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In Vitro Modulation of Fecal Microflora Growth Using Fermented "Pisang Mas" Banana and Red Guava Juices

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Abstract

Probiotic drink like yoghurt from dairy milk is one of the most popular functional food. However, some people are not able to consume dairy milk due to lactose intolerance. Thus, a substitute substrate such as fruit juice, is needed. It refers to prebiotic compounds that are found in some of fruits like banana and guava. The aim of this research was to determine the effect of fermented banana and guava juices on the viability of fecal microflora. The juices were made from banana or red guava fermented by *Lactobacillus casei* (15% v/v). In vitro test was conducted using indigenous human fecal bacteria (1% w/v). The viability of enteropathogens, lactic acid bacteria (LAB), and total microbes was determined by enumeration as well as the prebiotic index of the fermented juices. The *in vitro* test results showed that both fermented juices could elevate the total microbes and LAB as compared to control sample. The viability of the total microbes and LAB increased by 2.16 log CFU/mL and 2.90 log CFU/mL for fermented banana juice (FBJ), respectively. The same trend was also observed in fermented guava juice (FGJ) with an increase by 1.92 log CFU/mL for total microbes and 2.99 log CFU/mL for LAB. Interestingly, both fermented juice could decrease the population of most enteropathogens compared to control sample. FBJ could decrease the population of *E. coli, Klebsiella* sp. and *Salmonella* as low as 3.78, 3.32, and 1.37 log CFU/mL respectively. Meanwhile, FGJ could drop the number of *E. coli* (1.44 log CFU/mL), and *Klebsiella* sp. (1.29 log CFU/mL). Moreover, the prebiotic index for FBJ and FGJ were 2.57 and 2.16. In conclusion, both FBJ and FGJ were potential substrate for probiotic drink and had good effect for fecal microflora health.

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Introduction

High public awareness of benefit from functional food make the consumption of probiotic drink growing every year. The annual growth rate of probiotic global retail market between 2017 and 2022 is 5.8%.[1] Probiotics are defined as living microorganisms that can provide health benefits for the host. However, a product is considered as probiotic unless it reaches the minimum LAB viability of 6 log CFU/ml.[2,3] Usually, the carrier substrate of probiotic drink is milk which is fermented by lactic acid bacteria (LAB) such as *Lactobaccillus acidophilus, L. plantarum, L. casei, L. paracasei, L. delbrueckii, and L. reuteri*.[4,5,6,7,8] Compared with other kind of LAB, *L. casei* Shirota strain could produce the most acid than other LAB in the skim milk, MRSB (De Mann Rogosa Sharpe Broth) medium, and Jerusalem artichoke medium.[4,9]

Lactose is a carbohydrate that can be found in milk. It is used as the substrate for the fermentation of milk by lactic acid bacteria and is converted to lactic acid.[10,11] However, some people cannot consume milk due to lactose intolerance.[12] So to accommodate people with lactose intolerance, a substitute substrate without lactose is needed.

Fruit juice is a drink based on fruits which can be formulated with or without any additional ingredients.[13] Nowadays, fruit juice was used to produce probiotic drink. Some of the researches done utilized tomato, fig, pomegranate, blueberries and carrots.[5,6,7,8] *L. acidophilus, L. plantarum, and L. casei* were used to ferment tomato juice and increased the acidity up to 0.26% during 24 h.[6] On the other hand, *L. delbrueckii* could increase more acidity of fig juice than *L. plantarum* and *L. casei*.[7]

“Pisang mas” banana and red guava are tropical fruits commodity. These fruits contain compounds which can be potentially prebiotic in nature.[3,17,18,19,20] Prebiotics are nondigestible food ingredients which cannot be digested, but can stimulate the growth of probiotics in the colon such as inulin and oligofructose.[2,14,15,16] Banana contains 1% inulin and FOS (fructo oligosaccharides), while red guava contain of 4.99% dietary fiber including non-starch polysaccharides (NSP) group like pectin, cellulose, and hemicellulose. The amount of soluble fiber in red guava was 1.27% and the insoluble fiber was 3.72%.[3,17,18,19,20] Oligosaccharides are metabolized by lactic acid bacteria through glycolytic pathway (Embden-Meyerhof pathway) to produce lactic acid as the main end product.[21] In effect, pH in the colon decreases causing an unconducive growth environment for enteropathogens.[16] *In vitro* test of probiotic or prebiotic generally need animal colon surgery for knowing the profile of the microflora in the digestive system. An alternative of colon surgery is using fecal which is the final product of the digestive system. Fecal inoculation had been proven to be used for *in vitro* testing to represent colon conditions where probiotic and enteropathogens growth in same place.[15,22] The aim of this study was to determine the effect of fermented juice on the population of fecal microflora. In the future, fermented “pisang mas” banana and red guava juices can be applied as alternative substrate of probiotic drink.

Materials and Methods

Juice Production

Juices made from ripe banana (*Musa acuminata*) cultivar Mas and red guava (*Psidium guajava*) that were purchased from the Jember local market. Ripe banana was about 6–7 of ripeness level which had yellow to yellow flecked with brown peel.[23] Ripe red guava was harvested 2–3 months after fruition and had yellow peel.[24] The fruit juices were prepared using 1:4 ratio (fruit flesh:water), filtered using cheesecloth and pasteurized at 85 °C for 3 minutes.[25]

Starter Preparation

Lactobacillus casei was obtained from Nurhayati's culture collection isolated from fermented milk. Freeze-dried *L. casei* culture were activated with 2–3 transfer in De Mann Rogosa Sharpe Broth medium at 37 °C for 24 h.[6]

Fermentation Process of Fruit Juice

Production of fermented juice was start with inoculation starter *L. casei* 15% (v/v) in to 100 mL pasteurized juice. Incubation was carried out at 37 °C for 24 h in anaerobic condition with sealed glass jars.[6] The fermentation was finish after the juice had mild acidity taste with pH <4.5 and the LAB population reached more than 6 log CFU/mL.[2,3]

Chemical Analysis

pH value

The pH of the formulated juices from banana and guava was measured before and after fermentation during 24 h using Horiba pH meter F-51-HR4659.

Total Titratable Acidity

Total titratable acidity (TTA) represents the amount of organic acid like lactic acid in the sample. The analysis was carried out by adding 2 to 3 drops of phenolphthalein indicator to 10 mL of juice sample. The sample was titrated with 0.1N NaOH until the sample reached endpoint where its color becomes pink.26 The %TTA was computed using the following equation:

% TTA=(mL NaOH x N NaoH x 90 (lactic acid molecular weight) x dilution factor)/(mL sample x 1,000) x 100%

Enumeration of Microorganisms

Microbial evaluation was conducted after production of fermentated juice to know the viability of total microbe, LAB, and enteropathogens in juice. *In vitro* test using fecal determined the juice effect for the fecal microflora growth. The fecal was obtained from healthy human without antibiotic consumption for 3 months. Fecal solution made by mixing 10% (w/v) of fecal and sterile aquades with vortex. In vitro was done by adding 10% (v/v) fecal solution in each juice sample. The control was using fecal without juice addition. The sample were incubated anaerobically at 37 °C for 24 h.[16,27] The viability of three pathogenic microorganisms (*E. coli, Klebsiella sp.,* and *Salmonella*) that can usually be found in fecal deposits was determined using pour plate method in chromogenic agar medium, i.e. Salmonella Chromogenic Agar (SCA-Himedia M1078, India) and Hektoen Enteric Agar (HEA-Himedia GM467, India). Medium for probiotics (LAB) and total microbes were using De Mann Rogosa Sharpe Agar (MRSA-Merck 1.10660, Germany) and Plate Count Agar (PCA-Merck 1.05463, Germany), respectively. The dilution for plating was until 10-4 for enteropathogens and 10-6 for probiotics and total microbes. The viability of the microorganisms was calculated using the formula in Bacteriological Analytical Manual (BAM).[22,28]

$$N = \sum C / [1 x n_1 + 0.1 x n_2] x d$$

N	= number of colonies
$\sum C$	= total number of counted colonies
n_1	= number of plates in dilution 1
n_2	= number of plates in dilution 2
d	= dilution rate

Prebiotic Index (PI) Calculation

The prebiotic index was calculated based on the logarithmic number of probiotic and enteropathogens (pathogenic bacteria) growth on the total number of microbes.[16,22,29,30]

$$\mathrm{IP} = \frac{(\log_{10}\text{probiotic})_{t_x - t_0} - (\log_{10}\text{pathogenic bacteria})_{t_x - t_0}}{(\log_{10}\text{total microbial})_{t_x - t_0}}$$

t_x = time at the end of the treatment
t_0 = time at the end of the control

Statistical Analysis

Experimental design that was used was completely random design (CRD). The analysis was carried out in triplicate. The datas were presented as the mean value and error bars with standar deviation (SD) using Microsoft Office Excel 2007. Data analysis used ANOVA for mean differences with significance level 5% (P<0.05). Advanced analysis data used Least Significance Difference (LSD).

Results and Discussion

Chemical Properties of Fermented Juices

During fermentation, the pH value decreased of 1.21 in banana juice and 0.96 in guava juice, but the titratable acidity of both sample increased of 0.21% for banana juice and 0.18% for guava juice (Table 1). The reduction in pH was caused by organic acids such as lactic acid produced by LAB during fermentation as a result of metabolism through glycolytic pathway (Embden-Meyerhof pathway).[21,31] This was consistent with the decreasing of pH value 1.75 and increasing titratable acidity 0.03% in 24 hours fermentation of soymilk fermented by *L. casei* Shirota.[32] Decreasing pH in fermented banana juice was higher than fermented guava juice. It could be caused of the higher activity of *L. casei* in fermented banana juice so it produced more acid and made a big reduction of pH value. Higher activity of lactic acid bacteria could be related with inulin contain in banana juice. 5% (w/v) inulin addition in MRS medium had been proven increased the population of *Lactobacillus* sp. 0.24–1.07 log CFU/mL.[33]

Enumeration of Microbes on Fermented Juice

Total microbe and LAB viability of FBJ was higher than FGJ although it did not have significant differences (Table 2). Equivalent reason with chemical properties, inulin had been proven increased the population of *Lactobacillus* sp.[33]

Enteropathogens was not detected in both of juice. It meant that both of fermented juice were safe for consumption.

In Vitro Effect of Fermented Juice on the Total Fecal Microbial Count

Total fecal microbial count includes lactic acid bacteria (probiotics) and enteropathogens. The increasing of total microbes from *in vitro* test was the result of the comparison with the control or fecal without juice addition (Figure 1). Fermented banana juice (FBJ) and fermented guava juice (FGJ) increased total microbes by 2.16 log CFU/mL and 1.92 log CFU/mL, respectively. Banana juice (BJ) and guava juice (GJ) also increased the total microbes by 1.35 log CFU/mL and 1.41 log CFU/mL respectively.

Fermented juice increased the number of total microbes during fermentation as the increasing of LAB up to 2.3–3.9 x 10^8 CFU/mL in pomegranate juice.[5,6] Total microbes fermented juices were higher than unfermented juices. It could caused by L. casei starter that had good growth in fermented juices, so the LAB population of fermented juices were higher than unfermented juices like Figure 2. The higher number of LAB was equivalent with the higher number of total microbes. Unfermented juices

Table 1: pH value and acidity of fermented and unfermented juices

Sample	pH value	Acidity (%)
Banana juice (BJ)	5.08 ± 0.01^d	$0.13 \pm 0{,}01^a$
Fermented banana juice (FBJ)	3.87 ± 0.01^b	0.34 ± 0.04^b
Guava juice (GJ)	4.51 ± 0.02^c	0.15 ± 0.05^a
Fermented guava juice (FGJ)	3.55 ± 0.01^a	0.33 ± 0.02^b

Table 2. Microbe viability of fermented and unfermented juices

Kind of juice	Population of microbe ($\log_{10}$ CFU/mL)		
	Total microbe	LAB	Enteropathogens
Banana juice (BJ)	4.94 ± 0.03^a	nd	nd
Fermented banana juice (FBJ)	8.25 ± 0.12^b	7.53 ± 0.63^b	nd
Guava juice (GJ)	5.25 ± 0.57^a	1.29 ± 1.22^a	nd
Fermented guava juice (FGJ)	7.76 ± 0.16^b	7.38 ± 0.26^b	nd

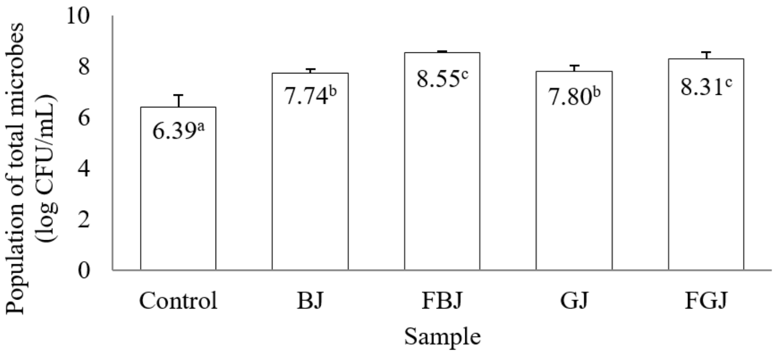

Fig.1: The population of total microbes respectively in control, BJ (banana juice), FBJ (fermented banana juice), GJ (guava juice), and FGJ (fermented guava juice)

could increase total of fecal microbe too because they already contained prebiotic. The increasing of microbes during fermentation indicated that microbes could use the contents of fruit juice like inulin, FOS, and non-starch polysaccharides (NSP) as carbon source. It showed that fruit juice could be used as a carrier substrate for probiotic drinks.6

In Vitro Effect of Fermented Juice on Fecal Lactic Acid Bacteria (LAB)

LAB is a group of non-pathogenic bacteria. It safe to be consumed because it known as Generally Recognize as Safe (GRAS).[4] The increasing of probiotics in BJ, FBJ, GJ, and FGJ respectively were 2.06, 2.90, 2.16, and 2.99 log CFU/mL as compared to control sample (Figure 2).

The increasing of LAB population after incubation *in vitro* test with fermented juice was higher than unfermented juice because of the fermentation process in juice production. Fermentation process gave time for the starter to grow up so the *L. casei* population in fermented juice would be higher. Unfermented juice treatments could increase the LAB population too because banana and red guava fruits were known to contains prebiotics which could stimulate the growth of probiotics.[14,16] Banana contains inulin and FOS, while red guava contains dietary fiber including non-starch polysaccharides (NSP) group like pectin, cellulose, and hemicellulose.[3,17,18,19,20]

In Vitro Effect of Fermented Juice on Fecal Enteropathogens

The depression of enteropathogens population like *E. coli, Klebsiella* sp, and *Salmonella* from *in vitro* test as compared to control sample can be seen in Figure 3. Fermented banana juice could reduce the population of *E. coli, Klebsiella* sp, and *Salmonella* by 3.78, 3.32, and 1.37 log CFU/mL, while fermented guava juice could reduce the population of *E. coli* and *Klebsiella* sp by 1.44 and 1.3 log CFU/mL. The diminish populations of *E. coli, Klebsiella* sp, and *Salmonella* in banana and guava juice were 0.37 and 1.96 log CFU/mL, 0.93 and 1.09 log CFU/mL, 0.43 and 0.43 log CFU/mL, respectively.

Unfermented juices could decrease fecal enteropathogens because they contained prebiotic that could stimulate the growth of fecal LAB. Generally, the depression of enteropathogens by fermented juice was higher than unfermented juice

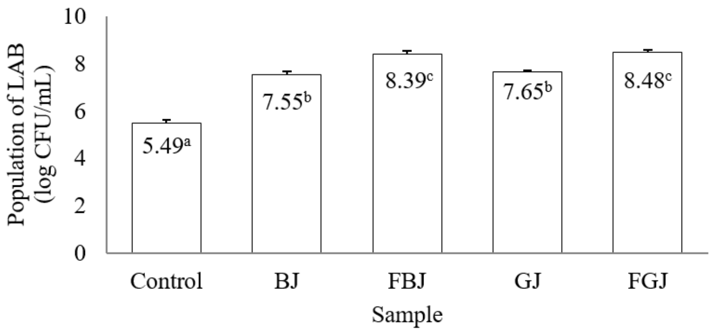

Fig. 2: The population of probiotics in control, BJ (banana juice), FBJ (fermented banana juice), GJ (guava juice), and FGJ (fermented guava juice)

Table 3: Prebiotic index of BJ (banana juice), FBJ (fermented banana juice), GJ (guava juice), and FGJ (fermented guava juice)

PI for	BJ	FBJ	GJ	FGJ
Escherichia coli	2.02 ± 0.85	3.24 ± 0.96	2.98 ± 0.57	2.50 ± 0.81
Klebsiella sp	2.65 ± 1.55	3.03 ± 1.00	2.41 ± 0.77	2.48 ± 1.03
Salmonella sp	2.15 ± 1.08	2.03 ± 0,51	1.90 ± 0.45	1.55 ± 0.90
Enteropathogens	2.12 ± 0.98	2.57 ± 0.41	2.52 ± 0.52	2.16 ± 0.91

in banana commodity. This could be caused by the higher number of LAB in FBJ than BJ. LAB would produce short chain fatty acid (SCFA) such as lactic, acetic and butyric acids that increased acidity and reduced the pH value of medium environment, while enteropathogens could not tolerate high number of acid.[16] Higher LAB population meant higher number of acid production that could make more unconducive environment for the enteropathogens. Probiotic (LAB) also produced antibiotic like acidophiline, bacitracin, and lactacin that could decrease enteropathogens population.[34]

Prebiotic Index (PI) of Fermented Juice

Prebiotic index represent the relationship between population of probiotic, enteropathogens, and total microbe.[35] Higher value of PI indicates higher number of increasing probiotic population or decreasing enteropathogens population.

The differences PI value for each enteropathogens (Table 3) showed that different bacteria had different response to a product because of their own characteristic. The prebiotic index (PI) of fermented banana juice (2.57) was higher than fermented guava juice (2.16). This was according to Figure 3 that showed the depression of enteropathogens from FBJ was higher than FGJ. It could cause by inulin in FBJ that was more effective to stimulate the growth and activity of LAB.[33] ANOVA test result showed that all of the sample had no differences. This indicated that both fermented and unfermented juice could increase the population of probiotics and reduce the population of enteropathogens.

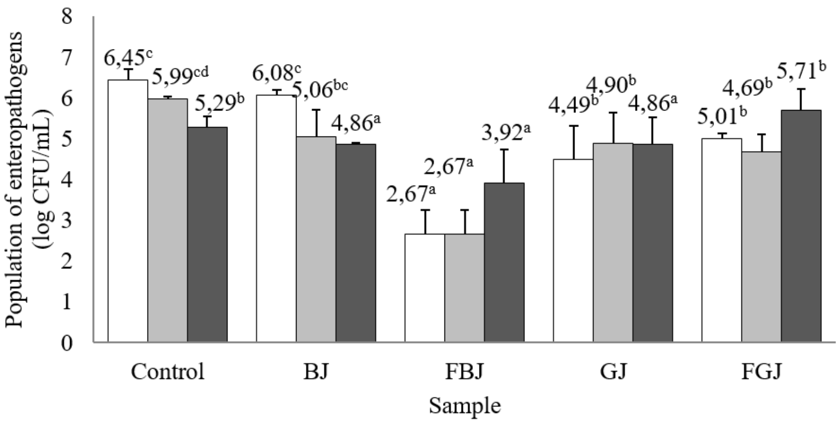

Fig.3: The population of enteropathogens ( ) *E. coli*, ( ) *Klebsiella* sp, ( ) *Salmonella* in control, BJ (banana juice), FBJ (fermented banana juice), GJ (guava juice) and FGJ (fermented guava juice)

Conclusion

During fermentation process in banana and guava juice, there were decreasing of pH value 0.96–1.21 and increasing of titratable acidity 0.18%–0.21%. Both fermented and unfermented banana-guava juices could increase LAB population of 2.06–2.99 log CFU/mL and decrease enteropathogens population of 0.37–3.78 log CFU/mL in human fecal as compared to control sample. The change of microbe population in femented juice was higher than unfermented juice. PI of FBJ and FGJ were 2.57 and 2.16, respectively. This was indicate that fermented juice could be substrate of probiotic drink as functional food.

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Conflict of Interest

The author(s) do not have any conflict of interest.

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