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Effects of Physicochemical, Fermentation, and Enzymatic Modifications on the Microstructural and Chemical Properties of Barley (*Hordeum vulgare*) Flour

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Abstract: Resistant starch, a dietary component with prebiotic potential, can be enhanced through starch modification techniques. This study investigated the effects of fermentation, enzymatic, and physicochemical modifications on the microstructural and chemical characteristics of barley flour (Hordeum vulgare). Modified samples were analysed for starch composition, reducing sugars, and granule morphology. Structural analysis showed pronounced disruption of starch granules and the formation of irregular aggregates, particularly following autoclaving, cooling, and enzymatic debranching treatments. Chemically, total starch content increased significantly, reaching 41.64% in the debranching pullulanase (DP) treatment, while amylose content peaked at 30.65% under annealing (ANN). In contrast, reducing sugar levels declined markedly, with the lowest values observed in autoclaving–cooling two-cycle (AC-2) and heat moisture treatment (HMT) samples (both 8.88%). These results demonstrate that targeted modification approaches can enhance starch resistance and improve the structural and functional properties of barley flour, supporting its potential use in health-oriented and functional food formulations.

Keywords: barley flour; fermentation; microstructure; pullulanase; resistant starch.

1. Introduction

Barley (*Hordeum vulgare*) is a nutrient-dense cereal grain recognised for its balanced macronutrient profile and health-promoting components. It serves as an important source of complex carbohydrates. It contains relatively high protein levels compared to other staple cereals, making it a promising alternative to rice, wheat, or oats in various food applications [1]. Whole barley also provides essential micronutrients and vitamins at higher concentrations than most other grains, while its abundant β-glucan, a soluble dietary fibre, contributes to

multiple physiological benefits [2]. The intake of β-glucan-rich barley products has been shown to improve lipid metabolism, reducing plasma triacylglycerides (TG), total cholesterol (TC), and LDL cholesterol while elevating HDL levels [3]. Moreover, the nutritional integrity of barley is largely retained during flour processing, allowing its incorporation into composite flours that enhance the nutritional quality of baked products [4,5].

The primary carbohydrate component of barley flour is starch, composed of two α-D-glucan polymers—amylose (approximately 25–28%) and amylopectin (75–85%) organised into semi-crystalline granules [6]. Amylose consists of mostly linear α-(1,4)-linked glucose chains, while amylopectin exhibits a highly branched α-(1,6)-linked structure. The ratio and structural arrangement of these polymers play crucial roles in starch digestibility and the formation of resistant starch (RS). Intrinsic factors, such as amylose content, chain-length distribution, lipid interactions, and non-starch components, determine the degree of retrogradation and the subsequent RS yield. In particular, Type III RS predominantly consists of retrograded amylose chains, typically with a degree of polymerisation around 100 glucose units, which facilitates stable double-helix crystallisation upon cooling [7].

Extrinsic factors, including heating, cooling, and storage parameters, also govern RS development, particularly under hydrothermal or enzymatic modification processes [8]. These treatments promote partial gelatinisation followed by molecular reassociation, enhancing the formation of ordered structures resistant to enzymatic hydrolysis. Li *et al*. [9] further demonstrated that increasing the amylose fraction and optimising process variables—such as water content, autoclaving temperature, enzymatic debranching, and controlled cooling—can significantly enhance RS yield. The linearity of amylose favours double-helix formation, especially during storage at low temperatures (≈4°C), where recrystallisation is thermodynamically favoured. This structural reorganisation increases gelatinisation temperature and thermal stability, though excessive hydrolysis or chain shortening may reduce the extent of retrogradation [10].

Despite extensive work on starch modification, most studies focus on isolated starch fractions rather than whole-grain or flour matrices, thereby neglecting the interactions between starch, proteins, and β-glucans that can influence retrogradation and RS formation [11,12]. Furthermore, direct comparisons between biological (fermentation, enzymatic) and physicochemical (hydrothermal, annealing, acid hydrolysis) modification approaches under standardised processing conditions are limited. Such comparisons are essential to elucidate how different modification mechanisms affect the structural and compositional transformations of starch in complex food systems [13]. Therefore, the present study aims to systematically compare fermentation, enzymatic, and hydrothermal modification methods of barley flour to identify which process most effectively enhances the structural characteristics associated with resistant starch. This approach provides new insights into optimising starch modification strategies to develop functional cereal-based ingredients with improved nutritional and technological properties.

2. Materials and Methods

2.1. Materials.

The bacterial culture used was *Lactobacillus plantarum* IIA-1A5, which originated from the Bacteriology Laboratory at BRIN Cibinong. The source material for starch was barley from Bantul Regency, Yogyakarta, Indonesia. The materials for chemical analysis were

Pullulanase enzyme (E2412) from SIGMA and 3,5-dinitrosalicylic acid (DNS) (D0550) from SIGMA.

2.2. The fermentation of barley starch modification.

Fermentation-based modification of barley starch was conducted following Winarti and Anggreini [12], with minor adjustments. Barley flour (250 g) was dispersed in sterile distilled water (1:3, w/v) and inoculated with *Lactobacillus plantarum* culture (5% w/w). The suspension was incubated at 37°C for 24 h, autoclaved at 121°C for 15 min, cooled at 4°C for 24 h, and oven-dried at 60°C for 24 h. The dried flour was ground, sieved (80 mesh), and stored in airtight containers.

2.3. The enzymatic barley starch modification.

Enzymatic debranching of barley starch was performed as described by Tu *et al*. [14]. Barley flour was suspended in distilled water (1:3, w/v), gelatinised by autoclaving at 121°C for 15 min, and cooled at 4°C for 24 h. The paste was then mixed with 0.1 M acetate buffer (pH 5.2) containing pullulanase enzyme (10.4 U/g flour) and incubated at 50°C for 24 h. The treated paste was re-autoclaved (121°C, 15 min), cooled (4°C, 24 h), and oven-dried (60°C, 24 h). The dried flour was ground, sieved (80 mesh), and stored in sealed containers.

2.4. The physicochemical barley starch modification.

Physicochemical modifications were carried out according to the procedures described by Putra [15] and other references [16–19]. Barley flour suspensions (1:3, w/v) were subjected to several treatments. The autoclaving–cooling (AC) treatment involved autoclaving at 121 °C for 15 min followed by cooling at 4 °C for 24 h; this cycle was repeated twice, and the samples were subsequently oven-dried at 60 °C for 24 h. In the microwave–cooling (MW) treatment, samples were heated in a microwave at 600 W for 5 min, then cooled at 4 °C for 24 h; this cycle was repeated twice before oven drying at 60 °C for 24 h. For heat–moisture treatment (HMT), the moisture content of the flour was adjusted to 20% and the samples were heated at 60 °C for 3 h. In the annealing (ANN) treatment, flour suspensions were incubated at 50 °C for 24 h. For acid hydrolysis (AH), the flour was mixed with 2.2 N HCl at a ratio of 1:1 (w/v) and incubated at 35 °C for 2 h with continuous stirring; the suspension was then neutralised to pH 6.0, washed, cooled at 4 °C for 24 h, and oven-dried at 60 °C for 24 h. All modified flours were subsequently ground, sieved through an 80-mesh screen, and stored in airtight containers until analysis.

2.5. Analysis of the appearance and structure of modified barley flour granules.

The microstructural characteristics of the modified barley flour granules were examined using scanning electron microscopy (SEM), following the procedure of Zeng *et al*. [20] with minor modifications. The samples were mounted on carbon-coated stubs and sputter-coated with a thin layer of gold to enhance conductivity. Imaging was performed using a Thermo Scientific Quattro S SEM operated at 10 kV. Micrographs were acquired at magnifications ranging from 1000× to 5000×, with a 10 µm scale bar. The resulting images were analysed to evaluate changes in granule morphology, surface texture, and aggregation behaviour associated with the modification treatment.

2.6. Analysis of total starch content of modified barley flour.

The total starch content of the modified barley flour was determined following the method described by Setiarto *et al*. [21], with minor modifications. Briefly, 1 g of modified barley flour was mixed with 100 mL of 95% ethanol and stirred using a magnetic stirrer to remove soluble impurities. The suspension was filtered through Whatman No. 1 filter paper and dried in a desiccator. The recovered starch was weighed after 24 h, finely ground, and 40 g of the flour was subsequently dispersed in 20 mL of distilled water. The mixture was autoclaved at 105°C for 1 h, cooled to room temperature, and diluted 40-fold with distilled water. For colourimetric determination, 0.5 mL of the diluted sample was mixed with 0.5 mL of 5% (w/v) phenol, followed by the addition of 2.5 mL of concentrated H_2SO_4. The mixture was vortexed, allowed to stand at room temperature for 10 min, vortexed again, and then allowed to stand for another 20 min. Absorbance was measured at 490 nm using a UV–Vis spectrophotometer (Shimadzu UV-1800, Japan). The instrument was calibrated using a glucose standard curve (0–100 µg/mL; $R^2 > 0.99$), and reagent blanks were included to correct the baseline. Total starch concentration was calculated from the standard curve and expressed as mg g^{-1} dry weight.

$$\text{Total starch content (\%)} = \frac{C \; x \; V \; x \; DF \; x \; 0.9}{W \; x \; 10}$$

Where: C= glucose concentration obtained from the standard curve (mg/mL); V= total extract volume (mL); DF= dilution factor; 0.9= conversion factor from glucose to starch (since hydrolysis of starch yields approximately 0.9 g glucose per g starch); W= sample weight (g, dry basis); The division by 10 converts mg/g to % (w/w).

2.7. Analysis of amylose and amylopectin content of modified barley flour.

The amylose and amylopectin contents of the modified barley flour were determined according to the method of Setiarto *et al*. [21], with slight modifications. Approximately 100 mg of sample was mixed with 1 mL of 95% ethanol and 9 mL of 1 N NaOH, then heated in a water bath at 95°C for 10 min to gelatinise the starch. The mixture was cooled to room temperature to form a homogeneous gel, then quantitatively transferred to a 100 mL volumetric flask and diluted to volume with distilled water. An aliquot of 5 mL of this solution was mixed with 1 mL of 1 N acetic acid, 2 mL of iodine solution (0.2% I_2 and 2.0% KI), and 5 mL of distilled water. The mixture was allowed to stand for 20 min at room temperature to allow colour development. Absorbance was measured at 625 nm using a UV–Vis spectrophotometer (Shimadzu UV-1800, Japan). The instrument was calibrated using amylose standard solutions (0–100 µg/mL; $R^2 > 0.99$), and reagent blanks were used for baseline correction. Amylose content was calculated from the calibration curve, while the difference between total starch and amylose values determined amylopectin content. Results were expressed as percentages of total starch (w/w, dry basis).

$$\text{Amylose content (\%)} = \frac{C \; x \; V \; x \; DF}{W \; x \; 10}$$

$$\text{Amylopectin content (\%)} = \text{Total starch (\%)} - \text{Amylose (\%)}$$

Where: C= amylose concentration obtained from the standard curve (mg/mL); V= total volume of the extract (mL); DF= dilution factor; W= sample weight (g, dry basis); The factor 10 converts mg/g to % (w/w).

2.8. Analysis of reducing sugar contents of modified barley flour.

The reducing sugar content of the modified barley flour was determined using the 3,5-dinitrosalicylic acid (DNS) method as described by Chang *et al*. [17], with slight modifications. Briefly, 1 g of the sample was mixed with 100 mL of 95% ethanol and stirred using a magnetic stirrer to remove soluble impurities. The suspension was filtered through Whatman No. 1 filter paper, and the residue was dried in a desiccator for 12 h. The dried material was ground to a fine powder, and 20 mg of the sample was dispersed in 10 mL of distilled water. The mixture was autoclaved at 105°C for 1 h, cooled to room temperature, and diluted tenfold prior to analysis. For reducing sugar determination, 1 mL of the diluted sample was mixed with 2 mL of DNS reagent, vortexed, and heated in boiling water for 5 min. After cooling to room temperature, 10 mL of distilled water was added, and the absorbance was recorded at 540 nm using a UV–Vis spectrophotometer (Shimadzu UV-1800, Japan). The instrument was calibrated using glucose standard solutions (0–500 µg/mL; $R^2 > 0.99$), and reagent blanks were included for baseline correction. Reducing sugar concentration was calculated from the calibration curve and expressed as µg glucose equivalents per mL of extract (µg/mL).

$$\text{Reducing sugar content (mg/g)} = \frac{C \; x \; V \; x \; DF}{W}$$

Where: C= concentration of reducing sugar (as glucose equivalent, mg/mL) obtained from the standard calibration curve; V= total volume of extract (mL); DF= dilution factor; W= weight of the sample (g, dry basis).

2.9. Statistical data analysis.

All measurements, including total starch, amylose, amylopectin, and reducing sugar contents of the modified barley flour, were performed in triplicate (n = 3), and results were expressed as mean ± standard deviation (SD). Statistical analyses were conducted using one-way analysis of variance (ANOVA) to assess significant differences among treatments at a 95% confidence level ($\alpha = 0.05$). When significant effects were detected, mean comparisons were performed using the Duncan test. Data analysis was carried out using IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA).

3. Results and Discussion

3.1. Physical appearance of modified barley flour granules.

The physical characteristics of TJM, particularly its appearance, were examined to observe the differences in barley flour after modification. The observations revealed a noticeable colour change in the barley flour following the fermentation (FLP), enzymatic (DP), and physicochemical treatments (AC-1, AC-2, MW, HMT, ANN, and HA), resulting in a brownish hue (Figure 1). This occurs due to a non-enzymatic browning reaction called the Maillard reaction [22]. The starch content influences the browning reaction in the flour [23].

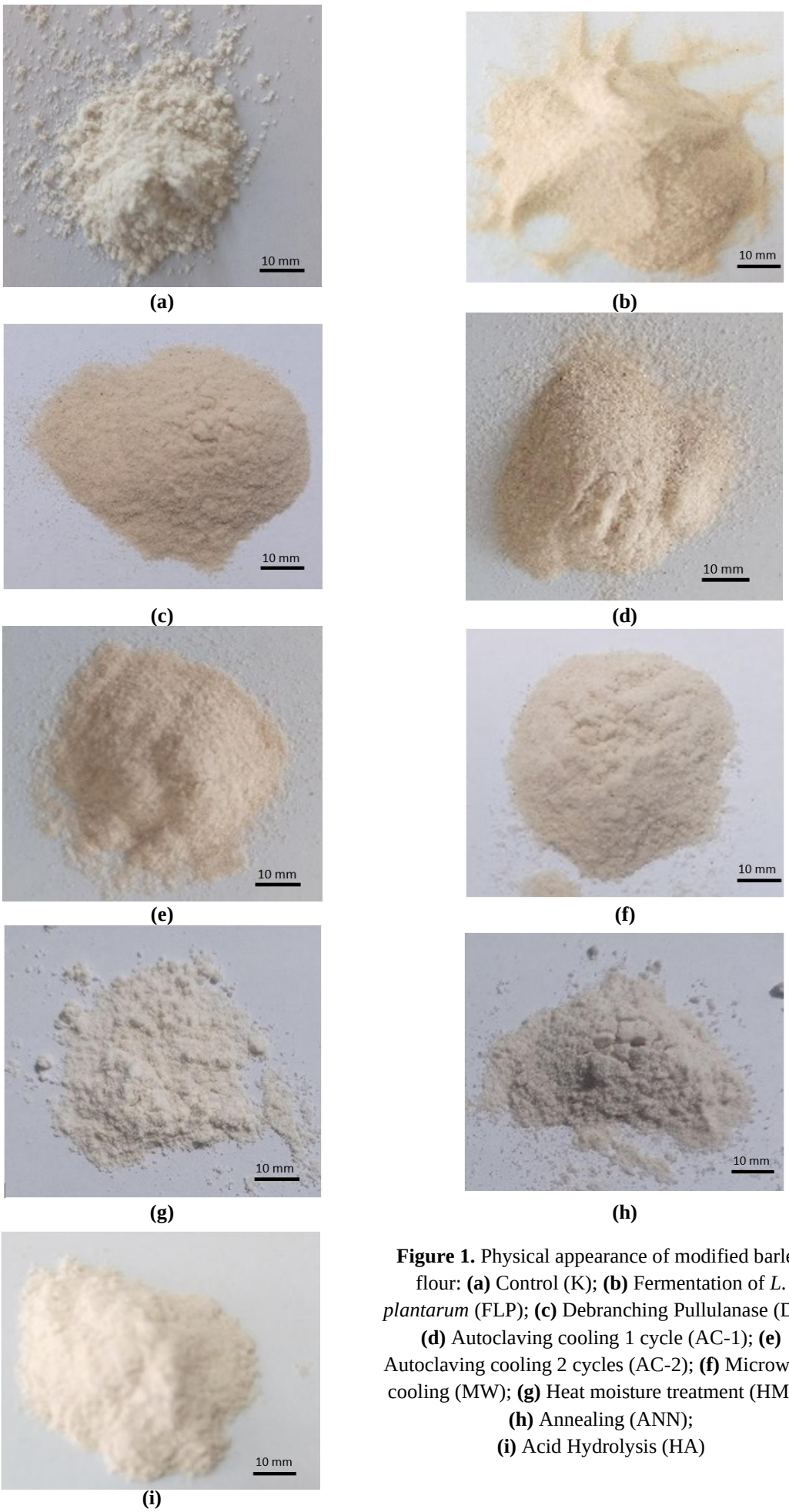


Figure 1. Physical appearance of modified barley flour: **(a)** Control (K); **(b)** Fermentation of *L. plantarum* (FLP); **(c)** Debranching Pullulanase (DP); **(d)** Autoclaving cooling 1 cycle (AC-1); **(e)** Autoclaving cooling 2 cycles (AC-2); **(f)** Microwave cooling (MW); **(g)** Heat moisture treatment (HMT); **(h)** Annealing (ANN); **(i)** Acid Hydrolysis (HA)

The Maillard reaction occurs during heating, typically at high temperatures and for prolonged exposure [24]. The Maillard reaction begins with the condensation of amino acids and sugars, followed by dehydration of their fragments. It concludes with aldol condensation and the formation of heterocyclic nitrogen compounds [22]. Based on this, it is explained that high temperatures, such as those experienced by HMT and ANN, trigger Maillard reactions.

Rannou *et al*. [22] It has been reported that water content affects the formation of Maillard reactions, with higher water content facilitating their occurrence. This indicates that Maillard reactions occurred in the FLP, DP, and AC-1 and AC-2 treatments. Dong *et al*. [25] reported that when heating using a microwave, the surface temperature of the flour becomes sufficiently hot to allow the MW treatment to form a Maillard reaction. Research by Nevara *et al*. [26] shows the effect of HMT on potato flour undergoing the Maillard reaction. Yonata *et al*. [27] It has been reported that the hydrolysis effect of acid solutions (HA) causes colour degradation in starch, and heating starch can also trigger the Maillard reaction.

3.2. Microstructure of modified barley flour granules.

Scanning electron microscopy (SEM) revealed distinct morphological alterations in barley starch granules depending on the modification technique employed (Figure 2). The autoclaving–cooling cycles produced the most extensive disruption, characterised by pronounced granule swelling, rupture, surface smearing, and loss of granular integrity. These features indicate near-complete gelatinisation followed by retrogradation [28]. Such disruption promotes the formation of amorphous regions and imperfect crystalline aggregates, structural attributes often associated with increased resistant starch formation.

The microwave–cooling treatment induced morphological effects that were comparable but less uniform. Localised fissures, porous domains, and partially collapsed granules were evident, reflecting internal steam pressure gradients. The heterogeneous nature of microwave heating resulted in a mixture of intact and disrupted granules, which may account for the irregularity in functional properties [29].

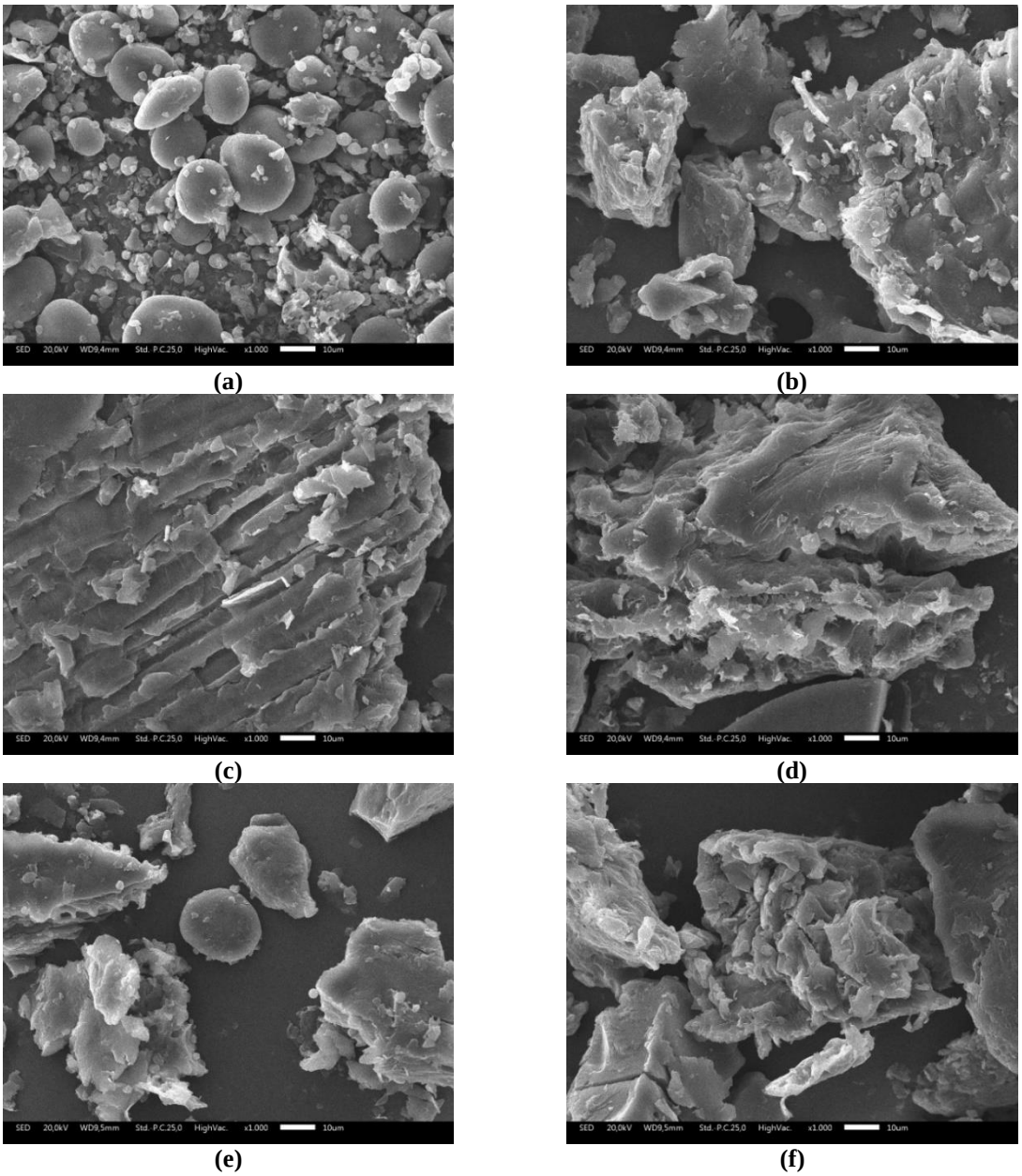
In contrast, heat–moisture treatment (HMT) largely preserved the granular architecture. The granules appeared compact but roughened, suggesting limited surface modification coupled with internal crystalline reorganisation and reduced swelling capacity [30]. Annealing caused even subtler changes, producing smoother surfaces and improved lamellar order without significant physical disruption. Both HMT and annealing therefore act as structural refinement processes, enhancing crystalline stability and promoting uniform, thermally stable granules [31].

Acid hydrolysis preferentially attacked amorphous regions, generating pitted surfaces and exposing crystalline remnants. Short hydrolysis durations increased relative crystallinity, whereas prolonged exposure led to fragmentation and partial loss of crystalline order [32]. Lactic acid bacteria (LAB) fermentation induced moderate but functionally significant changes. The combined action of organic acids and microbial enzymes enhanced porosity within the starch–protein matrix, occasionally accompanied by the deposition of exopolysaccharide films that modified particle interactions [33]. Pullulanase debranching, meanwhile, primarily influenced the molecular structure rather than the granular morphology, reducing amylopectin branching and promoting the formation of new crystalline regions during retrogradation [34].

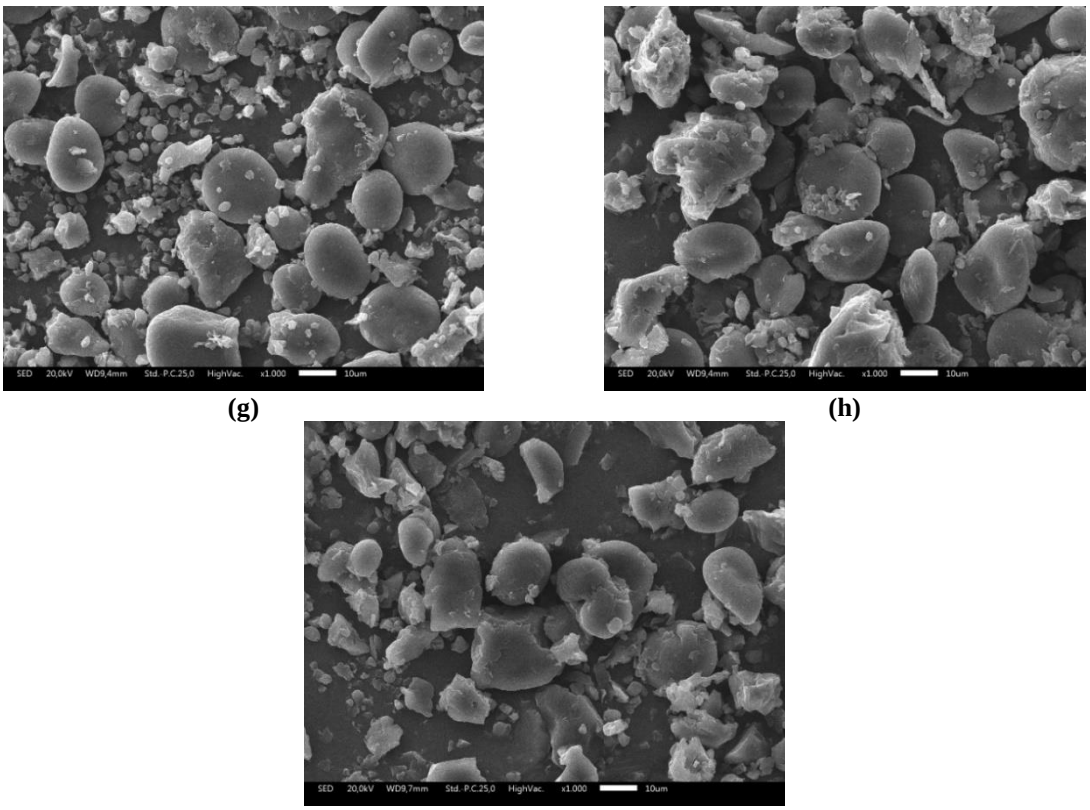
Overall, a gradient of microstructural disruption was observed across treatments. Autoclaving and microwave processes caused the most severe physical disintegration [35];

HMT and annealing enhanced crystalline order with minimal external damage; acid and enzymatic treatments modulated the crystalline–amorphous balance; and fermentation provided biologically mediated matrix remodelling [36]. These distinct structural outcomes underpin functional differences: severely disrupted granules (autoclaving, microwave) enhance water absorption and paste viscosity, whereas reorganised structures (HMT, annealing, pullulanase) improve thermal stability and resistant starch formation [37]. Fermentation further enhances nutritional functionality by reducing antinutritional factors and introducing microbial metabolites [38].

Nevertheless, SEM analysis provides qualitative insights limited to localised regions of observation. The heterogeneous effects of certain treatments—particularly microwave heating and fermentation—may not be fully captured by selected micrographs [39]. Moreover, treatment outcomes depend strongly on parameters such as moisture content, temperature, pH, and microbial activity, which collectively determine the balance between crystalline disruption and reorganisation (Figure 2). Interactions between starch and non-starch components of barley flour, especially proteins and β-glucans, also influence morphological evolution and warrant further investigation [40].



(a) **(b)** **(c)** **(d)** **(e)** **(f)**



(g) **(h)** **(i)**

Figure 2. Microstructure of modified barley flour by SEM magnification 1000 x: **(a)** Control (K); **(b)** Fermentation of *L. plantarum* (FLP); **(c)** Debranching Pullulanase (DP); **(d)** Autoclaving cooling 1 cycle (AC-1); **(e)** Autoclaving cooling 2 cycles (AC-2); **(f)** Microwave cooling (MW); **(g)** Heat moisture treatment (HMT); **(h)** Annealing (ANN); **(i)** Acid Hydrolisis (HA).

The physical structure of modified barley flour granules was examined by Scanning Electron Microscopy (SEM) to assess morphological alterations resulting from various modification treatments [21]. In the control sample, granules retained their typical neat and discrete morphology, indicative of native starch (Figure 2a). In contrast, marked structural changes were observed in the fermentation (FLP), enzymatic (DP), and physicochemical treatments (AC-1, AC-2, MW, HMT, ANN, and HA), resulting in aggregated, irregularly shaped granules (Figure 2b, c, d, e, f, g, h, i). These alterations are primarily attributed to gelatinisation and retrogradation processes occurring during modification [28].

Similar findings were reported by Setiarto [29], who found that *Lactobacillus plantarum* fermentation and pullulanase debranching caused starch swelling due to gelatinisation. Khan *et al*. [30] also observed that enzymatic hydrolysis resulted in irregular, deformed starch granules in corn flour. Hydrothermal treatments, such as autoclaving–cooling and microwave–cooling, induced structural disintegration and the formation of dense or sharp-edged granule fragments (Figure 2d,e,f). Setiarto [29] and Shen *et al*. [31] likewise demonstrated that hydrothermal processing of porang and oat flour resulted in gelatinisation, disrupting the original granular architecture. Chemical modification through acid hydrolysis similarly alters granule morphology (Figure 2i). Liang *et al*. [32] reported that acid-treated sorghum flour exhibited corroded, roughened surfaces, increased porosity, and cracked grains, features indicative of amorphous region degradation.

In barley (*Hordeum vulgare*) flour, diverse modification pathways—physical, enzymatic, and microbial—collectively reshape starch granule morphology, crystallinity, and the surrounding matrix organisation [33]. Autoclaving–cooling treatment induces partial gelatinisation followed by retrogradation, disrupting native granules and forming dense,

compact aggregates often associated with resistant starch type III (Figure 2d and 2e). SEM images typically show rough, irregular surfaces and reduced granule integrity, signifying crystalline rearrangements [34]. Heat–Moisture Treatment (HMT) promotes starch reorganisation under limited moisture and elevated temperature without inducing full gelatinisation (Figure 2g). The resulting granules exhibit smoother, compact surfaces and tighter molecular packing, reflecting improved crystalline order and reduced susceptibility to enzymatic attack [35]. Annealing preserves granule shape while enhancing internal molecular alignment within amorphous and crystalline domains (Figure 2h), producing minimal morphological changes but increased birefringence and crystallinity indicative of higher thermal stability [36]. Microwave–cooling treatment generates rapid heating and cooling cycles, resulting in localised gelatinisation and microcracking (Figure 2f). Uneven expansion and contraction lead to porous or fractured surfaces that enhance enzymatic accessibility and may facilitate the formation of resistant starch, depending on processing intensity [37].

Fermentation modifies the starch–protein matrix through microbial enzyme activity and acid production, degrading the surrounding network and increasing porosity (Figure 2b). SEM micrographs commonly reveal widened intergranular spaces, surface pits, and partial erosion of starch granules, consistent with microbial and biochemical remodelling [38]. A combined autoclaving–cooling and pullulanase debranching treatment further enhances microstructural reorganisation. The initial thermal cycle promotes gelatinisation and retrogradation, while subsequent enzymatic cleavage of α-1,6-glycosidic bonds facilitates linear chain alignment and crystalline aggregation (Figure 2c). The resulting microstructure shows dense, blocky remnants and well-organised aggregates, consistent with increased crystallinity and the formation of resistant starch [39].

Overall, each modification strategy produces distinct microstructural signatures. Thermal gelatinisation–retrogradation cycles (autoclaving, microwave) cause extensive granule disruption; controlled hydrothermal treatments (HMT, annealing) promote molecular reorganisation and crystalline refinement; acid and enzymatic processes selectively target amorphous or branched regions; and fermentation introduces gradual, biologically mediated remodelling [31,35,37]. Understanding these morphological responses enables the rational selection and combination of treatments to tailor barley flour functionality. Disrupted structures enhance hydration and pasting behaviour, whereas reorganised and crystalline structures confer improved thermal stability, enzymatic resistance, and potential for low-glycemic or functional food applications [40]. Future studies should integrate SEM characterisation with quantitative analyses of crystallinity, digestibility, and rheology to fully elucidate the structure–function relationships underlying modified barley flour systems.

3.3. Total starch content of modified barley flour.

Starch, the primary carbohydrate in barley flour, consists of two glucose polymers: amylose, a mostly linear α-(1,4)-D-glucan, and amylopectin, a highly branched α-(1,4)/α-(1,6)-D-glucan stored within starch granules [41]. Significant differences ($p < 0.05$) were observed in the total starch content among the various modification treatments—fermentation (FLP), enzymatic (DP), and physicochemical (AC-1, AC-2, MW, HMT, ANN, HA)—compared with the unmodified control (Figure 3). The control sample exhibited a total starch content of 30.62%, lower than the typical range reported for barley varieties from China (45.7–66.4%) [42] and Austria (51.96–59.48%) [43]. These variations reflect genotypic and environmental influences on starch biosynthesis [42].

Among all treatments, pullulanase debranching (DP) yielded the highest total starch content (41.65%), significantly higher than that of the control and other modifications. This aligns with Harder *et al*. [43], who found that pullulanase-treated barley starch exhibited enhanced starch recovery and structural reorganisation. The enzymatic cleavage of α-1,6 linkages in amylopectin during pullulanase treatment facilitates the formation of more linear chains, which readily realign into ordered crystalline regions during retrogradation, thereby contributing to the formation of resistant starch (RS type III). Other treatments, including microwave–cooling and autoclaving–cooling cycles, produced moderate increases in starch content, consistent with findings by Li *et al*. [44], who reported similar improvements in sorghum starch after thermal–cooling treatments. These changes are primarily due to partial gelatinisation followed by retrogradation, which reorganises starch molecules into less digestible crystalline aggregates.

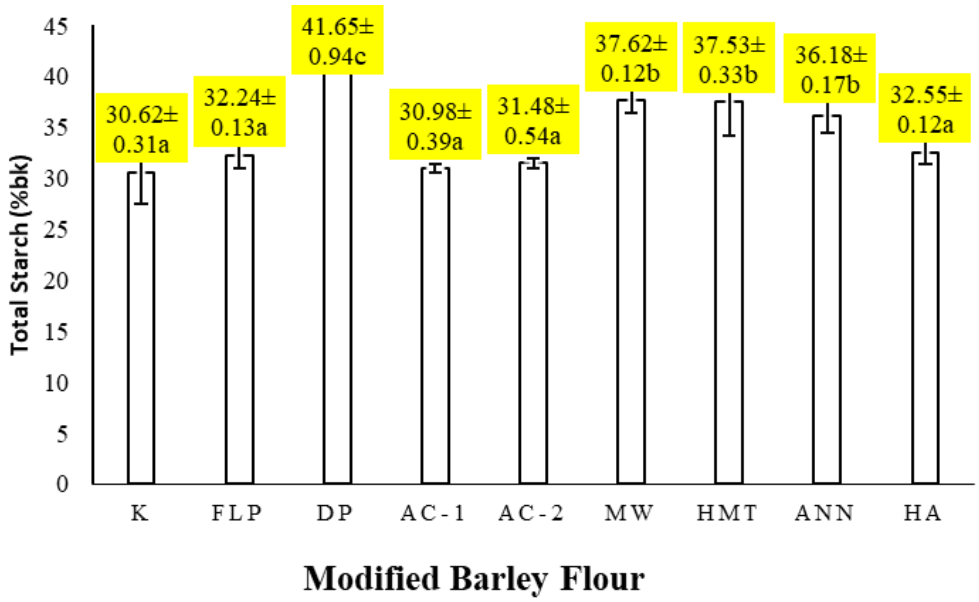


Figure 3. Total starch content of modified barley flour: **(a)** Control (K); **(b)** Fermentation of *L. plantarum* (FLP); **(c)** Debranching Pullulanase (DP); **(d)** Autoclaving cooling 1 cycle (AC-1); **(e)** Autoclaving cooling 2 cycles (AC-2); **(f)** Microwave cooling (MW); **(g)** Heat moisture treatment (HMT); **(h)** Annealing (ANN); **(i)** Acid Hydrolysis (HA). Note: The same letters on the bar chart indicate values that are not significantly different at a 95% confidence level (α=5%). The treatments produced distinct effects on total starch content, with enzymatic (DP) and hydrothermal (ANN, HMT, MW) modifications generally resulting in higher total starch compared to the control, while fermentation (FLP) showed a moderate increase.

The total starch content of barley flour is significantly affected by physical (thermal) and enzymatic modifications, as well as fermentation processes. These treatments influence starch structure, crystallinity, and enzymatic accessibility, leading to either apparent changes in measured starch levels or to actual degradation via hydrolysis or microbial metabolism [45]. Autoclaving–Cooling (AC) induces starch gelatinisation during heating, followed by retrogradation upon cooling. This process enhances the formation of resistant starch (RS type III), which is less susceptible to enzymatic hydrolysis and may therefore be underestimated in standard starch assays. Although the true starch mass remains largely conserved, the increase in crystalline, enzyme-resistant domains lowers apparent starch values [46].

Heat–Moisture Treatment (HMT), performed under restricted moisture and elevated temperature, reorganises starch granules without inducing gelatinisation. The resulting compact molecular arrangement increases resistance to enzymatic digestion but does not chemically degrade starch. Consequently, the apparent starch content may decline slightly due

to reduced solubility and reduced enzyme accessibility, rather than an actual loss of material [47]. Annealing, a milder hydrothermal process conducted below gelatinisation temperature, similarly enhances crystalline order and reduces digestibility without major alteration of total starch [36]. Microwave treatment exerts dual effects depending on exposure intensity. Moderate microwaving promotes partial gelatinisation and subsequent retrogradation, thereby enhancing resistant starch formation, whereas excessive heating can induce polymer depolymerisation and dextrinisation, leading to a reduction in measurable starch [48].

Fermentation, in contrast, causes a genuine decrease in total starch. Microorganisms such as lactic acid bacteria and yeasts utilise starch and its hydrolysates as carbon sources, enzymatically degrading granules and reducing starch mass [49]. The extent of this reduction depends on microbial strain, fermentation duration, and process conditions. Amylopectin typically degrades more rapidly than amylose, leading to altered amylose-to-amylopectin ratios and starch loss. A combined Autoclaving–Cooling with Pullulanase Debranching treatment integrates thermal and enzymatic modification. Pullulanase specifically cleaves α-1,6-glycosidic bonds in amylopectin, generating linear chains that retrograde more readily into crystalline RS type III. Although the starch remains chemically present, these resistant fractions are less hydrolysed by assay enzymes, leading to a perceived decline in total starch [50].

Technological modifications influence both true and apparent starch content. Fermentation results in actual starch degradation through microbial metabolism [51], whereas treatments such as autoclaving–cooling, HMT, and enzymatic debranching primarily reorganise starch structure into enzyme-resistant forms, reducing apparent digestibility rather than total mass. Understanding these distinctions is essential for designing functional barley-based products with targeted nutritional properties, particularly those aimed at lowering glycemic response or enriching resistant starch fractions.

In summary, enzymatic debranching using pullulanase was the most effective strategy for enhancing total starch content and promoting resistant starch formation. Physicochemical treatments (microwave, autoclaving–cooling) also improved starch structure but to a lesser extent, while fermentation and hydrothermal treatments primarily induced compositional and matrix-level changes rather than substantial increases in total starch. The combined analysis indicates that molecular restructuring via enzymatic modification offers the most efficient pathway to develop barley flour with improved starch functionality and potential low-glycemic properties.

3.4. Amylose and amylopectin content of modified barley flour.

Starch, the main carbohydrate in barley flour, consists of two polysaccharides: amylose, a predominantly linear α-(1,4)-linked glucan, and amylopectin, a highly branched polymer with α-(1,6) linkages [41]. The proportions of these two components determine starch functionality, particularly its gelatinisation, retrogradation, and the capacity to form resistant starch (RS) (Figure 4). The control barley flour contained 21.87% amylose, consistent with values reported for barley cultivars from Canada (26.6–28.8%) [52] and Europe (17.6–29.3%) [53]. Environmental and genetic factors, such as endosperm development and growing temperature, are known to influence this variability [42].

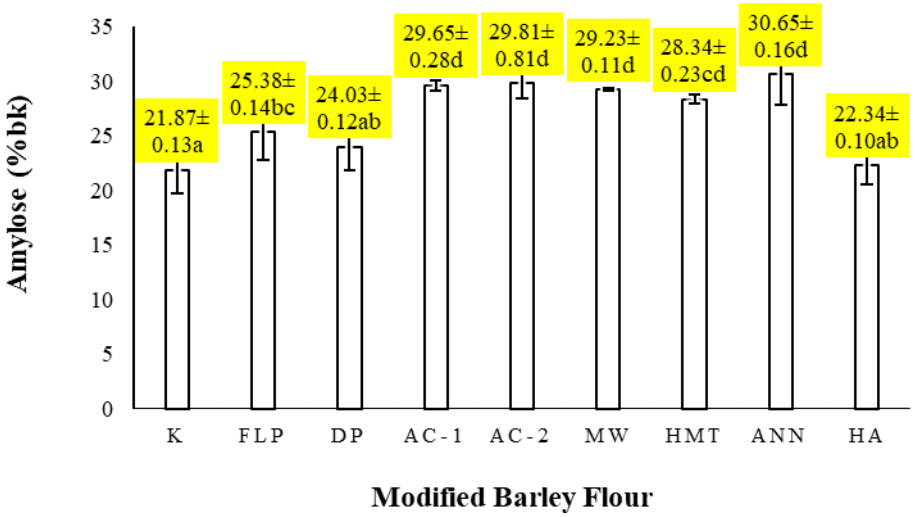


Figure 4. Amylose content of modified barley flour: (**(a)** Control (K); **(b)** Fermentation of *L. plantarum* (FLP); **(c)** Debranching Pullulanase (DP); **(d)** Autoclaving cooling 1 cycle (AC-1); **(e)** Autoclaving cooling 2 cycles (AC-2); **(f)** Microwave cooling (MW); **(g)** Heat moisture treatment (HMT); **(h)** Annealing (ANN); **(i)** Acid Hydrolysis (HA). Note: The same letters on the bar chart indicate values that are not significantly different at a 95% confidence level (α=5%). Treatments involving annealing and autoclaving–cooling showed the highest amylose levels, while fermentation and enzymatic modification resulted in comparatively lower values. Bars sharing the same letters are not significantly different at the 95% confidence level (α = 0.05).

Significant differences ($p < 0.05$) in amylose content were observed among all modification treatments (Figure 4). Annealing produced the highest amylose level (30.65%) and the lowest amylopectin level (5.53%), indicating substantial molecular reorganisation that favoured the formation of linear glucan chains. Similarly, autoclaving–cooling cycles (AC-2 and AC-1) significantly increased amylose to 29.81% and 29.65%, respectively, with corresponding reductions in amylopectin to 1.67% and 1.33% (Figures 4 and 5). These changes reflect amylopectin depolymerisation and retrogradation-driven recrystallisation, processes closely linked to the formation of resistant starch (RS type III).

Microwave–cooling and heat–moisture treatment (HMT) also moderately increased amylose content, suggesting that controlled hydrothermal conditions promote molecular rearrangement without extensive degradation. In contrast, fermentation (FLP) and enzymatic debranching (DP) resulted in smaller yet significant increases in amylose proportion. Fermentation-induced hydrolysis of amylopectin and microbial enzymatic activity contributed to the relative enrichment of amylose fractions, while pullulanase treatment cleaved α-1,6 linkages, generating linear chains that can retrograde more readily.

Comparative analysis across treatments indicates that annealing and two-cycle autoclaving–cooling were the most effective in increasing amylose content and thus promoting resistant starch formation (Figure 5). These methods favour structural reorganisation and crystallisation over hydrolytic degradation, aligning with findings from Cozzolino *et al.* [54], Zhou *et al.* [55], and Shen *et al.* [31] on hydrothermally modified cereal flours. Microwave and HMT treatments provided intermediate improvements, while fermentation and enzymatic debranching enhanced linear chain formation through biochemical pathways rather than thermal restructuring.

The control barley flour contained 21.87% amylose, which falls within the range reported in previous studies—26.6–28.8% for Canadian barley [52] and 17.6–29.3% for European varieties [53]. Such variation is commonly attributed to genetic background and

environmental factors, including temperature and rainfall, which influence endosperm development [42].

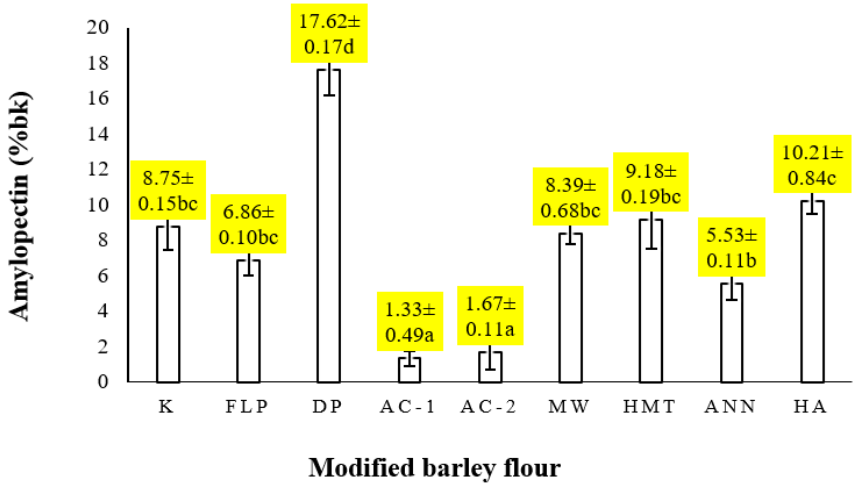


Figure 5. Amylopectin content of modified barley flour: (**(a)** Control (K); **(b)** Fermentation of *L. plantarum* (FLP); **(c)** Debranching Pullulanase (DP); **(d)** Autoclaving cooling 1 cycle (AC-1); **(e)** Autoclaving cooling 2 cycles (AC-2); **(f)** Microwave cooling (MW); **(g)** Heat moisture treatment (HMT); **(h)** Annealing (ANN); **(i)** Acid Hydrolysis (HA). Note: The same letters on the bar chart indicate values that are not significantly different at a 95% confidence level (α=5%). Treatments that increased amylose content (e.g., ANN, AC-1, and AC-2) correspondingly showed a marked reduction in amylopectin levels, whereas fermentation and pullulanase treatments maintained moderate values. Bars sharing the same letters are not significantly different at the 95% confidence level (α = 0.05).

Among all treatments, significant differences ($p < 0.05$) were observed in amylose and amylopectin contents (Figures 4–5). The annealing treatment produced the highest amylose content (30.65%) and the lowest amylopectin level (5.53%), followed by the two-cycle and one-cycle autoclaving–cooling treatments, with amylose values of 29.81% and 29.65%, and corresponding amylopectin values of 1.67% and 1.33%, respectively. These results align with previous findings in Australian [54] and Chinese barley [55], and are comparable to amylose increases observed in sorghum [44] and oat flours [31] following hydrothermal treatments.

The rise in amylose content and concomitant reduction in amylopectin can be attributed to partial depolymerisation of amylopectin branches into shorter, linear chains that behave as amylose. The autoclaving–cooling cycles promote gelatinisation, followed by amylose retrogradation, forming more crystalline fractions of resistant starch. However, excessive cycling may lead to the depolymerisation of amylose into simple sugars [56]. Different modification techniques influenced amylose–amylopectin distribution through distinct mechanisms [57]. Autoclaving–cooling promoted molecular reorganisation and recrystallisation, resulting in higher apparent amylose due to enhanced extractability [58]. Heat–moisture treatment (HMT) caused limited structural rearrangement without degradation, slightly increasing apparent amylose by partially linearising amylopectin chains [59]. Annealing, conducted below gelatinisation temperature, improved crystalline order but did not drastically alter the absolute levels of starch fractions [36]. Microwave treatment induced moderate depolymerisation, producing shorter glucan chains that may appear as amylose in analytical assays [48].

Fermentation and enzymatic debranching (pullulanase treatment) acted primarily through biochemical hydrolysis. Microbial enzymes in fermentation preferentially degraded branched amylopectin, increasing the relative proportion of amylose [60], whereas pullulanase hydrolysed α-1,6 linkages, generating linear chains that structurally and functionally resemble

amylose [61]. These linearised chains enhance the potential for the formation of resistant starch (RS type III) upon cooling. Collectively, these findings demonstrate that thermal–physical methods (particularly annealing and autoclaving–cooling cycles) are most effective in promoting amylose enrichment and starch retrogradation. At the same time, biochemical treatments (fermentation and enzymatic debranching) contribute complementary modifications by generating linear chain precursors. The resulting increase in amylose proportion and reduction in amylopectin branching are key determinants of the nutritional functionality and digestibility of modified barley flour.

3.5. Reducing sugar content of modified barley flour.

Reducing sugars comprise monosaccharides and some oligosaccharides that possess a free carbonyl group capable of acting as a reducing agent [62]. Variations in reducing sugar content are closely associated with starch hydrolysis and the formation of resistant starch [24]. As shown in Figure 6, all modification methods—fermentation (FLP), enzymatic (DP), and physicochemical treatments (AC-1, AC-2, MW, HMT, ANN, and HA)—significantly affected ($p < 0.05$) the reducing sugar levels compared to the control. The control barley flour exhibited 9.49% reducing sugars, consistent with the 7–9% range reported by Ojha *et al*. [63] for barley from Nepal.

Overall, most modification treatments reduced sugar content, particularly AC-2, MW, HMT, and HA (8.88–8.95%), while FLP, AC-1, and ANN showed no significant difference ($p > 0.05$) from the control (9.01–9.22%). Only the pullulanase-debranching (DP) treatment produced a significant increase (9.63%), indicating enhanced hydrolysis of α-1,6-glycosidic linkages that released short-chain dextrins and aldose groups [64]. The decrease in reducing sugars after most treatments is partly due to their participation in Maillard reactions during heating, in which the carbonyl groups of reducing sugars react with amino groups to form early browning products [24].

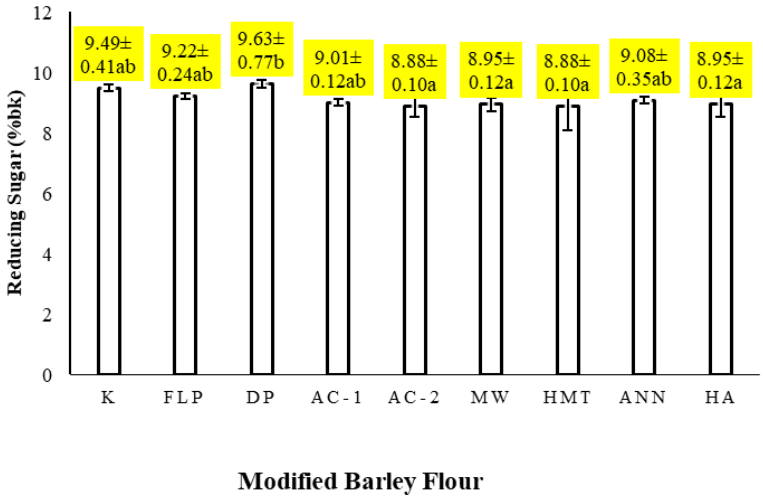


Figure 6. Reducing sugar content of modified barley flour: **(a)** Control (K); **(b)** Fermentation of *L. plantarum* (FLP); **(c)** Debranching Pullulanase (DP); **(d)** Autoclaving cooling 1 cycle (AC-1); **(e)** Autoclaving cooling 2 cycles (AC-2); **(f)** Microwave cooling (MW); **(g)** Heat moisture treatment (HMT); **(h)** Annealing (ANN); **(i)** Acid Hydrolysis (HA). Note: The same letters on the bar chart indicate values that are not significantly different at a 95% confidence level (α=5%). Reduced sugar levels were generally observed after modification, particularly in AC-2, MW, HMT, and HA treatments, indicating enhanced starch retrogradation and resistant starch formation. The DP treatment showed a slight increase in reducing sugars due to enzymatic cleavage of α-1,6-glycosidic bonds in amylopectin, whereas fermentation and annealing caused minor or no significant changes.

From a mechanistic perspective, autoclaving–cooling (AC) promotes starch gelatinisation, followed by retrogradation, thereby enhancing the formation of resistant starch (RS type III) while limiting enzymatic hydrolysis and reducing sugar release [65, 66]. Heat-moisture treatment (HMT) and annealing (ANN) further strengthen molecular ordering within starch granules, thereby decreasing solubility and enzyme accessibility, which explains their lower reducing sugar values [67]. Microwave (MW) treatment partially gelatinises starch, briefly enhancing sugar release, but excessive heating may degrade sugars through caramelisation or Maillard reactions, lowering detectable levels [44]. Fermentation (FLP) exhibits a dual effect: microbial amylases initially increase reducing sugar formation, but subsequent microbial metabolism depletes these sugars, resulting in a net decline [68]. Conversely, pullulanase debranching (DP) distinctly elevates reducing sugars by cleaving α-1,6 bonds in amylopectin, generating linear dextrins that are readily hydrolysed [69]. However, if these fragments undergo retrogradation during subsequent cooling, their contribution to reducing sugar levels may decline, while resistant starch levels increase [70,71].

Collectively, these findings indicate that treatments that enhance molecular order (HMT, ANN, AC-2) or promote retrogradation result in lower reducing sugar levels and higher resistant starch formation. In contrast, enzymatic debranching (DP) temporarily increases reducing sugars but ultimately facilitates the structural rearrangements required for the development of resistant starch upon cooling. Thus, autoclaving–cooling and pullulanase debranching emerge as the most effective combinations for enhancing resistant starch, balancing initial hydrolysis with subsequent recrystallisation into enzyme-resistant structures.

4. Conclusions

This study demonstrated that physicochemical, fermentation, and enzymatic modifications markedly altered the structural and compositional properties of barley flour (*Hordeum vulgare*). Among the treatments, autoclaving–cooling (particularly two cycles) and enzymatic debranching with pullulanase were the most effective, increasing total starch and amylose contents to approximately 41.6% and 30.6%, respectively, while reducing sugar levels declined to around 8.9%. These compositional shifts indicate enhanced formation of resistant starch, supported by observed granule disruption and crystalline reorganisation. Physicochemical modifications (autoclaving–cooling, microwave, HMT) primarily affected granule morphology and crystallinity, whereas enzymatic and fermentation treatments induced more selective molecular rearrangements within the starch matrix. Collectively, these results highlight that the structural pathways leading to resistant starch formation differ across modification types, with physicochemical and enzymatic methods showing the greatest efficacy. However, the nutritional implications remain inferential, as direct assessments of digestibility and prebiotic functionality were not performed. Future research should integrate in vitro digestion assays, glycaemic response modelling, and prebiotic evaluations, alongside process optimisation for moisture, duration, and sequential treatments, to validate and scale these modifications for functional food applications.

Authorship Contribution

Conceptualisation, L.A., R.H.B.S., and D.A.; methodology, L.A., R.H.B.S.; software, L.A., R.H.B.S.; validation, R.H.B.S., D.A., T.K.; formal analysis, L.A.; R.H.B.S.; D.A.; investigation, R.H.B.S., D.A., T.K., D.Y., A.F., A.F.; resources, L.A., R.H.B.S., A.F.; data

curation, L.A., D.A.,D.Y., T.K.; writing—original draft preparation, L.A., R.H.B.S. A.F.; writing—review and editing, L.A., R.H.B.S., D.A.; visualization, L.A., R.H.B.S.; supervision, R.H.B.S., and D.A.; project administration, L.A.; funding acquisition, L.A. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

References

1. Blattner, F.R. Taxonomy of the Genus *Hordeum* and Barley (*Hordeum vulgare*). In The Barley Genome. Compendium of Plant Genomes; Stein, N., Muehlbauer, G.J., Eds.; Springer International Publishing: Cham, **2018**; pp. 11-23, https://doi.org/10.1007/978-3-319-92528-8_2.
2. El-Hashash, E.F.; El-Absy, K.M. Barley (*Hordeum vulgare* L.) Breeding. In Advances in Plant Breeding Strategies: Cereals: Volume 5, Al-Khayri, J.M., Jain, S.M., Johnson, D.V., Eds.; Springer International Publishing: Cham, **2019**; pp. 1-45, https://doi.org/10.1007/978-3-030-23108-8_1.
3. Ferdiansyah, M.K. Pengaruh Konsumsi Serat Pangan Barley Pada Metabolisme Lipid. *J. Ilmu Pangan Hasil Pertanian* **2018**, *2*, 72–81, https://doi.org/10.26877/jiphp.v2i1.2441.
4. Narwal, S.; Gupta, O.P.; Pandey, V.; Kumar, D.; Ram, S. 9 - Effect of storage and processing conditions on nutrient composition of wheat and barley. In Wheat and Barley Grain Biofortification; Gupta, O.P., Pandey, V., Narwal, S., Sharma, P., Ram, S., Singh, G.P., Eds.; Woodhead Publishing: **2020**; pp. 229-256, https://doi.org/10.1016/B978-0-12-818444-8.00009-2.
5. Quddos, A.; Nadeem, M.; Ahsan, S.; Khaliq, A.; Chughtai, M.F.J.; Rebezov, M.; Terent'ev, S.; Tryabas, Y.; Ermolaev, V.; Iskakova, G.; Konovalov, S.; Gayvas, A.; Shariati, M.A. Genotype by Environment Interactions in Barley (Hordeum vulgare L.) Cultivars for Nutritional Quality Assessment. *Agrivita J. Agric. Sci.* **2021**, *43*, 569–580, https://doi.org/10.17503/agrivita.v43i3.2925.

6. Rosell, C.M.; Koehler, P. Chemistry of Cereal Grains. In Handbook on Sourdough Biotechnology, Gobbetti, M., Gänzle, M., Eds.; Springer International Publishing: Cham, **2023**; pp. 25-66, https://doi.org/10.1007/978-3-031-23084-4_3.
7. Raigond, P.; Ezekiel, R.; Raigond, B. Resistant starch in food: a review. *J. Sci. Food Agric.* **2015**, *95*, 1968–1978, https://doi.org/10.1002/jsfa.6966.
8. Liu, H.; Guo, X.; Li, W.; Wang, X.; lv, M.; Peng, Q.; Wang, M. Changes in physicochemical properties and in vitro digestibility of common buckwheat starch by heat-moisture treatment and annealing. *Carbohydr. Polym.* **2015**, *132*, 237-244, https://doi.org/10.1016/j.carbpol.2015.06.071.
9. Li, C.; Dhital, S.; Gidley, M.J. High-amylose wheat bread with reduced in vitro digestion rate and enhanced resistant starch content. *Food Hydrocoll.* **2022**, *123*, 107181, https://doi.org/10.1016/j.foodhyd.2021.107181.
10. Thuy, N.M.; Too, B.C.; Vuong, K.M.; Lan, P.T.T.; Tuyen, P.T.T.; Tram, N.B.; Vy, L.T.T.; Tuyen, L.N.; Tai, N.V. Resistant starch in various starchy vegetables and the relationship with its physical and chemical characteristics. *J. Appl. Biol. Biotechnol.* **2022**, *10*, 181-188, http://dx.doi.org/10.7324/JABB.2021.100122.
11. Behera, S.S.; Ray, R.C.; Zdolec, N. *Lactobacillus plantarum* with Functional Properties: An Approach to Increase Safety and Shelf-Life of Fermented Foods. *BioMed Res. Int.* **2018**, *2018*, 9361614, https://doi.org/10.1155/2018/9361614.
12. Winarti, S.; Anggreini, R.A. Karakteristik Dan Aktivitas Prebiotik Pati Resisten Dari Tepung Umbi Uwi (Dioscorea alata) Termodifikasi. *J. Teknol. Pangan* **2020**, *13*, https://doi.org/10.33005/jtp.v13i2.1796.
13. Wang, S.; Li, C.; Copeland, L.; Niu, Q.; Wang, S. Starch Retrogradation: A Comprehensive Review. *Comp. Rev. Food Sci. Food Safe* **2015**, *14*, 568–585, https://doi.org/10.1111/1541-4337.12143.
14. Tu, D.; Ou, Y.; Zheng, Y.; Zhang, Y.; Zheng, B.; Zeng, H. Effects of freeze-thaw treatment and pullulanase debranching on the structural properties and digestibility of lotus seed starch-glycerin monostearin complexes. *Int. J. Biol. Macromol.* **2021**, *177*, 447–454, https://doi.org/10.1016/j.ijbiomac.2021.02.168.
15. Putra, R.P. Potensi Prebiotik Tepung Pisang yang Dimodifikasi Menggunakan Pemanasan Autoklaf Dilanjutkan dengan Retrogradasi. *J. Pendidik. Teknol. Pertan.* **2020**, *6*, 349–360.
16. Wang, X.; Nie, Y.; Xu, Y. Industrially produced pullulanases with thermostability: Discovery, engineering, and heterologous expression. *Bioresour. Technol.* **2019**, *278*, 360–371, https://doi.org/10.1016/j.biortech.2019.01.098.
17. Chang, R.; Tian, Y.; Lu, H.; Sun, C.; Jin, Z. Effects of fractionation and heat-moisture treatment on structural changes and digestibility of debranched waxy maize starch. *Food Hydrocoll.* **2020**, *101*, 105488, https://doi.org/10.1016/j.foodhyd.2019.105488.
18. Chung, H.-J.; Liu, Q.; Hoover, R. Impact of annealing and heat-moisture treatment on rapidly digestible, slowly digestible and resistant starch levels in native and gelatinized corn, pea and lentil starches. *Carbohydr. Polym.* **2009**, *75*, 436–447, https://doi.org/10.1016/j.carbpol.2008.08.006.
19. Sudheesh, C.; Sunooj, K.V.; Bhavani, B.; Aaliya, B.; Navaf, M.; Akhila, P.P.; Sabu, S.; Sasidharan, A.; Sinha, S.K.; Kumar, S.; Sajeevkumar, V.A.; George, J. Energetic neutral atoms assisted development of kithul (Caryota urens) starch–lauric acid complexes: A characterisation study. *Carbohydr. Polym.* **2020**, *250*, 116991, https://doi.org/10.1016/j.carbpol.2020.116991.
20. Zeng, L.; Zhang, L.; Li, K.; He, J.; Xin, H.; Wang, Q. Effect of gelatinization processing on the antioxidant, digestion, and physicochemical properties of wheat starch enhanced with tannic acid. *LWT* **2020**, *125*, 109228, https://doi.org/10.1016/j.lwt.2020.109228.
21. Setiarto, R.H.B.; Muhammad, I.; Dimas, A.; Nunuk, W.; Masrukhin. Improvement of Prebiotic Properties and Resistant Starch Content of Corn Flour (*Zea mays* L.) Momala Gorontalo Using Physical, Chemical and Enzymatic Modification. *Trop. Life Sci. Res.* **2023**, *34*, 255–278, https://doi.org/10.21315/tlsr2023.34.2.13.
22. Rannou, C.; Laroque, D.; Renault, E.; Prost, C.; Sérot, T. Mitigation strategies of acrylamide, furans, heterocyclic amines and browning during the Maillard reaction in foods. *Food Res. Int.* **2016**, *90*, 154-176, https://doi.org/10.1016/j.foodres.2016.10.037.
23. Arp, C.G.; Correa, M.J.; Ferrero, C. High-Amylose Resistant Starch as a Functional Ingredient in Breads: a Technological and Microstructural Approach. *Food Bioprocess Technol.* **2018**, *11*, 2182–2193, https://doi.org/10.1007/s11947-018-2168-4.
24. Massarolo, K.C.; Ferreira, C.F.J.; de Borba, V.S.; Kupski, L.; Furlong, E.B. Particle size and physical-chemical characteristics of hydrothermally treated cornmeal on resistant starch content. *Food Chem.* **2019**, *283*, 39-45, https://doi.org/10.1016/j.foodchem.2019.01.018.

25. Dong, L.; Qiu, C.; Wei, F.; Yu, Z.; Zhang, Y.; Wang, S. The Effect of Microwave Baking Conditions on the Quality of Biscuits and the Control of Thermal Processing Hazards in the Maillard Reaction. *Front. Nutr.* **2022**, *9*, 825365, https://doi.org/10.3389/fnut.2022.825365.
26. Nevara, G.A.; Yea, C.S.; Karim, R.; Muhammad, K.; Mohd Ghazali, H. Effects of moist-heat treatments on color improvement, physicochemical, antioxidant, and resistant starch properties of drum-dried purple sweet potato powder. *J. Food Process Eng.* **2019**, *42*, e12951, https://doi.org/10.1111/jfpe.12951.
27. Yonata, D.; Triwitono, P.; Lestari, L.A.; Pranoto, Y. Physicochemical and Structural Properties of Resistant Starch Prepared from Tacca Tuber Starch using Autoclaving-Cooling and Acid Hydrolysis Treatments. *Trends Sci.* **2025**, *22*, 8822, https://doi.org/10.48048/tis.2025.8822.
28. Espinosa-Solis, V.; Zamudio-Flores, P.B.; Espino-Díaz, M.; Vela-Gutiérrez, G.; Rendón-Villalobos, J.R.; Hernández-González, M.; Hernández-Centeno, F.; López-De la Peña, H.Y.; Salgado-Delgado, R.; Ortega-Ortega, A. Physicochemical Characterization of Resistant Starch Type-III (RS3) Obtained by Autoclaving Malanga (*Xanthosoma sagittifolium*) Flour and Corn Starch. *Molecules* **2021**, *26*, 4006, https://doi.org/10.3390/molecules26134006.
29. Setiarto, R.H.B.; Adyeni, W.D.; Puspawati, N.N.; Wardana, A.A.; Anshory, L.; Khusniati, T. Physicochemical, enzymatic and fermentation modifications improve resistant starch levels and prebiotic properties of porang (*Amorphophallus oncophyllus*) flour. *Int. J. Food Sci. Technol.* **2024**, *59*, 9353-9367, https://doi.org/10.1111/ijfs.17580.
30. Khan, A.; Rahman, U.U.; Siddiqui, S.; Irfan, M.; Shah, A.A.; Badshah, M.; Hasan, F.; Khan, S. Preparation and characterization of resistant starch type III from enzymatically hydrolyzed maize flour. *Mol. Biol. Rep.* **2019**, *46*, 4565-4580, https://doi.org/10.1007/s11033-019-04913-5.
31. Shen, H.; Yu, J.; Bai, J.; Liu, Y.; Ge, X.; Li, W.; Zheng, J. A new pre-gelatinized starch preparing by spray drying and electron beam irradiation of oat starch. *Food Chem.* **2023**, *398*, 133938, https://doi.org/10.1016/j.foodchem.2022.133938.
32. Liang, W.; Lin, Q.; Zeng, J.; Gao, H.; Muratkhan, M.; Li, W. Understanding the improvement of sorghum starch acid hydrolysis modification by E-beam irradiation: A supramolecular structure perspective. *Food Chem*. **2024**, *437*, 137820, https://doi.org/10.1016/j.foodchem.2023.137820.
33. Yan, X.; McClements, D.J.; Luo, S.; Ye, J.; Liu, C. A review of the effects of fermentation on the structure, properties, and application of cereal starch in foods. *Crit. Rev. Food Sci. Nutr.* **2025**, *65*, 2323–2342, https://doi.org/10.1080/10408398.2024.2334269.
34. Kumar, Y.; Singh, L.; Sharanagat, V.S.; Patel, A.; Kumar, K. Effect of microwave treatment (low power and varying time) on potato starch: Microstructure, thermo-functional, pasting and rheological properties. *Int. J. Biol. Macromol.* **2020**, *155*, 27-35, https://doi.org/10.1016/j.ijbiomac.2020.03.174.
35. Gunaratne, A. Heat-Moisture Treatment of Starch. In Physical Modifications of Starch; Sui, Z., Kong, X., Eds.; Springer Nature Singapore: Singapore, **2023**; pp. 49-72, https://doi.org/10.1007/978-981-99-5390-5_4.
36. Yao, T.; Sui, Z.; Janaswamy, S. Annealing. In Physical Modifications of Starch; Sui, Z., Kong, X., Eds.; Springer Nature Singapore: Singapore, **2023**; pp. 73-89, https://doi.org/10.1007/978-981-99-5390-5_5.
37. Li, H.-T.; Zhang, W.; Bao, Y.; Dhital, S. Enhancing enzymatic resistance of starch through strategic application of food physical processing technologies. *Crit. Rev. Food Sci. Nutr.* **2024**, *64*, 11826–11849, https://doi.org/10.1080/10408398.2023.2245031.
38. Sharma, R.; Bhandari, M.; Sharma, S.; Bhardwaj, R. Compositional, structural and functional characteristics of millets as modified by bioprocessing techniques: A review. *J. Food Process. Preserv.* **2022**, *46*, e16885, https://doi.org/10.1111/jfpp.16885.
39. Saeid, A.; Akter, F.; Ali, M.A.; Rahman, M.H. Enzymatic Modification of Starch: Amylases and Pullulanase. In Advanced Research in Starch; Mazumder, N., Rahman, M.H., Eds.; Springer Nature Singapore: Singapore, **2024**; pp. 95-115., https://doi.org/10.1007/978-981-99-9527-1_3.
40. Bereka, T.Y.; Zhou, X.; Sun, Z.; Xin, X.; Jin, Z. Impact of pullulanase concentration on resistant starch formation in whole oat grain flour through dual extrusion–debranching. *J. Sci. Food Agric.* **2025**, *105*, 7242-7249, https://doi.org/10.1002/jsfa.14430.
41. Pereira, B.L.B.; Leonel, M. Resistant starch in cassava products. *Food Sci. Technol*. **2014**, *34*, 298–302, https://doi.org/10.1590/fst.2014.0039.
42. Zhu, F. Barley Starch: Composition, Structure, Properties, and Modifications. *Comp. Rev. Food Sci. Food Safe* **2017**, *16*, 558–579, https://doi.org/10.1111/1541-4337.12265.

43. Harder, H.; Khol-Parisini, A.; Zebeli, Q. Treatments with organic acids and pullulanase differently affect resistant starch and fiber composition in flour of various barley genotypes (*Hordeum vulgare* L.). *Starch - Stärke* **2015**, *67*, 512-520, https://doi.org/10.1002/star.201400254.
44. Li, R.; Dai, L.; Peng, H.; Jiang, P.; Liu, N.; Zhang, D.; Wang, C.; Li, Z. Effects of microwave treatment on sorghum grains: Effects on the physicochemical properties and in vitro digestibility of starch. *J. Food Process. Eng.* **2021**, *44*, e13804, https://doi.org/10.1111/jfpe.13804.
45. Zailani, M.A.; Kamilah, H.; Husaini, A.; Awang Seruji, A.Z.R.; Sarbini, S.R. Starch Modifications via Physical Treatments and the Potential in Improving Resistant Starch Content. *Starch - Stärke* **2023**, *75*, 2200146, https://doi.org/10.1002/star.202200146.
46. Oktaviani, S.R.; Faridah, D.N.; Wulandari, N.; Afandi, F.A.; Jayanegara, A. Resistant starch content of dual modification autoclaving-cooling and pullulanase debranching on various carbohydrate sources: a systematic review. *Int. J. Food Sci. Technol.* **2023**, *58*, 6890-6901, http://dx.doi.org/10.1111/ijfs.16525.
47. Dubey, N.; Yadav, N.; Kaur, D. Physicochemical, Functional, and Structural Characterization of Quinoa (*Chenopodium quinoa*) Starch Modified by Heat Moisture Treatment, Annealing, and Gelatinization-Retrogradation. *Starch - Stärke* **2025**, *77*, e70080, https://doi.org/10.1002/star.70080.
48. Kurek, M.A.; Wojtasik-Kalinowska, I.; Marcinkowska-Lesiak, M.; Onopiuk, A.; Szpicer, A.; Kultys, E.; Zalewska, M. Effect of Microwaves on Food Carbohydrates. In Microwave Processing of Foods: Challenges, Advances and Prospects: Microwaves and Food; Pratap Singh, A., Erdogdu, F., Wang, S., Ramaswamy, H.S., Eds.; Springer International Publishing: Cham, **2024**; pp. 221-249 https://doi.org/10.1007/978-3-031-51613-9_12.
49. Katina, K.; Poutanen, K.; Gobbetti, M. Nutritional Aspects of Cereal Fermentation with Lactic Acid Bacteria and Yeast. In Handbook on Sourdough Biotechnology, Gobbetti, M., Gänzle, M., Eds.; Springer International Publishing: Cham, **2023**; pp. 303-324, https://doi.org/10.1007/978-3-031-23084-4_10.
50. Wang, Y.; Tian, Y.; Christensen, S.J.; Blennow, A.; Svensson, B.; Møller, M.S. An enzymatic approach to quantify branching on the surface of starch granules by interfacial catalysis. *Food Hydrocoll.* **2024**, *146*, 109162, https://doi.org/10.1016/j.foodhyd.2023.109162.
51. Wijaya, A.; Muhandri, T.; Hasanah, U.; Haryo Bimo Setiarto, R. Physical, Chemical, Fermentation and Enzymatic Modification Technology to Increase Resistant Starch and Improve Prebiotic Properties of High Carbohydrate Foods. *Rev. Agric. Sci.* **2024**, *12*, 377-400, https://doi.org/10.7831/ras.12.0_377.
52. Ma, J.; Jiang, Q.-T.; Wei, L.; Wang, J.-R.; Chen, G.-Y.; Liu, Y.-X.; Li, W.; Wei, Y.-M.; Liu, C.; Zheng, Y.-L. Characterization of shrunken endosperm mutants in barley. *Gene* **2014**, *539*, 15-20, https://doi.org/10.1016/j.gene.2014.02.004.
53. Källman, A.; Bertoft, E.; Koch, K.; Sun, C.; Åman, P.; Andersson, R. Starch structure in developing barley endosperm. *Int. J. Biol. Macromol.* **2015**, *81*, 730-735, http://dx.doi.org/doi:10.1016/j.ijbiomac.2015.09.013.
54. Cozzolino, D.; Roumeliotis, S.; Eglinton, J. Relationships between starch pasting properties, free fatty acids and amylose content in barley. *Food Res. Int.* **2013**, *51*, 444–449, https://doi.org/10.1016/j.foodres.2013.01.030.
55. Zhou, X.; Yu, W.; Li, C. Protein content correlates with the *in vitro* starch digestibility of raw barley flour. *Food Biosci*. **2021**, *43*, 101292, https://doi.org/10.1016/j.fbio.2021.101292.
56. Faridah, D.N.; Silitonga, R.F.; Indrasti, D.; Afandi, F.A.; Jayanegara, A.; Anugerah, M.P. Verification of autoclaving-cooling treatment to increase the resistant starch contents in food starches based on meta-analysis result. *Front. Nutr*. **2022**, *9*, 904700, https://doi.org/10.3389/fnut.2022.904700.
57. Sinhmar, A.; Pathera, A.K.; Sharma, S.; Nehra, M.; Thory, R.; Nain, V. Impact of Various Modification Methods on Physicochemical and Functional Properties of Starch: A Review. *Starch - Stärke* **2023**, *75*, 2200117, https://doi.org/10.1002/star.202200117.
58. Ma, M.; Wen, Y.; Zhang, C.; Xu, Z.; Li, H.; Sui, Z.; Corke, H. Extraction and characterization of starch granule-associated surface and channel lipids from small-granule starches that affect physicochemical properties. *Food Hydrocoll.* **2022**, *126*, 107370, https://doi.org/10.1016/j.foodhyd.2021.107370.
59. Fonseca, L.M.; Halal, S.L.M.E.; Dias, A.R.G.; Zavareze, E.d.R. Physical modification of starch by heat-moisture treatment and annealing and their applications: A review. *Carbohydr. Polym.* **2021**, *274*, 118665, https://doi.org/10.1016/j.carbpol.2021.118665.
60. Li, T.; Huang, J.; Tian, X.; Zhang, C.; Pan, Y.; Pu, H. Physicochemical evolution of sorghum grain starch under the condition of solid-state fermentation of Baijiu. *Int. J. Biol. Macromol.* **2024**, *282*, 137225, https://doi.org/10.1016/j.ijbiomac.2024.137225.

61. Gaenssle, A.L.O.; van der Maarel, M.J.E.C.; Jurak, E. The influence of amylose content on the modification of starches by glycogen branching enzymes. *Food Chem.* **2022**, *393*, 133294, https://doi.org/10.1016/j.foodchem.2022.133294.
62. Başkan, K.S.; Tütem, E.; Akyüz, E.; Özen, S.; Apak, R. Spectrophotometric total reducing sugars assay based on cupric reduction. *Talanta* **2016**, *147*, 162-168, https://doi.org/10.1016/j.talanta.2015.09.049.
63. Ojha, P.; Gautam, N.; Subedi, U.; Dhami, N.B. Malting quality of seven genotypes of barley grown in Nepal. *Food Sci. Nutr.* **2020**, *8*, 4794–4804, https://doi.org/10.1002/fsn3.1743.
64. Agustina; Faridah, D.N.; Jenie, B.S.L. PENGARUH RETROGRADASI DAN PERLAKUAN KELEMBABAN PANAS TERHADAP KADAR PATI RESISTEN TIPE III DALUGA. *J. Teknol. Industri Pangan* **2016**, *27*, 78–86, https://doi.org/10.6066/jtip.2016.27.1.78.
65. Souilah, R.; Djabali, D.; Belhadi, B.; Mokrane, H.; Boudries, N.; Nadjemi, B. In vitro starch digestion in sorghum flour from Algerian cultivars. *Food Sci. Nutr.* **2014**, *2*, 251-259, https://doi.org/10.1002/fsn3.104.
66. Wei, Z.; Zheng, Y.; Hao, J.; Deng, Y.; Zhang, Y.; Tang, X.; Liu, G.; Li, P.; Zhong, L.; Zhao, Z.; Wang, J.; Liao, N.; Wang, Z.; Zhang, M. Effects of soybean oil and whey protein complexation by temperature treatments on the structural, physicochemical and digestive characteristics of autoclaved rice. *Food Struct.* **2022**, *31*, 100252, https://doi.org/10.1016/j.foostr.2022.100252.
67. Roder, N.; Gerard, C.; Verel, A.; Bogracheva, T.Y.; Hedley, C.L.; Ellis, P.R.; Butterworth, P.J. Factors affecting the action of α-amylase on wheat starch: Effects of water availability. An enzymic and structural study. *Food Chem.* **2009**, *113*, 471-478, https://doi.org/10.1016/j.foodchem.2008.07.106.
68. Zhang, G.; Zhang, L.; Ahmad, I.; Zhang, J.; Zhang, A.; Tang, W.; Ding, Y.; Lyu, F. Recent advance in technological innovations of sugar-reduced products. *Crit. Rev. Food Sci. Nutr.* **2024**, *64*, 5128-5142, https://doi.org/10.1080/10408398.2022.2151560.
69. Yin Tan, W.; Li, M.; Devkota, L.; Attenborough, E.; Dhital, S. Mashing performance as a function of malt particle size in beer production. *Crit. Rev. Food Sci. Nutr.* **2023**, *63*, 5372-5387, https://doi.org/10.1080/10408398.2021.2018673.
70. Bhati, N.; Bhatt, P.; Kumar, V.; Tomar, S.; Sethi, V.A.; Mittal, A.; Maguluri, L.P.; Goel, R.; Tyagi, L.K. Eco-Friendly Enzymatic Starch Modification: Precision Strategies for Tailored Industrial and Biomedical Functionalities. *Starch - Stärke* **2025**, *77*, e70087, https://doi.org/10.1002/star.70087.
71. Xiao, Z.; Wu, S.; Liang, H.; Li, B.; Li, J. Effects of oligosaccharides, sodium carboxymethyl cellulose and d-allulose as a compound improver on the quality of sugar-reduced bread. *J. Sci. Food Agric.* **2025**, *105*, 3024–3035, https://doi.org/10.1002/jsfa.14065.

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