation, followed by pressure, heat, and cooling treatment. The combination of fermentation and pressure–heat–cooling has been reported to increase RS content in sorghum flour by 8.1-fold. Similarly, the combination of mixed LAB culture fermentation and autoclave heating increased the RS content of plantain flour from 5.87%-6.45% to 12.99%-13.71% [8].

Two LAB species identified as having amylolytic characteristics during *growol* fermentation are *Lactobacillus plantarum* and *Lactobacillus rhamnosus*. *L. plantarum* produces lactic acid through glucose metabolism and synthesizes amylase and amylopullulanase enzymes. The use of *L. rhamnosus* SKG 34 was based on its homofermentative characteristics. The involvement of microbial enzymes plays a crucial role in determining the proportions of amylose and amylopectin. Enzymatic treatment leads to the formation of amylopectin with an increased number of α-(1,6) linkages and a higher proportion of short-chain amylose and amylopectin molecules. These shorter chains are unable to form junction zones, thereby resulting in an increase in RS [9]. RS type III from cassava starch can be enhanced through pressure heating and further optimized by lactic acid addition. The utilization of LAB aims to naturally produce lactic acid during fermentation, which promotes amylopectin linearization.

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Heat Moisture Treatment (HMT) is classified as a hydrothermal starch modification technique capable of inducing partial gelatinization of starch, which subsequently results in the formation to retrograded starch upon cooling [10]. The principle of hydrothermal treatment involves the application of controlled heat and limited moisture to modify starch structure. The HMT process is conducted using limited moisture contents (18, 21, 24, and 27%) and heating at temperatures above the gelatinization temperature. The combination of fermentation and physical modification increased the RS content of sorghum flour from 4.85% to 39.06%, whereas fermentation without physical treatment increased RS content from 4.85% to 27.31% [11].

Based on these findings, the combination of fermentation and physical modification has strong potential to enhance RS content in flour. To date, the production of resistant starch from millet grains through combined fermentation and HMT has not been reported. Therefore, it is expected that this combined modification approach will increase RS type III content in millet flour, resulting in a functional food ingredient. This study aimed to evaluate and analyze the effects of fermentation modification and Heat Moisture Treatment (HMT) on the resistant starch content of millet flour and to identify the optimal modification technique for producing millet flour with the highest RS type III (RS3) content.

Research Methods

This study employed a Completely Randomized Design (CRD) with five fermentation treatments: fermentation with *Lactobacillus plantarum* FNCC followed by HMT, and fermentation with *Lactobacillus rhamnosus* SKG 34 followed by HMT, spontaneous/natural fermentation followed by HMT, no fermentation with HMT, control (without fermentation and HMT). Each treatment was replicated three times, resulting in a total of 15 experimental units.

Preparation of Modified Millet Flour

Fermentation Millet Grain

The primary material used in this study was proso millet obtained from a traditional market in Denpasar, Indonesia. For the fermentation treatments, 300 g of millet were soaked in 400 mL of sterile distilled water, followed by the addition of starter cultures according to two treatment levels, while no culture was added for the natural fermentation treatment. The millet was then fermented at 27°C for 48 hours. For the control and non-fermented treatments, the millet grains were thoroughly washed and directly dried in an oven at 50°C for 4 hours, after which they were milled into flour.

Preparation Millet Flour

Following fermentation, millet grains were removed from the fermentation medium, washed thoroughly, and dried in oven dehydrator at 50°C for 5 h. The dried grains were then ground using a laboratory mill and sieved through an 80-mesh sieve. The resulting flour was subsequently subjected to HMT according to the experimental treatments.

Heat Moisture Treatment (HMT)

A 100 gram millet flour was first analyzed for water content to determine the amount of water required for HMT preparation. The moisture content of the flour was adjusted to 30% by adding distilled water to increase humidity. The flour was then wrapped in aluminum foil, placed in a sealed container, and stored at 4°C for 12 hours. Thermal treatment was subsequently conducted by oven heating at 110°C for 8 hours while the flour remained wrapped in aluminum foil and placed in a heat-resistant container [8]. After HMT, the flour was equilibrated at room temperature for 2 hours and then dried using a tray dryer at 45°C for 3 hours. The modified millet flour was reground and sieved through an 80-mesh sieve.

Data Analysis

The samples analyzed in this study were millet flour produced according to the applied treatments. A simple random sampling technique was applied to the flour samples, which were considered homogeneous experimental units under the Completely Randomized Design (CRD). The parameters observed in this study included starch content and resistant starch content [12], as well as amylose content [13]. All data were analyzed using analysis of variance (ANOVA). When significant differences were observed, Duncan's Multiple Range Test (DMRT) was applied at a 95% confidence level using SPSS version 16.0. The resistant starch content before and after Heat Moisture Treatment (HMT) was analyzed using a Paired T-test to determine differences in resistant starch values.

Results and Discussion

The fermentation process resulted in a reduction in starch content due to the presence of water-soluble starch fractions that dissolved during fermentation. The starch content of millet flour ranged from 47.33% to 64.53%. Analysis of variance revealed a significant difference ($p < 0.05$) among treatments. The lowest starch content (47.33%) was observed in flour subjected to fermentation, whereas the highest starch content was observed in non-fermented flour. Starch degradation occurred as fermentation promoted the breakdown of complex compounds, particularly starch, into simpler compounds such as sugars and organic acids. This phenomenon was confirmed by the decrease in pH values of the fermentation medium throughout the fermentation process.

The proportion of amylopectin and amylose plays a crucial role in determining the functional properties of starch. In general, starch contains a higher proportion of amylopectin than amylose. The ratio of amylose to amylopectin influences starch solubility and the degree of gelatinization. Lower amylose levels promote stickiness and surface gloss, whereas an increased amylose proportion result in drier texture, darker appearance, and lower palatability [14]. The secretion of enzyme and organic acids by Lactic acid bacteria drives starch depolymerization and changes the structure and relative proportions of amylose and amylocpectin [15].

The amylose content of millet flour ranged from 2.96% to 3.35%, and analysis of variance indicated no significant difference ($p > 0.05$) in amylose content among the treatments. Amylose plays a crucial role in the formation of RS3; amylose content can be increased through enzymatic debranching, where enzymes, including pullulanase and isoamylase, selectively hydrolyse α-1,6-D-glycosidic linkages, thereby transforming amylopectin side chains into linear short-chain amylose (SCA) [16].

Table 1. Physicochemical Properties of Millet Flour

Treatment	Starch content (%)	Amylopectin (%)	Amylose (%)	Amylose: Amylopectin	RS (%)
P1	53.93 ± 9.38^c	50.60 ± 11.79^d	3.33 ± 0.61	6.16 : 93.84	3.51 ± 0.01^b
P2	60.04 ± 0.08^b	56.69 ± 1.01^c	3.35 ± 0.32	5.58 : 94.42	3.85 ± 0.24^a
P3	61.91 ± 0.60^b	58.78 ± 0.47^b	3.13 ± 0.08	5.06 : 94.94	3.47 ± 0.06^b
P4	64.53 ± 0.05^a	61.56 ± 0.06^a	2.96 ± 0.04	4.59 : 95.41	3.37 ± 0.09^c
P5	47.33 ± 0.02^d	44.32 ± 0.02^e	3.01 ± 0.71	6.36 : 93.64	3.15 ± 1.51^d

Notes:
P1: Fermentation with *L. plantarum* FNCC followed by HMT;
P2: Fermentation with *L. rhamnosus* SKG 34 followed by HMT;
P3: Natural fermentation followed by HMT;
P4: No fermentation with HMT;
P5: control (Without fermentation, without HMT)
Different letters within the same row indicate significant differences ($p < 0.05$).

The highest amylopectin content was observed in the treatment without fermentation and HMT, which differed significantly ($p < 0.05$) from treatment P3 (natural fermentation without HMT). Based on these results, the HMT process successfully increased the amylopectin content of millet flour. This finding is consistent with previous reports stating that one of the advantages of HMT is its ability to increase starch content in flour-based materials.

The highest resistant starch content in millet flour was observed in the treatment involving fermentation with *Lactobacillus rhamnosus* SKG 34, followed by HMT, which was significantly different ($p < 0.05$) from millet flour subjected to fermentation with Lactobacillus plantarum, followed by HMT. The significant increase in resistant starch content of millet flour is presumably attributed to the role of bacteria used during the fermentation process. Naturally fermented flour exhibited a resistant starch content of 3.47%, which was significantly different ($p < 0.05$) from millet flour fermented with *L. plantarum* (3.51%) and *L. rhamnosus* SKG 34 (3.85%) (Table 2). *Lactobacillus rhamnosus* has the ability to produce Pullulanase. Fermentation of waxy rice flour with *L. rhamosus* has been reported to increase RS content from 17.34% to 30.12% [17]. An increase in resistant starch content may occur due to the debranching of α-1,6 amylopectin linkages by pullulanase enzymes produced during lactic acid bacteria fermentation [18]. Pullulanase enzymes hydrolyse α-1,6 branching bonds randomly within the internal structure of amylopectin. These enzymes are heat-stable and act on outer branch chains consisting of two or more glucose units [19]. Pullulanase has been shown to be effective in enhancing the amylose level in starch. Physical, chemical, enzymatic, and fermentation modification strategies significantly enhanced the resistant starch characteristics and prebiotic potential of porang flour. Among the treatments, debranching pullulanase (DP) was identified as the most effective approach, producing starch granules with sharply defined surface morphology, a total starch content of 39.81%, an amylose content of 3.73%, and an amylopectin content of 36.08% [20].

Table 2. Comparison of RS of Millet Flour Before and After HMT

Treatment	RS without HMT (%)	RS with HMT (%)
P1	2.94*	3.51*
P2	2.94*	3.85*
P3	3.27*	3.47*
P4	3.15*	3.37*

Notes:
P1: Fermentation with *L. plantarum*;
P2: Fermentation with *L. rhamnosus* SKG 34;
P3: Natural fermentation;
P4: No fermentation.
Based on a paired t-test ($p < 0.05$), indicating a significant difference between millet flour before and after HMT.

The HMT process (30% moisture content, 12 h cooling at 4°C, and thermal treatment at 110°C for 8 h) effectively increased the resistant starch content of millet flour. A significant difference ($p < 0.05$) was observed between millet flour before and after HMT treatment. Millet flour fermented with *L. rhamnosus* SKG 34 exhibited the highest increase in resistant starch content compared to other treatments (Table 2). Flour can be classified into five categories based on resistant starch content: very low (<1%), low (1–2.5%), moderate (2.5–5%), high (5–15%), and very high (>15%). Based on this classification, all millet flour samples in this study were categorized as having a moderate resistant starch content (2.5–5%) [21].

Conclusion

Fermentation methods using different bacterial isolates, as well as natural fermentation, significantly influenced changes in the starch composition, amylopectin, and resistant starch content of millet flour. Each treatment produced different values for starch, amylopectin, and resistant starch content. Fermentation using *Lactobacillus rhamnosus* SKG 34 followed by HMT produced the highest resistant starch content, reaching 3.85%. Treatments without HMT and with HMT resulted in significantly different resistant starch contents in millet flour. Fermentation followed by HMT effectively increased the resistant starch content of millet flour, particularly in flour fermented using

L. rhamnosus SKG 34. Amylose content plays a crucial role in the formation of resistant starch in flour. Therefore, further studies are recommended to investigate pre-processing and modification strategies that can optimize amylose content in millet flour and enhance its resistant starch formation.

Author's Contribution

I. D. P. K. Pratiwi: conceptualized the study, designed the methodology, conducted the experiments, collected and analyzed the data, and drafted the manuscript. K. A. Nocianitri: supervised the research, contributed to data interpretation, reviewed and revised the manuscript, and approved the final version. Both authors read and approved the final manuscript.

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