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Starch Digestibility and Prebiotic Properties of Jack Bean Flour Modified by Physical, Chemical, Enzymatic, and Fermentation Methods

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Abstract: This study investigated the effects of various physical, chemical, and enzymatic modification techniques on the *in vitro* starch digestibility and prebiotic properties of jack bean flour. The modification methods included autoclaving–cooling (AC-1C and AC-2C), annealing (ANN), microwave–cooling (MC), heat–moisture treatment (HMT), acid hydrolysis (AH), debranching with pullulanase (DP), and fermentation combined with one-cycle autoclaving–cooling (FAC). The novelty of this work lies in the comprehensive comparison of multiple modification approaches to identify an optimal method for enhancing both resistant starch formation and prebiotic functionality in jack bean flour, a legume that remains underutilized. Overall, the modification treatments significantly increased resistant starch and reduced rapidly digestible starch ($p < 0.05$). Among all treatments, FAC produced the most favorable functional profile, exhibiting superior resistance to simulated gastric conditions, promoting the growth and activity of *Lactobacillus plantarum* IIA-1A5, and suppressing *Enteropathogenic Escherichia coli*. These findings demonstrate that FAC-modified jack bean flour offers enhanced starch resistance and prebiotic potential, underscoring its promise as a novel prebiotic ingredient.

Keywords: enzymatic; fermentation; modified jack bean flour; physical; prebiotic properties; starch digestibility.

1. Introduction

The jack bean (*Canavalia ensiformis*) is a legume that grows well on suboptimal land in Indonesia and is primarily used as fertilizer and animal feed, due to its limited use as food [1]. Its restricted use is due to the presence of toxic and antinutritional compounds, including trypsin inhibitors, hydrogen cyanide, phytic acid, concanavalin A, and tannins [1–5]. Several studies have demonstrated that pretreatments, such as soaking in sodium bicarbonate [4, 6] or

thermal processing [4, 7], can significantly reduce hydrogen cyanide levels. Alkaline soaking promotes the hydrolysis of cyanogenic glycosides by β-glucosidase, enabling the release and removal of HCN during washing [4, 8], while thermal treatments enhance loss through dissolution and volatilization [4, 9]. Despite its anti-nutritional factors, jack bean has a favorable nutritional profile, characterized by high protein and carbohydrate content and low fat levels [5, 10]. Its starch content reaches 36.9%, of which 10.8% is resistant starch (RS) [11], highlighting its potential as a raw material for RS development [12].

Resistant starch functions as a dietary component that modulates gut microbiota activity and stimulates the production of short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate [13,14]. These metabolites support immune modulation, intestinal epithelial integrity, and overall gastrointestinal health. The relevance of dietary factors to gut health is underscored by increasing digestive health concerns, including the high burden of colorectal cancer (CRC) in Indonesia, where CRC ranks among the top diagnosed malignancies and contributes substantially to national mortality [15]. This context underscores the importance of developing functional ingredients to enhance intestinal health.

Previous studies have shown that starch modification can alter its digestibility profile—namely VRDS, RDS, SDS, and RS—and that physical, chemical, enzymatic, and fermentation-based approaches can increase RS levels [16-18]. Techniques such as annealing (ANN), autoclaving–cooling (AC), acid hydrolysis (AH), debranching with pullulanase (DP), and fermentations followed by thermal treatments have demonstrated varying effects on starch structure and digestibility in different plant sources [19–22]. These findings indicate that the type of modification strongly influences RS formation. However, despite jack bean's favorable native RS content, systematic data on how various modification techniques affect the starch digestibility profile and prebiotic potential of jack bean flour remain limited.

Modification can also influence the prebiotic functionality of starch. To be considered a prebiotic, a component must resist gastric digestion, selectively stimulate beneficial gut bacteria, and not promote enteropathogens [23, 24]. Modified starches from taro, daluga, and sago have demonstrated enhanced resistance to digestion and improved growth of probiotic strains compared with their native counterparts [19, 25–27]. Nonetheless, intervention studies assessing the prebiotic potential of modified jack bean starch are lacking. Given this gap, the present study aims to evaluate the effects of various physical, chemical, enzymatic, and fermentation-based modifications on the composition, starch digestibility profile, and prebiotic properties of jack bean flour. This study is expected to provide foundational data for developing jack bean as a novel source of resistant starch with potential functional and prebiotic benefits.

2. Materials and Methods

2.1. Materials.

The main ingredient in this research was jack bean, obtained from PT Yasa Jamur Sriwijaya in West Java, Indonesia. Probiotic bacteria *Lactobacillus plantarum* IIA-1A5 and EPEC (*Enteropathogenic Escherichia coli*) were provided by the Livestock Product Technology Laboratory, Faculty of Animal Science, IPB University, Indonesia.

2.2. Preparation of jack bean flour.

The preparation of jack bean flour followed the method of Putro *et al*. [6] with minor modifications. Seeds were cleaned, soaked in a 1% $NaHCO_3$ solution (1:3, w/v) for 24 h at

room temperature, dehulled, chopped, and subsequently dried at 40°C. The dried seeds were milled and sieved through an 80-mesh screen, and the resulting flour was packed in impermeable plastic bags and stored at 25°C for further analysis.

2.3. Physical modifications of jack bean flour.

Physical modifications of jack bean flour included autoclaving–cooling (AC), annealing (ANN), microwave–cooling (MC), and heat moisture treatment (HMT), adapted from previous studies [10, 28, 29]. AC was performed on a 20% (w/v) suspension at 121°C for 15 min, ANN on a 30% (w/v) suspension at 50°C for 24 h, MC on a 30% (w/v) suspension at 800 W for 10 min, and HMT by adjusting flour moisture to 20% before heating at 120°C for two h. After each treatment, suspensions were cooled at 4°C, oven-dried at 60°C for 24 h, then milled and sieved through an 80-mesh screen.

2.4. Chemical modification of jack bean flour.

Chemical modification of jack bean flour was conducted following the method of Sudheesh *et al*. [30] with minor adjustments. Flour was treated with 2.2 N HCl (1:2, w/v) at 35°C for 2 hours, then neutralized with 1 M NaOH to a pH of 6, and washed with distilled water. The paste was then cooled at 4°C for 24 hours, oven-dried at 60°C for 24 hours, milled, and sieved through an 80-mesh screen.

2.5. Enzymatic modification of jack bean flour.

Enzymatic modification of jack bean flour was conducted following the method of Setiarto *et al*. [20] with slight modifications. A 20% (w/v) suspension was gelatinized by heating at 70°C for 5 min, then autoclaved at 121°C for 15 min, and finally cooled to 4°C for 24 h. The paste was adjusted to 50°C, mixed with 0.1 M acetate buffer (pH 5.2) and pullulanase (10.4 U/g flour), and incubated at 50°C and 110 rpm for six hours. Enzyme activity was terminated by one cycle of autoclaving and cooling, followed by milling and sieving through an 80-mesh screen.

2.6. Fermentation modification of jack bean flour.

Fermentation modification of jack bean flour was carried out as described by Setiarto *et al*. [21] with slight modifications. Sterilized flour was suspended in sterile distilled water (1:3, w/v) and inoculated with 5% *Lactobacillus plantarum* IIA-1A5 previously activated in MRS broth. The mixture was incubated at 37°C for 18 h, followed by one autoclaving–cooling cycle, then milled and sieved through an 80-mesh screen.

2.7. In vitro starch digestibility analysis.

In vitro starch digestibility was determined according to the procedure of Setiarto *et al*. [20], with modifications to provide full methodological detail. Modified jack bean flours (100 mg dry basis) were suspended in 10 mL of sodium acetate buffer (0.1 M, pH 5.2). The digestion mixture contained porcine pancreatin (2% w/v; equivalent to approximately 4 mg enzyme per sample) and amyloglucosidase (210 U; 0.7 mL of a 300 U/mL stock), resulting in a final sample-to-enzyme ratio of 100 mg flour: 4 mg pancreatin: 210 U amyloglucosidase. The suspension was incubated at 37°C with continuous shaking (150 rpm). Aliquots (0.5 mL) were

withdrawn at 0, 1, 20, and 120 minutes and immediately heated in a boiling water bath for 5 minutes to inactivate enzymes. Samples were centrifuged, and the glucose concentration in the supernatant was determined by the DNS method, with absorbance measured at 540 nm using a UV–Vis spectrophotometer (Shimadzu UV-1280, Tokyo, Japan).

A glucose standard curve (0–1.0 mg/mL) was prepared by serially diluting a D-glucose stock solution (1 mg/mL) to obtain standards at 0.1, 0.25, 0.5, 0.75, and 1.0 mg/mL. Each standard underwent the same DNS heating procedure as the samples. The calibration equation (typically y = ax + b) was generated from triplicate measurements and used to convert absorbance values to glucose concentration. Starch digestibility fractions were quantified based on glucose released at specific time points and converted to starch equivalents (glucose × 0.9). Starch fractions were categorized as follows: very rapidly digestible starch (VRDS, digested at 1 min), rapidly digestible starch (RDS, 1–20 min), slowly digestible starch (SDS, 20–120 min), and resistant starch (RS, undigested after 120 min). All measurements were performed in triplicate. Data were analyzed using a one-way ANOVA to compare digestibility fractions among treatments, followed by the Duncan test at $p < 0.05$. The following equations, as presented by Setiarto *et al*. [21], were used in the calculations:

$$VRDS\ (\%) = \frac{G1\ X\ 0.9\ X\ F}{W}\ X\ 100 \quad (1)$$

$$RDS\ (\%) = \frac{(G20-G1)\ X\ 0.9\ X\ F}{W}\ X\ 100 \quad (2)$$

$$SDS(\%) = \frac{(G120-G20)\ X\ 0.9\ X\ F}{W}\ X\ 100 \quad (3)$$

$$RS(\%) = 100 - VRDS - RDS - SDS \quad (4)$$

Where G1 = The absorbance of glucose after 1 min incubation; G20 = The absorbance of glucose after 20 min incubation; G120 = The absorbance of glucose after 120 min incubation; F = 100/absorbance, W: sample weight, and 0.9 is used to represent an experimental factor to convert monosaccharides into polysaccharides.

2.8. Prebiotic properties analysis.

2.8.1. Resistance to simulated gastric juice.

Flour digestion by gastric juice was performed as described by Setiarto *et al*. [20]. Jack bean flour was dissolved in sterile distilled water (1% w/v). Simulated gastric juice was prepared by suspending the following chemicals in 1 L of distilled water: NaCl (8g/L), KCl (0,2 g/L), Na_2HPO_4 x $2H_2O$ (8,25 g/L), NaH_2PO_4 (14,35 g/L), $CaCl_2$ x $2H_2O$ (0,1 g/L), and $MgCl_2$ x $6H_2O$ (0,18 g/L). The acidity of the gastric juice was adjusted to pH 2 using 5 M HCl. A flour sample (1%, w/v) was dispersed in gastric juices and incubated in a water bath at 37±1°C for 6 hours. They were analyzed at 0, 0.5, 1, 2, 4, and 6 hours post-injection. The percentage (%) of hydrolysis of jack bean flour is calculated using the equation according to Lu *et al*. [31]:

$$Resistance\ hyrolysis(\%) = 100\% - \frac{Reducing\ sugar\ content(\%db)}{Total\ sugar\ content(\%db)} x\ 100\% \quad (5)$$

2.8.2. Probiotic growth.

Jack bean flour was evaluated for its prebiotic potential using a lactic acid bacteria (LAB) viability assay, as described by Setiarto *et al*. [20]. A 2.5 mL culture of *L. plantarum*

IIA-1A5, aged 24 h and containing 10^5 CFU/mL, was inoculated into the growth medium supplemented with the sample. After 24 h of incubation, 1 mL of the culture was transferred into 9 mL of 0.85% NaCl solution for serial dilution. Each dilution was spread-plated onto MRSA medium. The inoculated MRSA plates were incubated at 37°C, and colony counts were determined after 48 h, expressed as CFU/mL.

2.8.3. Prebiotic effects and index.

Prebiotic effect and prebiotic index assays of jack bean flour were conducted using a modified method of Setiarto *et al*. [21]. The assessment was performed by comparing changes in colony counts of *Lactobacillus plantarum* IIA-1A5 grown on m-MSRB medium alone and on m-MSRB medium supplemented with jack bean flour at 2.5%. The 2.5% concentration was selected based on previous studies reporting that carbohydrate substrates within the 1–3% (w/v) range provide sufficient fermentable carbon to support detectable differences in *L. plantarum* growth without causing osmotic stress or nutrient imbalance. In particular, 2.5% has been shown to elicit measurable, reproducible growth responses in *L. plantarum* IIA-1A5, making it a suitable benchmark concentration for prebiotic evaluation. After 24 hours of incubation at 37°C, cultures were enumerated on MRSA medium to determine viable cell counts. The same procedure was applied using commercial fructooligosaccharides (FOS) as a positive control, and the prebiotic effect and index were calculated using the following equations:

$$\text{Prebiotic effect} = \log(\text{CFU/mL})\ \text{2,5\% Sample} - \log(\text{CFU/mL})\ \text{mMRSB} \quad (6)$$

$$\text{Prebiotic index} = \frac{\log(\text{CFU/mL})\ \text{2,5\% Sample} - \log(\text{CFU/mL})\ \text{mMRSB}}{\text{Sample weight (g)}} \quad (7)$$

2.8.4. Prebiotic activity against pathogens.

Prebiotic activity was assessed following the method of Setiarto *et al*. [20]. For this assay, 2% (v/v) culture of *L. plantarum* IIA-1A5 was inoculated into m-MRSB supplemented with either 2.5% (w/v) glucose or 2.5% (w/v) jack bean flour, and viable counts were determined on MRSA medium at 0 and 24 h of incubation. A similar procedure was applied to the diarrhea-causing bacterium *Enteropathogenic Escherichia coli* (EPEC), where 2% (v/v) inoculum was cultured in m-TSB containing 2.5% (w/v) glucose or 2.5% (w/v) jack bean flour, followed by enumeration on TSA medium after 0 and 24 h of incubation at 37 °C. The prebiotic activity value was then calculated using the following equation:

$$Prebiotic\ activity\ value = \left\{\frac{N\log\frac{CFU}{ml}Sample_{t1} - N\log\frac{CFU}{ml}Sample_{t0}}{N\log\frac{CFU}{ml}Glucose_{t1} - N\log\frac{CFU}{ml}Glucose_{t0}}\right\} - \left\{\frac{E\log\frac{CFU}{ml}Sample_{t1} - E\log\frac{CFU}{ml}Sample_{t0}}{E\log\frac{CFU}{ml}Glucose_{t1} - E\log\frac{CFU}{ml}Glucose_{t0}}\right\} \quad (8)$$

Where N = number of *L. plantarum* IIA-1A5 (log CFU/mL), E = number of *Enteropathogenic Escherichia coli* (log CFU/mL), t0 = start of incubation time (0 hours), t1 = end of incubation time (24 hours).

2.9. Statistical analysis.

All experimental data were statistically analyzed using Analysis of Variance (ANOVA) in SPSS version 27.0. Determinations of *in vitro* starch digestibility, resistance against simulated gastric juice, probiotic growth, prebiotic effects, prebiotic index, and prebiotic

activity were performed in triplicate (n=3). The Duncan test was used to determine the significance of the difference between means at a 5% significance level.

3. Results and Discussion

3.1. In vitro starch digestibility of jack bean flour.

The starch digestibility profile of modified jack bean flour is summarised in Table 1. Starch fractions are categorized as VRDS, RDS, SDS, and RS based on their susceptibility to enzymatic hydrolysis, with lower VRDS/RDS and higher SDS/RS generally reflecting reduced digestibility. Physical, chemical, enzymatic, or fermentation treatments can shift these proportions by increasing crystalline order and restricting enzyme accessibility. Such increases in crystallinity reduce the exposure of glycosidic bonds to hydrolytic enzymes, thereby lowering digestibility [21]. Functionally, higher VRDS and RDS fractions may support rapid energy release. In contrast, higher SDS and RS fractions are relevant to formulations intended to moderate postprandial glycemic responses in individuals at risk of diabetes [32, 33].

Across treatments, modified jack bean flour showed a consistent decrease in VRDS and RDS, accompanied by increases in SDS and RS, indicating a reduction in digestibility relative to native jack bean flour. The VRDS levels in ANN-, HMT-, and AH-modified samples did not differ significantly from those in native flour, likely due to partial gelatinization during these treatments. Partial gelatinization promotes interactions between amylose and amylopectin, leading to imperfect crystallite formation and, in turn, reducing susceptibility to enzymatic attack. This mechanism is consistent with the observations of Zou *et al*. [34], who reported that imperfect crystallites are less readily hydrolyzed. Similar non-significant reductions in VRDS have also been documented in ANN-modified pea starch and HMT-modified field bean starch [35, 36], supporting the trends observed in this study.

Table 1. Starch digestibility rate profile in modified jack bean flour (n = 3).

Samples	***In vitro* starch digestibility profile (%db)**			
	VRDS	**RDS**	**SDS**	**RS**
Native	44.31±0.64^{f}	22.63±0.99^{d}	17.42±0.55^{ab}	15.65±1.13^{a}
AC-1C	41.56±0.70^{de}	11.86±0.88^{ab}	19.09±1.54^{abc}	27.49±1.09^{de}
AC-2C	38.55±0.71^{c}	12.07±0.58^{ab}	20.53±1.50^{cde}	28.85±0.45^{e}
ANN	43.93±1.60^{f}	18.95±2.70^{c}	19.34±1.49^{bcd}	17.78±0.38^{b}
MC	40.66±0.57^{d}	9.78±0.90^{a}	23.72±1.23^{f}	25.85±1.23^{d}
HMT	43.29±0.59^{ef}	22.08±1.13^{d}	16.24±2.12^{a}	18.39±1.28^{bc}
AH	43.33±0.96^{ef}	19.67±1.95^{cd}	16.96±1.14^{ab}	20.04±0.32^{c}
DP	33.91±1.61^{a}	12.23±2.76^{ab}	23.27±2.55^{ef}	30.60±1.61^{f}
FAC	36.13±1.47^{b}	13.47±2.04^{b}	22.21±1.66^{def}	28.19±0.71^{e}

Note: AC-1C = autoclaving-cooling one cycle; AC-2C = autoclaving-cooling two cycles; ANN = annealing; MC = microwave-cooling; HMT = heat moisture treatment; AH = acid hydrolysis; DP = debranching pullulanase; FAC = fermentation followed by AC 1 cycle; VRDS = very rapidly digestible starch; RDS = rapidly digestible starch; SDS = slowly digestible starch; RS = resistant starch. The mean ± SD of triplicate analyses for each component was reported. Numbers in a column that do not share the same alphabetical letter represent significant differences ($p < 0.05$).

The greatest reduction in RDS was observed in MC-modified jack bean flour. Microwave treatment induces rapid gelatinization, followed by recrystallization, thereby enhancing the formation of ordered crystalline regions within the granule [29]. Higher microwave power increases molecular mobility and promotes retrogradation, thereby reducing the proportion of rapidly digestible fractions [37–39]. Thermal processes such as MC may also cleave α-1,6-glycosidic bonds more readily than α-1,4-bonds, generating shorter linear chains

[40]. These short-chain amylose fractions retrograde efficiently into tightly packed double helices stabilized by hydrogen bonding, thereby reducing susceptibility to enzymatic hydrolysis [41, 42]. This mechanism is consistent with findings reported for MC-modified chestnut starch, where significant decreases in RDS were also observed relative to the native starch [29].

In contrast, HMT- and AH-modified jack bean flours showed lower SDS levels than the native flour. For HMT, this decrease is likely associated with imperfect double-helix formation and partial structural disruption induced by high-temperature conditioning. Such disruption weakens molecular interactions, increasing enzymatic accessibility and thereby reducing the proportion of slowly digestible fractions, as described by Gong *et al*. [43]. A similar pattern was observed in AH-modified flour. Acid hydrolysis only mildly affects relative crystallinity (RC) in some starches because densely packed crystallites can resist acid penetration [44]. Reduced RC indicates weak crystal-cluster stability, where abundant short chains inhibit the formation of a robust crystalline network [45]. The present findings are in agreement with Deng *et al*. [46] and Polnaya *et al*. [47], who reported no significant differences in SDS levels between native and HMT- or AH-modified starches.

RS levels in all modified jack bean flours were higher than in the native sample, with DP showing the highest RS content ($p < 0.05$). Modification enhances RS by promoting amylose chain re-association into double helices and crystalline complexes that resist enzymatic attack [48]. Pullulanase treatment of DP specifically hydrolyses α-1,6 branches of amylopectin, producing shorter linear amylose chains that readily retrograde into enzyme-resistant structures. Bodjrenou *et al*. [49] demonstrated that pullulanase-assisted modification can generate large, stable crystalline domains, consistent with the elevated RS observed here. The RS level of DP-modified jack bean flour (30.60%) exceeded that of DP-modified black chickpea starch (12.3%) [50]. Still, it remained lower than that of DP-modified porang flour (56.73%) [21], indicating that the starch source strongly influences the extent of RS formation.

3.2. Resistance of jack bean flour against simulated gastric juice.

The resistance of modified jack bean flour to artificial gastric acid is shown in Table 2. *In vitro* acid resistance was assessed by incubating for 6 hours at pH 2, simulating gastric conditions. Resistance to gastric acidity is an essential criterion for prebiotic candidates, as the material must remain structurally intact long enough to reach the intestine and serve as a substrate for beneficial gut microbes [51]. During incubation, decreasing pH and prolonged exposure increased hydrolysis, reflected by higher levels of reducing sugars released. The rise in reducing sugars indicates progressive cleavage of glycosidic bonds, producing additional reducing ends as digestion proceeds [52]. Accordingly, a lower proportion of hydrolyzed starch under acidic conditions indicates greater resistance to gastric juice and a higher likelihood of survival through the stomach.

Table 2. Resistance of modified jack bean flour against simulated gastric juice hydrolysis (n = 3).

Samples	Resistance of jack bean flour towards simulated gastric juice (%)					
	Incubation time (hours)					
	0	0.5	1	2	4	6
Native	100±0.00^{a}	87.18±0.98^{a}	87.03±0.15^{a}	86.01±0.14^{a}	84.05±0.85^{a}	83.96±0.74^{a}
AC-1C	100±0.00^{a}	91.51±1.69^{c}	91.41±0.36^{d}	90.72±0.38^{c}	90.49±0.51^{dc}	90.48±0.39^{c}
AC-2C	100±0.00^{a}	91.95±0.84^{c}	91.76±0.74^{d}	91.00±1.61^{c}	90.76±0.26^{dc}	90.50±0.52^{c}
ANN	100±0.00^{a}	88.67±2.51^{ab}	88.12±0.65^{b}	87.99±0.59^{b}	87.10±0.78^{c}	86.64±0.34^{b}
MC	100±0.00^{a}	90.35±0.22^{bc}	90.17±0.49^{c}	89.90±1.28^{c}	89.87±0.15^{d}	89.80±0.67^{c}
HMT	100±0.00^{a}	86.80±0.60^{a}	86.58±0.60^{a}	86.18±1.10^{a}	85.88±0.72^{b}	85.79±0.79^{b}

Samples	Resistance of jack bean flour towards simulated gastric juice (%)					
	Incubation time (hours)					
	0	0.5	1	2	4	6
AH	100±0.00^{a}	87.55±1.06^{a}	86.67±1.26^{a}	85.41±1.00^{a}	84.49±0.29^{a}	84.23±0.68^{a}
DP	100±0.00^{a}	91.91±0.51^{c}	91.64±0.38^{d}	91.49±0.67^{c}	91.24±0.26^{c}	90.99±1.39^{c}
FAC	100±0.00^{a}	91.61±1.15^{c}	91.61±0.46^{d}	91.34±0.41^{c}	90.55±0.77^{dc}	90.42±0.25^{c}

Note: AC-1C = autoclaving-cooling one cycle; AC-2C = autoclaving-cooling two cycles; ANN = annealing; MC = microwave-cooling; HMT = heat moisture treatment; AH = acid hydrolysis; DP = debranching pullulanase; FAC = fermentation followed by AC 1 cycle. The mean ± SD of triplicate analyses for each component was reported. Numbers in a column that do not share the same alphabetical letter represent significant differences ($p < 0.05$).

Native jack bean flour showed high susceptibility to hydrolysis in simulated gastric juice, consistent with its elevated VRDS and RDS fractions and low RS content. Prolonged exposure to pH 2 further increased its hydrolysis rate. In contrast, jack bean flour modified by AC-1C, AC-2C, DP, and FAC demonstrated more than 90% resistance to hydrolysis after 2 hours of incubation, indicating substantially improved acid stability. Among the treatments, DP produced the highest RS level ($p < 0.05$), reflecting the formation of more compact and stable double-helix structures that are less accessible to acid hydrolysis. This observation aligns with findings by Yousefi *et al*. [53], who reported that starches with higher RS and lower glycaemic index release less glucose under simulated gastric conditions.

Physical modification techniques that promote crystalline reorganization are known to enhance starch resistance to gastric acid and limit hydrolysis during digestion [54]. The acid resistance observed in the present study was higher than that reported for HMT- and AC-modified taro starch by Setiarto *et al*. [25], which exhibited >87% resistance after 2 hours. Comparable studies also showed that DP-modified corn flour and porang flour displayed 93.67% and 98.60% resistance, respectively [20, 21], values slightly higher than those obtained for DP-modified jack bean flour. A material can be considered a potential prebiotic source when it demonstrates ≥88% resistance to gastric acid hydrolysis, allowing it to reach the colon for microbial fermentation [54]. Based on this criterion, DP- and FAC-modified jack bean flours exhibited strong acid resistance and therefore show promising properties as prebiotic candidates, although further *in vivo* validation is still required.

3.3. Probiotic growth of jack bean flour.

The growth response of *L. plantarum* IIA-1A5 on media containing modified jack bean flour is shown in Figure 1. Resistant starch may function as a prebiotic when the gut microbiota ferments it, thereby stimulating the proliferation of beneficial bacteria [55]. After 24 hours of incubation, *L. plantarum* IIA-1A5 exhibited increased growth across all modified samples. The highest cell density was observed in media containing 2.5% FAC (3.10 log CFU/mL), while the lowest was recorded in media containing 2.5% AC-1C (2.32 log CFU/mL). As expected, media supplemented with FOS yielded significantly higher cell counts than all modified jack bean flour samples ($p < 0.05$). Resistant starch serves as an important carbon source during fermentation and can enhance LAB growth. Prior work has shown that the optical density of pure *Lactobacillus* and *Bifidobacterium* cultures increases significantly in the presence of resistant starch compared with media lacking it [55]. Similarly, Zi-Ni *et al*. [27] reported that sago starch modified through combined DP and AH treatments resulted in higher RS-supported greater *L. plantarum* FTCC0350 cell density than DP treatment alone. Consistent with these findings, the present study demonstrates that increased RS content in modified jack bean flour is positively associated with the growth of *L. plantarum* IIA-1A5.

However, media containing the prebiotic reference (FOS) promoted markedly higher growth than any modified jack bean flour. This is likely due to substrate preference among LAB. Sorndech *et al*. [56] noted that *Lactobacillus* species preferentially utilize lower-molecular-weight carbohydrates, whereas Bifidobacterium more effectively metabolizes higher-molecular-weight prebiotics. Moreover, RS type 3 is especially associated with stimulating *Bifidobacterium* proliferation [57]. Given that *L. plantarum* IIA-1A5 favors low-molecular-weight substrates such as FOS, its growth was expectedly higher in FOS-supplemented media than in media containing modified jack bean flour.

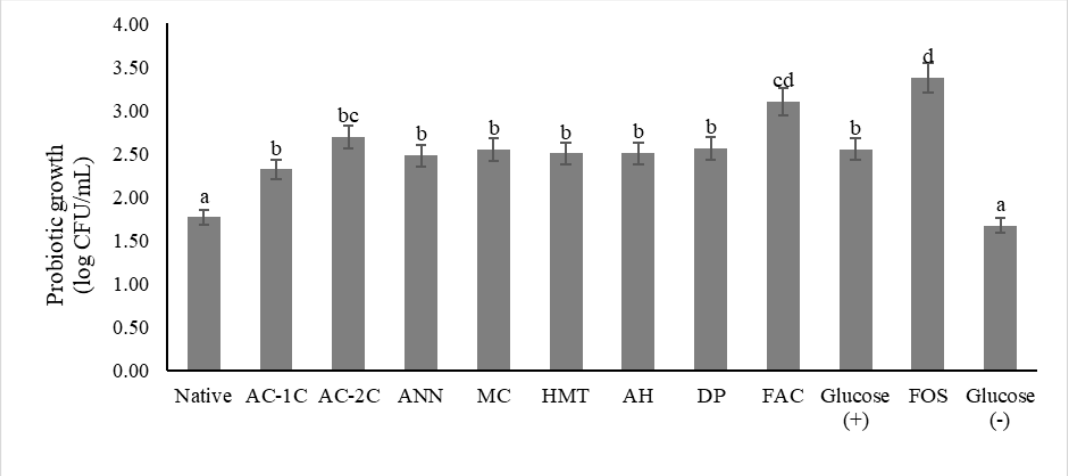


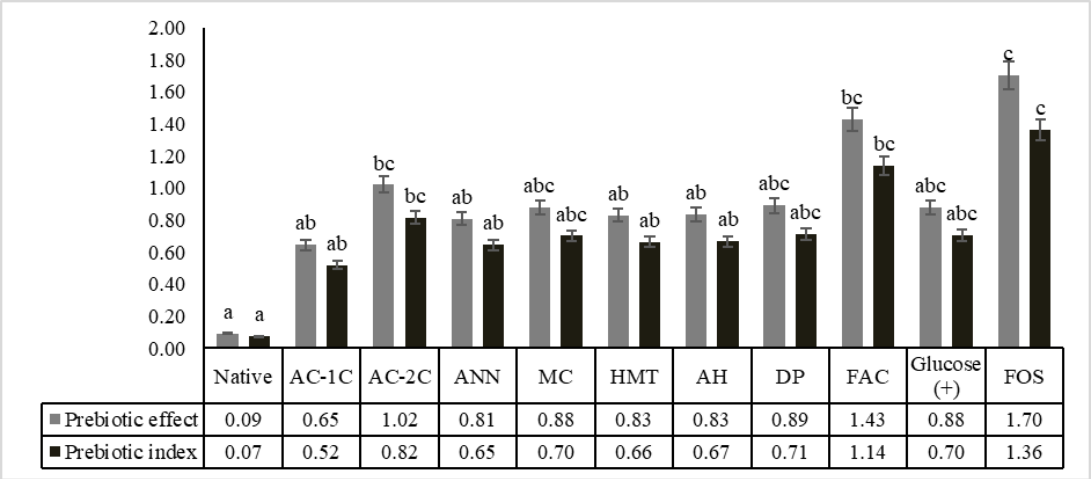
Figure 1. Growth of *L. plantarum* IIA-1A5 of modified jack bean flour. Different letters on bars indicate significant differences ($p < 0.05$). AC-1C = autoclaving-cooling one cycle; AC-2C = autoclaving-cooling two cycles; ANN = annealing; MC = microwave-cooling; HMT = heat moisture treatment; AH = acid hydrolysis; DP = debranching pullulanase; FAC = fermentation followed by AC 1 cycle; FOS = fructooligosaccharide; Glucose (+) = with carbon/glucose source; Glucose (-); without carbon source. Error bars indicate standard deviation.

Native jack bean flour is thought to have a higher RS type 2 content than modified jack bean flour. Zhou *et al*. [58] reported that RS type 2 shows a higher molecular weight than RS type 3. The low utilization of *L. plantarum* IIA-1A5 in native jack bean flour is likely to result in lower probiotic growth. Differences in the molecular weight of the starch used as a carbon source can affect probiotic preferences during fermentation. This is supported by research by Sorndech *et al*. [56], who reported that *Lactobacillus* growth was significantly higher in media containing RS type 3 as a carbon source than in media containing RS type 2. A similar study by Zi-Ni *et al*. [27] reported that unmodified sago starch had lower percentages and cell densities and was significantly different from modified sago starch in *L. plantarum* FTCC0350 cultures.

3.4. Prebiotic effect and prebiotic index of jack bean flour.

The prebiotic effect and prebiotic index of modified jack bean flour are presented in Figure 2. The prebiotic effect describes the ability of a substrate to consistently enhance probiotic cell populations relative to a carbohydrate-free control, independent of inoculum size [21]. In contrast, the prebiotic index quantifies probiotic growth relative to the concentration of the test substrate [59]. An increase in the prebiotic effect typically corresponds to a higher prebiotic index, as improved fermentability supports greater bacterial proliferation [60]. Among all treatments, FAC produced the highest values for both parameters ($p < 0.05$), with a prebiotic effect of 1.43 and a prebiotic index of 1.14. A higher prebiotic effect suggests that *L.*

plantarum IIA-1A5 can efficiently metabolize the substrate, while a higher prebiotic index indicates an enhanced ability to support probiotic growth. These findings imply that FAC-modified jack bean flour demonstrates promising prebiotic properties, although further *in vivo* validation is required to confirm its physiological relevance.



	Native	AC-1C	AC-2C	ANN	MC	HMT	AH	DP	FAC	Glucose (+)	FOS
Prebiotic effect	0.09	0.65	1.02	0.81	0.88	0.83	0.83	0.89	1.43	0.88	1.70
Prebiotic index	0.07	0.52	0.82	0.65	0.70	0.66	0.67	0.71	1.14	0.70	1.36

Figure 2. Prebiotic effect and index of modified jack bean flour. Different letters on bars indicate significant differences ($p < 0.05$). AC-1C = autoclaving-cooling one cycle; AC-2C = autoclaving-cooling two cycles; ANN = annealing; MC = microwave-cooling; HMT = heat moisture treatment; AH = acid hydrolysis; DP = debranching pullulanase; FAC = fermentation followed by AC 1 cycle; FOS = fructooligosaccharide; Glucose (+) = with carbon/glucose source. Error bars indicate standard deviation.

These findings indicate that L. plantarum IIA-1A5 more readily ferments modified jack bean flour than its native form. Zi-Ni *et al*. [27] similarly reported that *L. plantarum* FTCC0350 showed greater fermentability of sago starch with elevated RS levels than unmodified starch. This aligns with evidence that *Lactobacillus* species can utilize RS type 3, where retrograded starch enhances fermentability relative to native starch. Comparable results were reported by Khayrah *et al*. [51], who found that unmodified cempedak flour exhibited a negative prebiotic effect (–0.390), while modified cempedak flour showed a positive prebiotic effect (0.180) toward *L. rhamnosus* R23.

However, the growth of *L. plantarum* IIA-1A5 in media containing modified jack bean flour remained significantly lower than in FOS-supplemented media ($p < 0.05$). This difference is likely attributable to variations in substrate composition and degree of polymerization (DP). RS typically has a higher DP (20–30) than FOS (DP 2–10) and inulin (DP 2–70) [25, 61]. Modification treatments may decrease DP, but not to the extent of producing low-molecular-weight oligosaccharides. Setiarto *et al*. [62] showed that fermentation of taro slices for 24 hours significantly reduced DP compared with native starch. Shorter oligosaccharide chains are more rapidly metabolized by microbes, contributing to FOS's superior performance as a carbon source [63].

The prebiotic index is a key parameter in determining whether a food ingredient exhibits prebiotic potential. A value greater than 1 indicates a positive effect on probiotic growth, whereas a value approaching 1 suggests limited efficacy [25, 64]. In this study, FAC-modified jack bean flour achieved the highest prebiotic index among all treatments. It exceeded that of FAC-modified porang flour (0.84) reported by Setiarto *et al*. [21], although it remained lower than the value reported for FAC-modified taro flour (1.23) [19]. Nevertheless, a limitation of this *in vitro* study is that increased resistant starch content does not necessarily

translate into a proportional increase in prebiotic activity *in vivo*. Therefore, further animal or human studies are required to confirm its physiological relevance.

3.5. Prebiotic activity of jack bean flour.

The prebiotic activity of *L. plantarum* IIA-1A5 against EPEC in modified jack bean flour is shown in Figure 3. Among all treatments, FAC exhibited the highest prebiotic activity, while AC-1C showed the lowest compared with the native flour ($p < 0.05$). A high prebiotic activity value indicates that the prebiotic substrate effectively promotes probiotic growth while inhibiting pathogen proliferation. In contrast, low or negative values suggest poor support for probiotics and a possible advantage for pathogen growth [65]. In this study, the prebiotic activity of AC-2C was higher than that of AC-1C, aligning with Setiarto *et al*. [25], who reported a similar pattern in modified taro starch. These findings further demonstrate that an increase in resistant starch content alone does not guarantee improved prebiotic performance. Consistent with Zi-Ni *et al*. [27], high RS levels may not always correspond to stronger prebiotic activity, emphasizing that the functional quality of prebiotics depends on factors beyond RS concentration.

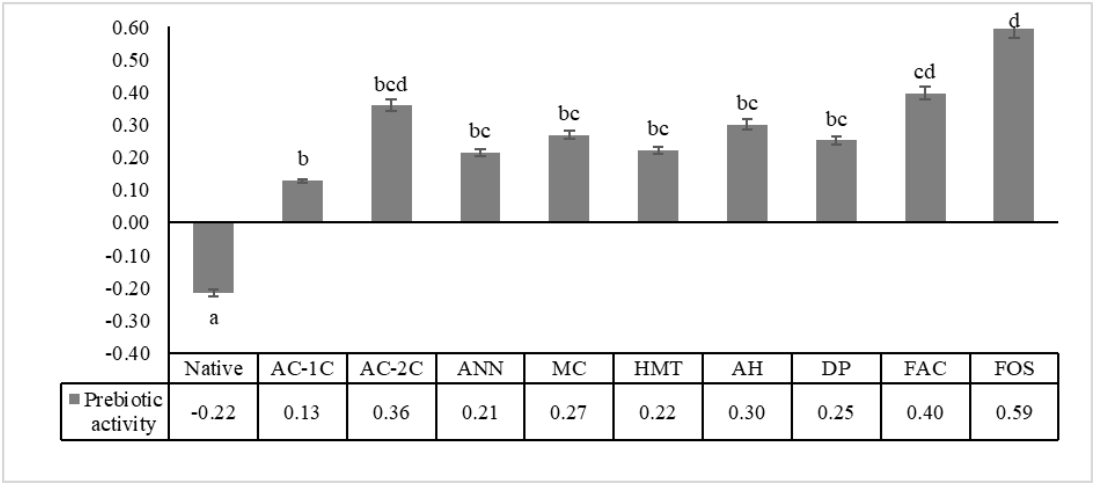


Figure 3. Prebiotic activity of modified jack bean flour. Different letters on bars indicate significant differences ($p < 0.05$)**.** AC-1C = autoclaving-cooling one cycle; AC-2C = autoclaving-cooling two cycles; ANN = annealing; MC = microwave-cooling; HMT = heat moisture treatment; AH = acid hydrolysis; DP = debranching pullulanase; FAC = fermentation followed by AC 1 cycle; FOS = fructooligosaccharide. Error bars indicate standard deviation.

The highest prebiotic activity was observed in the commercial prebiotic (FOS), which exceeded that of all modified jack bean flour samples. However, the prebiotic activity value of FOS in this study was lower than that reported by Setyawan *et al*. [66] for *L. plantarum* Dad-13 against *E. coli* InaCC B-4 (1.02). Zi-Ni *et al*. [27] similarly found that *Lactobacillus* and *Bifidobacterium* cultures exhibit positive prebiotic activity values when grown on commercial prebiotics such as FOS and inulin against *E. coli*. The magnitude of prebiotic activity is strongly influenced by the probiotic species and strain used in testing [26]. Fermentation of RS by lactic acid bacteria generates short-chain fatty acids (SCFAs), which can selectively suppress pathogen growth [67]. Zi-Ni *et al*. [27] also reported that commercial prebiotics caused a greater reduction in medium pH than RS, indicating that substrates with higher fermentability produce more organic acids and thus lower pH more effectively. A food ingredient can be considered to have prebiotic activity if it exhibits an activity value above 0.25 and is selectively metabolized by probiotics but not by pathogens such as EPEC [21]. In this study, the modified

jack bean flour treatments AC-2C, MC, AH, DP, and FAC all demonstrated prebiotic activity values exceeding 0.25, suggesting potential as prebiotic sources.

Although the modified jack bean flours showed increased resistant starch levels and positive *in vitro* indicators of prebiotic activity, these results should be viewed as preliminary. *In vitro* assays are useful for assessing microbial utilization but cannot fully simulate the complexity of the human gastrointestinal tract. Thus, the prebiotic functionality of modified jack bean starch remains putative until it is validated in *in vivo* models or human clinical trials. Moreover, practical application may be influenced by factors such as variability in modification efficiency, stability during food processing, sensory quality, and the presence of residual anti-nutritional compounds. Further research is needed to optimize processing conditions, evaluate performance in real food matrices, and confirm physiological effects under biologically relevant conditions.

4. Conclusions

This study demonstrates that diverse modification techniques, including physical (autoclaving–cooling cycles, annealing, microwave–cooling, and heat-moisture treatment), chemical (acid hydrolysis), enzymatic (pullulanase debranching), and fermentation-based approaches (LAB fermentation followed by AC-1C), produce distinct effects on the functional characteristics of jack bean flour. All treatments increased resistant starch content to varying degrees, with the highest improvement observed in debranched preparations. The best modification method for prebiotic properties in jack bean flour was FAC modification, with resistance to artificial gastric acid juice in 2 hours of incubation of 91.34%, growth of *L. plantarum* IIA-1A5 of 3.10 log CFU/mL, prebiotic effect of 1.43, prebiotic index of 1.14, and prebiotic activity against *Enteropathogenic Escherichia coli* (EPEC) of 0.40. While these findings suggest that FAC-modified jack bean flour may hold potential as a prebiotic ingredient, this conclusion remains preliminary. The outcomes are based solely on *in vitro* assays and therefore cannot fully predict physiological performance or *in vivo* health benefits. Additionally, the absence of structural characterization, XRD, and DSC limits the ability to relate functional changes to underlying molecular transformations. Future research should incorporate more comprehensive analyses, including structural profiling, simulated colon fermentation models, and *in vivo* studies to confirm functional efficacy and safety. Evaluating formulation stability, sensory attributes, and interactions with diverse probiotic strains will further support potential application in functional foods.

Author Contributions

Conceptualization, R.H.B.S. and A.W.; methodology, R.H.B.S., A.W., T.M., U.H., and L.A.; software, A.W. and L.A.; validation, R.H.B.S., T.M., U.H., and L.A.; formal analysis, A.W. and L.A.; investigation, R.H.B.S., A.W., T.M., U.H., and L.A.; resources, R.H.B.S.; data curation, A.W. and L.A.; writing—original draft preparation, A.W., R.H.B.S., T.M., and U.H.; writing—review and editing, A.W., R.H.B.S., T.M., and U.H.; visualization, A.W.; supervision, R.H.B.S., T.M., U.H., and L.A.; project administration, R.H.B.S., T.M., and U.H. All authors have read and agreed to the published version of the manuscript.

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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 no conflict of interest.

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