

# Prebiotic products based on kepok banana starch (*Musa paradisiaca* Formatypica) and moringa (*Moringa oleifera*) as functional food for female wistar rats

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**Abstract.** Background: Colon cancer ranks as the 5<sup>th</sup> most common cancer worldwide, with over one million new cases reported between 2015 and 2018. Resistant starch found in Kepok Banana extract may have potential in colon cancer prevention. Additionally, diets rich in iron and vitamin C contribute to chronic disease prevention. Moringa leaves, rich in vitamin C, enhance iron absorption. This study evaluated the growth of *Lactobacillus* and *Shigella spp* in female rats given Moringa-Banana starch (MBS) as a prebiotic supplement. Results showed significant differences in gut microbiota with MBS consumption, with higher concentrations of beneficial bacteria and reduced levels of pathogenic bacteria observed in the group receiving the highest MBS dose. These findings suggest that MBS could be a potential prebiotic candidate for further development.

## 1 Introduction

Colon cancer is the 5<sup>th</sup> most common cancer worldwide, with over one million new cases between 2015 and 2018. The possibility of this incident will increase annually [1]. Undigested starch (resistant starch) in the small intestine offers several physiological benefits to human health, including preventing colon cancer, lowering blood sugar levels, prebiotic effects, cholesterol reduction, inhibition of fat accumulation, promotion of satiety, enhanced mineral absorption, and a reduced risk of gallstone formation [2]. According to several studies, undigested starch can also increase iron absorption in the body. Meanwhile, easily digestible starch can inhibit iron absorption, suggesting that iron intake should not coincide with digestible starch. Resistant starch, which has the potential to prevent colon cancer, is found in Kepok Banana extract.

Chronic disease can be avoided by eating a diet rich in iron and vitamin C. Vitamin C-rich moringa leaves can aid in iron absorption. Previous research revealed that resistant starch from bananas contains 15% of whole starch, while moringa leaves and bananas contain 7.45 mg of Fe. In the small intestine, resistant starch is not absorbed and remains intact until it

reaches the intestine, where it is fermented by beneficial bacteria such as *Bifidobacteria* and *Lactobacilli*. This process highlights the potential of resistant starch as a prebiotic. Prebiotics, which are indigestible food elements, play a crucial role in modulating the complex microbial community by selectively promoting the growth and/or activity of beneficial microbes, ultimately benefiting the host [3]. Green Kepok bananas are a source of type 2 resistant starch. Other common sources of type 2 RS include fruits, nuts, grains, and tubers.

Kepok bananas contain resistant starch type 2 (RS2) because their granular starch has a natural crystalline structure. This form of starch comprises more than 50% of the total starch in raw Kepok bananas. The starch in raw Kepok bananas remains in a form that digestive enzymes cannot digest [4]. The green Kepok banana has the greatest resistant starch content, ranging from 35.14 to 45.87%, according to Marsono's [4] research findings. Research on the Kepok banana and Moringa powder related to LAB and pathogens has never been studied before. This study measured the growth of *Lactobacillus* and *Shigella spp* in female rats given Moringa-Banana starch (MBS). The study results show that MBS can become a candidate for high Fe prebiotics and as a standardized supplement. The study aims to determine the effect of MBS supplementation on changes in rat microbiota growth in feces and in the intestines. The study examined changes in intestinal pH and the blood profile of the rats.

## 2 Materials and methods

### 2.1 Sample preparation and analysis

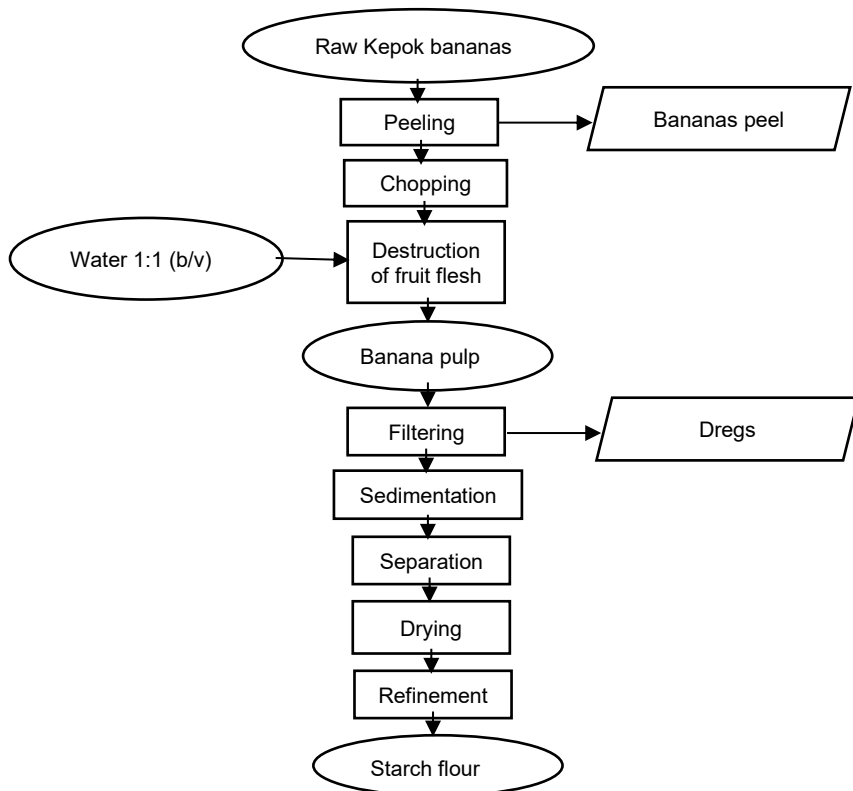
This study employed MBS, a prebiotic supplement combining Kepok Banana starch and Moringa leaf extract. The flow diagram of the banana starch extraction process is shown in Figure 2. Banana Selection: Choose a raw, high-quality kepok banana with undamaged dark green skin. This type of banana has a higher starch content, making it suitable for use as flour. Peeling: Peel the banana and cut it into small pieces to make it easier to process. Washing and Soaking: Wash the cut bananas with clean water and soaked in calcium hydroxide ( $\text{Ca}(\text{OH})_2$ ) to keep their bright color. To reduce sap content, the bananas may also be soaked in water. Drying: After washing, the bananas are dried either in the sun or in an oven at a 50-60°C for 10-12 hours until completely dried. Grinding: Fully dried bananas are ground using a blender or food processor until they become a fine powder. Filtration: The ground banana flour is then sifted using an 80 mesh sieve to remove coarse particles and obtain a finer consistency. The stability analysis of resistant starch in MBS was conducted following the method developed by AOAC 2002.02 and AACC 32.40, using the K-RAPRS 11/19 reagent. Vitamin C stability was assessed as described in [5]. All analyses were performed in triplicate. The resistant starch and vitamin C levels in MBS stored at 40 degrees Celsius were 21.49% and 35.07 mg, respectively [4,5].

The production of resistant starch from banana flour involves modification techniques such as autoclaving-cooling. Banana flour is mixed with water to form a suspension with a moisture content of 30–50%. It is then heated in an autoclave at 120°C for 15-30 minutes to allow gelatinization. Cooling: the preheated suspension is then cooled (usually at 4°C) for several hours to promote retrogradation or the formation of new starch structures. This process can be repeated several times (1-4 cycles) to increase resistant starch levels. Drying and Storage: After retrogradation, the mixture is dried again to achieve low moisture content, ground into flour, and filtered through a 100-mesh sieve. The resulting flour exhibits a resistant starch level. Research shows that a higher number of autoclaving-cooling cycles leads to the greater resistant starch content [5].

The process of making Moringa leaf extract powder involves several steps [6]. First, young Moringa leaves are selected for optimal quality. The leaves are then washed

thoroughly with clean water to remove dirt and pesticides. To produce the extract in powder form, the leaves may be dried under sunlight or using artificial drying methods. Once dried, the leaves are ground into a fine powder using a blender or grinder. Finally, the ground leaves are placed in a container, and sterile distilled water (aquades) is added as the solvent for extraction. The extraction process involves soaking moringa leaf powder in the solvent for 4-6 hours. Filtration: After the extraction is complete, strain the moringa leaves using gauze or a filter to separate the extracted liquid from the leaf pulp. Drying of Extract (for powder): To produce extract powder, the liquid is dried in an oven at 40-60°C for 10 hours.

Mixing of Kepok Banana Resistant Starch Extract with Moringa Leaf Extract. Drying Ingredients: Before mixing, banana starch powder and moringa leaf powder are thoroughly dried in the oven. Sieve: Sift both powders (banana starch and moringa powder) to remove any large particles or debris that could interfere with the mixing process. Powder Mixing: Mix banana starch and moringa powder in the appropriate ratio, with 70-80% banana and 30-20% moringa powder. A mixer is used to mix the two ingredients evenly. Addition of Iron Preservatives and Strengtheners: To improve the quality and shelf life of the product, add natural preservatives such as sorbic acid or potassium sorbate (0.1-0.3% of the total mixture). These preservatives help to increase the absorption of iron in the body and help maintain the green color of moringa powder, preventing it from turning brown easily. Packaging: Once the powder mixture is homogeneous, store it in an airtight, sealed container in a cool, dry place. Avoid direct exposure to sunlight to maintain product quality [6,7].



**Fig. 1.** The Flow Diagram of Banana Starch Extraction Process [6]

## 2.2 Diet and experimental model

This research employed experimental animals, female white rats of the Wistar strain, aged 6–8 weeks, with a body weight of 80–150 g. The rats were obtained from local breeders from PT Kencana, Bandung, West Java. Furthermore, the condition of the rats was verified with a Veterinary Certificate number: TN.01.01.11/2088-DKPP/V/2023, issued by the Food Security and Agriculture Service of the Bandung City Government. Thirty-two healthy female Wistar rats were randomly divided into four equal groups: K1 = control; K2 = 0.5 g MBS/rat/day; K3 = 1 g MBS/rat/day; K4 = 2 g MBS/rat/day. During the acclimatization period, the rats were fed standard feed for 2 weeks, followed by additional feed and supplements as listed for an additional 6 weeks (50 days). During the acclimatization period, the rats were given a standard AD II diet, which contained 51% carbohydrates, 15% crude protein, 7% crude fat, and 6% crude fiber, and provided with mineral water. Standard feed was given at a rate of 30 g, with additional feed offered continuously until the rats stopped eating on their own. The Moringa-Kepok Banana starch dosage was based on the effective dose identified in previous research for increasing intestinal microbiotas.

### 2.2.1 Blood, microbiota, and weight measurements

Blood sampling was performed at the end of the research. Blood samples were collected from the rat's tail and transferred into an Eppendorf tube. Blood, feces, and cecum specimens were obtained by anesthetizing the rats, with assistance from an analyst at the Experimental Animal Development Laboratory.

Hematological examination in rats begins with intracardiac blood collection (through the heart), a method commonly used in animal laboratories to obtain sufficient blood volume. The procedure follows standard procedures, beginning with following stages: 1). Rat Preparation: The rats are first anesthetized. After being unconscious, they are placed in a supine position on a surgical tray to facilitate access to the heart. 2) Blood Collection Process: The location of the heart is determined by palpation or based on anatomical signs in the chest cavity. A sterile syringe needle is carefully inserted into the heart through the gap between the ribs. This procedure is conducted slowly to minimize the risk of tissue damage. The volume of blood taken was 3 ml. 3). Blood Sample Handling: The blood taken is immediately put into a vacutainer tube containing an anticoagulant, such as EDTA. The blood tube is shaken slowly with a figure-eight movement to mix the anticoagulant with the blood completely. Blood samples are stored at 4°C if not immediately processed for analysis. 4). Hematology Examination: This stage was conducted to analyze parameters such as hematocrit, number of erythrocytes, leukocytes, and leukocyte differential. Analysis was carried out using the ABX Micros 60 Hematology Analyzer automatic hematology tool [8]. Blood specimens were examined at the FKUB Clinical Pathology Laboratory for Hematology Analysis. At the same time, the cecum and feces were analyzed at the FKUB Microbiology Laboratory to identify microscopically growing bacterial colonies.

### 2.2.2 Cecum examination stage

The cecum contents were removed and collected in a sterile tube containing 9 ml of saline solution (NaCl 0.85%) for a  $10^{-1}$ , then homogenized. Next, 0.1 ml of the cecum content suspension was put into a dilution tube containing 0.9 ml of saline, achieving a  $10^{-2}$  dilution. The mixture was vortexed until homogeneous, then further diluted to a  $10^{-6}$  dilution. The total LAB population was determined by taking 0.1 ml of a diluted sample (dilutions  $10^{-3}$  to  $10^{-6}$ ) spread on the surface of MRS Agar media, which had been supplemented with BromoCresol Purple (BCP). The plate was then incubated anaerobically for 24 hours at 37

°C using an anaerobic gas pouch in an anaerobic chamber. Bacterial colonies that grew were counted using the dilution method, assuming that each colony originated from one single cell. The total bacterial population was calculated by multiplying the number of growing colonies by the dilution factor.

Analyzing the type and amount of intestinal microbiota was performed using fresh stool samples collected in Eppendorf tubes and immediately frozen at -8 °C. TPC was conducted using the Pour Plate method with *Lactobacillus* spp. as the target bacteria, cultured on Modified Bromocresol Purple-Sucrose (MBS) Agar. The *E. coli* activity test was carried out using the Biochemical Test ImViC Coliform Bacteria with Bacterial Inoculation on SIM medium. The total bacterial population was determined using the total plate count (TPC) method on Plate Count Agar (PCA) media. A stool sample of 1 g was suspended in 9 mL of physiological saline (0.9% NaCl) in each test tube, followed by serial dilution. A 0.1 mL of the dilution sample was taken and then inoculated using the pour plate technique on a petri dish containing PCA medium. The samples were incubated at 37 °C for 48 hours. The bacterial colonies that grew were counted using a colony counter, and the total bacterial colonies were calculated. [9,10].

### 2.2.3 Isolation and characterization of anaerobic bacteria

Fecal bacteria were isolated using a serial dilution technique in 0.9% NaCl solvent. A 0.1 mL aliquot of the diluted samples was inoculated into sterile Petri dishes. The medium used was peptone yeast glucose (PYG) agar to isolate facultative and obligate anaerobic bacteria, and the pour plate technique was used. Incubation was performed in an anaerobic jar for 48 hours at 37 °C. Colony characterization was carried out by observing the visible morphological characters. The characteristics observed included shape, configuration, elevation, optical characteristics, texture, color, edges, and the presence of clear zones. Purification was conducted using a four-way streak plate technique until a pure culture was obtained. Pure isolates were cultured on agar media as stock isolates and stored in the cool room at 4 °C for use in subsequent tests.

The body mass was evaluated each week using an analytical scale. Meanwhile, measurements of microbiota in feces and cecum (large intestine) were carried out once, at the end of the intervention. Examinations of blood samples for complete blood profiles, along with feces and cecum were conducted after 50 days of rat maintenance and intervention. Additionally, surgery on the cecum was performed to determine the growth of microbiota.

## 2.3 Statistical analysis

The data obtained was collected, coded, and entered into a computer file using the SPSS for Windows Release 24.0 application. Data analysis utilized The Shapiro-Wilk test for normality and Independent sample t-tests for group comparisons. The One-Way ANOVA was employed to observe the general differences in the mean of every variable for the rats in all groups. Next, a post-doc (Tukey) test was conducted to find out which groups were different.

## 3 Results and discussion

In healthy humans, resistant starch (RS) refers to starch and its breakdown products that bypass the small intestine and enter the colon, where they can be fermented by specific microbes [11]. Previous studies suggest that probiotics, particularly *Lactobacillus* sp. strains can improve small intestinal mucosa, increase energy efficiency, and significantly alter body

weight in both humans and animals. In this research, the body weight of rats significantly increased with higher MBS (Table 1), and this aligns with the growth of microbiota. This effect is attributed to the gut microbiota's ability to produce short-chain fatty acids (SCFAs), regulate bile acid metabolism, and prevent metabolic endotoxemia [12]. The study found no significant difference in blood profile data (albumin, hemoglobin, and erythrocyte). The p-value indicated no difference, suggesting that MBS did not have a hematotoxin effect on Wistar white rats after 50 days of administration [13].

**Table 1.** Average data of body weight and blood profile in the treatment group sample

Variable	Treatment (mean±SD)				P Value
	K1	K2	K3	K4	
Weight (g)	127.0±5.9 <sup>a</sup>	130.2±4.5 <sup>b</sup>	139.1±8.2 <sup>c</sup>	130.7±6.9 <sup>b</sup>	0.01*
Albumin (mg/dL)	3.3±0.2 <sup>a</sup>	3.2±0.3 <sup>a</sup>	3.0±0.2 <sup>a</sup>	3.1±0.4 <sup>a</sup>	0.29
Hemoglobin (mg/dL)	14.6±0.9 <sup>a</sup>	13.7±0.5 <sup>a</sup>	14.5±0.6 <sup>a</sup>	13.8±0.5 <sup>a</sup>	0.89
Erythrocyte (10 <sup>6</sup> /mm <sup>3</sup> )	7.7±0.5 <sup>a</sup>	7.4±0.5 <sup>a</sup>	8.1±0.7 <sup>a</sup>	7.4±0.2 <sup>b</sup>	0.12

Note: Different superscript letters are significantly different (p < 0.05), analyzed using one-way-ANOVA and Tukey's honestly significant difference test

There is a significant difference in the weight between rats with treatment and without treatment (Table 1). Consumption of certain prebiotics, such as resistant starch, oligo-fructose, xylooligosaccharides, and fructooligosaccharides, has shown some beneficial effects on malnutrition and related comorbidities. Therefore, it can improve glucose tolerance and lipid metabolism-balanced body weight [14].

**Table 2.** Bacterial analysis in the treatment group sample

Variable		Treatment (mean±SD)				P-Value
		K1	K2	K3	K4	
Pathogens (10 <sup>3</sup> CFU/g)	Feces	2.3±1.2 <sup>a</sup>	5.1±3.1 <sup>a</sup>	4.1±2.9 <sup>a</sup>	3.2±2.0 <sup>a</sup>	0.50
	Intestine	4.3±3.3 <sup>a</sup>	3.9±2.1 <sup>b</sup>	4.7±2.7 <sup>c</sup>	7.2±3.9 <sup>d</sup>	0.03*
LAB (10 <sup>6</sup> CFU/g)	Feces	2.6±0.6 <sup>a</sup>	1.4±0.2 <sup>b</sup>	2.3±0.6 <sup>b</sup>	1.7±0.7 <sup>b,c</sup>	0.03*
	Intestine	1.7±0.6 <sup>a</sup>	2.0±0.8 <sup>b</sup>	2.6±0.2 <sup>c</sup>	2.0±0.7 <sup>b</sup>	0.72

Note: Different superscript letters are significantly different (p < 0.05), analyzed using one-way-ANOVA and Tukey's honestly significant difference test

There is a significant difference in the growth of lactic acid bacteria in the feces, with K2, K3, and K4 showing significantly higher levels than K1 (Table 2). During fermentation, lactic acid bacteria, including *S. thermophilus* and *Lactobacillus bulgaricus*, proliferate rapidly in the intestine and feces, with the highest concentrations found in the cecum. Bacterial counts in the feces are lower, as several types of bacteria cannot survive outside the cecum. MBS exhibits anti-inflammatory, immune system-stimulating, and antibacterial properties, which contributes to a significant difference in bacterial growth in the intestinal tract and feces [15]. Both feces and the intestines include pathogenic bacteria, such as *Proteus* and *Shigella* species, with their quantities decreasing as the dose of MBS treatment increases.

Furthermore, to assess the magnitude of the influence of each treatment, additional tests were conducted to determine the Effect Size (ES). ES of the intervention/exposure to the outcome is expressed by the correlation summary number (r family) e.g., Pearson's Product Moment r, Spearman's Rank r, R, RR, OR) or the difference summary number (d family, e.g., d = mean difference, d = proportion difference) which may be standardized or non-standardized [16].

Effect size is a statistical measure used to quantify the magnitude of the effect of an intervention or the difference between two groups. In the context of providing interventions, effect size helps determine the extent of change occurring and its relevance, without relying

solely on p-value [16]. Cohen's d is used to calculate the size of the effect between two groups, typically after conducting an Independent T-test [16].

$$d = \frac{2 \times t}{\sqrt{df}}$$

Information:

d = Cohen's interpretation

t = calculation between 2 sample averages

df = degrees of freedom

Interpretasi Cohen's d: 0.2: Small effect.; 0.5: Medium effect.; 0.8: Great effect.

**Table 3.** Result of effect size analysis

Group	Variable Effect Size	Results of Analysis	Information
K1-K2	Body weight	-0.6	
	<i>Lactobacillus, sp</i>	0.68	Medium effect
	<i>Enterobacter</i>	0.98	Powerful effect
K1-K3	Body weight	1.20*	Powerful effect, increased
	<i>Lactobacillus sp</i>	0.95*	Moderate effect, increased
	<i>Enterobacter</i>	-1.86	
K1-K4	Body weight	0.63	Powerful effect
	<i>Lactobacillus</i>	0.79*	Powerful effects, increase
	<i>Enterobacter</i>	-1.17	

The measurement of effect size was conducted on weight and microbiota parameters because the main purpose of this study was to determine the effect of the MBS administration on the risk of infection and microbiota growth. As shown in Table 3, the administration of MBS had a strong influence, particularly in the K3 group, where body weight demonstrated a significant increase, with an effect size value of 1.20. Additionally, the growth of *Lactobacillus* exhibited an effect size (ES) of 0.95.

Based on data on *Lactobacillus* growth in cecum after the intervention, it is proven that the dose of 1 g of MBS/rat/day (K3) has an optimal effect. This aligns with the theory that explains that the resistant starch from MBS can promote the growth of beneficial bacteria like *Lactobacillus* while inhibiting the growth of pathogenic bacteria such as *Enterobacter*, *Salmonella*, and *Escherichia coli*. This effect is attributed to prebiotic properties and fermentation mechanisms in the colon. Resistant starch, a complex carbohydrate, is not digested in the small intestine but fermented by the microbiota in the colon. This fermentation produces short-chain fatty acids (SCFAs), including butyric acid, acetate, and propionate, which support the growth of *Lactobacillus* and *Bifidobacterium*. These beneficial bacteria create an acidic environment that inhibits the growth of pathogens such as *Salmonella* and *E. coli*. Resistant starches also increase the expression of mucosal proteins, such as MUC2, thereby improving the integrity of the intestinal epithelium. This prevents colonization of pathogens and reduces inflammation caused by infections from *Salmonella* or *E. coli*. Good bacteria that multiply due to resistant starch produce antimicrobial metabolites, such as bacteriocin, which directly inhibit pathogens [17].

Resistant starch in bananas turns out to be able to increase the production of mucosal proteins, but also increase the production of antimicrobial metabolites. One of the important metabolites is bacteriocin which is an antimicrobial peptide produced by intestinal bacteria that inhibits the growth of pathogens.[18,19]

## 4 Conclusion

This study observed the effect of MBS on body weight and growth of *Lactobacillus* sp. The results showed that the optimal condition for body weight and growth of *Lactobacillus* sp. growth was achieved with an MBS dose of 1 g/rat/day. The growth concentration of *Lactobacillus* was  $2.3 + 0.6 \cdot 10^6$  CFU/ml and  $2.6 + 10^6$  CFU/ml in the cecum after 50 days. This study demonstrates that MBS can be used as a prebiotic candidate, offering the potential to produce optimal probiotics in the intestines.

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