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In Vitro and *In Vivo* Prebiotic Activities of Purified Oligosaccharides Derived from Various Local Bananas (*Musa* sp.): Tanduk, Uli, Raja Sereh, and Cavendish

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Banana is a good source of prebiotic, and in Indonesia it is consumed as staple food. The aims of this research were to evaluate the activity of purified oligosaccharides (POS) as prebiotic from various different local bananas (*Musa* sp.): *Tanduk* (T), *Uli* (U), *Raja Sereh* (RS), and Cavendish (C); and to investigate their capacity in promoting the growth of *Lactobacillus* sp., *in vivo. In vitro* investigation including purification of oligosaccharides from various different local bananas were by 80% ethanol extraction. Subsequently, absolute ethanol was reconstituted before precipitation/centrifugation for glucose removal. Water was also removed by freeze drying. POS from the four bananas were analyzed by Thin Layer Chromatography (TLC). Prebiotic activity of POS was investigated by measurement of Prebiotic Activity Score (PAS). *In vivo* investigation was conducted as followed, Balb/c mice were grouped into 6 groups with different prebiotics supplementation: negative control (4 mice, standard feed), positive control (6 mice, 15 mg of inulin g^{-1} day^{-1}), and samples (5 mice, 150 mg of T, U, RS, or C banana g^{-1} day^{-1}) for 40 days. Following 40 days after treatment, fecal viable counts of *Lactobacillus* sp. and *Enterobacteriaceae* of Balb/c mice was measured (CFU g^{-1}) and analysed. PAS value revealed a positive correlation between the oligosaccharides from bananas and *Lactobacillus paracasei*, with PAS value for T (0.05), RS (0.15), U (0.33), and C (0.77). Overall data suggest that fecal viable counts of *Lactobacillus* sp. increased after 25 days administration of U, RS, and C banana when compared to controls. Contrastingly, the fecal viable counts of *Enterobacteriaceae* decreased after 40 days administration of U, RS, and C banana compared to the control. Different types of local bananas demonstrate diverse prebiotic activities, U and C promote *Lactobacillus* sp. growth and reduce *Enterobacteriaceae* count. PAS value of U and C suggest potential prebiotic activity, whereas T and RS do not.

Key words: banana, lactic acid bacteria, oligosaccharides, PAS, prebiotics

Pisang merupakan sumber prebiotik, dan di Indonesia pisang dikonsumsi sebagai makanan pokok. Tujuan penelitian ini untuk mengevaluasi aktivitas dari oligosakarida yang sudah dipurifikasi (POS) sebagai prebiotik dari berbagai macam pisang lokal (*Musa* sp.): Tanduk (T), Uli (U), Raja Sereh (RS), dan Cavendish (C); dan untuk mengetahui kapasitas pisang dalam memicu pertumbuhan *Lactobacillus* sp., *in vivo.* Percobaan *In vitro* dilakukan sebagai berikut: Oligosakarida dari berbagai macam pisang lokal yang berbeda dipurifikasi dengan 80% etanol. Selanjutnya, etanol absolut ditambahkan sebelum presipitasi/sentrifugasi untuk menghilangkan glukosa. Air dihilangkan dengan metode *freeze drying.* POS dari empat pisang dianalisis dengan *Thin Layer Chromatography* (TLC). Aktivitas prebiotik POS diukur dari hasil *Prebiotic Activity Score* (PAS). Sedangkan percobaan *In vivo* sebagai berikut: Mencit Balb/c dibagi menjadi 6 kelompok, dengan pemberian suplemen prebiotik yang berbeda: kontrol negatif (4 mencit, pakan biasa), kontrol positif (6 mencit, 15 mg inulin/g/hari), dan sampel (5 mencit, 150 mg pisang T, U, RS, dan C g^{-1} $hari^{-1}$) selama 40 hari. Setelah 40 hari perlakuan, dihitung jumlah *Lactobacillus* sp. dan *Enterobacteriaceae* dari sampel feses mencit dalam satuan CFU g^{-1} dan dianalisa. Nilai PAS menunjukkan korelasi positif antara oligosakarida dari pisang dan *Lactobacillus paracasei*, dengan nilai PAS T (0,05), RS (0,15), U (0,33), dan C (0,77). Secara keseluruhan data dari perhitungan sampel feses untuk *Lactobacillus* sp. meningkat setelah 25 hari pemberian pisang U, RS, dan C dibandingkan dengan kontrol. Sebaliknya, hasil perhitungan sampel feses untuk *Enterobacteriaceae* menurun setelah 40 hari pemberian pisang U, RS, dan C dibandingan dengan kontrol. Perbedaan jenis pisang lokal menunjukkan aktivitas prebiotik yang berbeda, U dan C memicu pertumbuhan *Lactobacillus* sp. dan menurunkan perhitungan *Enterobacteriaceae*. Berdasarkan nilai PAS dari U dan C diduga berpotensi memiliki aktivitas prebiotik, dimana T dan RS tidak.

Kata kunci: bakteri asam laktat, oligosakarida, PAS, pisang, prebiotik

Nutraceuticals and functional foods have become important tools for consumers to manage their health and well-being. Many food components, especially food oligosaccharides and polysaccharides (including dietary fiber), have been shown to exhibit prebiotic activity. However, not all dietary carbohydrates are prebiotics. Clear criteria of prebiotics have been established. These criteria are 1) resistance to gastric acidity, degree of hydrolysis by mammalian enzymes, and degree of gastrointestinal absorption; 2) degree of fermentation by intestinal microflora, and 3) selective stimulation on the growth and/or activity of the intestinal bacteria that contribute to health and well-

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being of host organism (Thammarutwasik *et al*. 2009; Gibson *et al*. 2004). Prebiotic was first defined by Roberfroid and Gibson (1995), indigestible food ingredients that favorably affect the host by selectively promote growth and/or activity of beneficial bacteria in the colon, and thus improving the host health.

Indigestible oligosaccharides, particularly fructo-oligosaccharides, are prebiotics. Intake of prebiotics can significantly modulate the colonic microbiota by increasing the number and activities of specific bacteria, such as *Lactobacilli* and *Bifidobacteria* and inhibit the growth or activities of pathogenic bacteria such as *Enterobacteriaceae* causing change in the microbiome. Prebiotics are known to indirectly promote physiological support of the gastrointestinal tract's. (Gibson and Wang 1994; Roberfroid 2007).

Acccording to Kahlon and Smith (2007) and Gibson and Wang (1994), banana is a source of prebiotics. Indonesia is the leading banana producer in Asia, producing more than 50 % of Asia's supply of bananas. Indonesia has approximately 200 different kinds of local bananas (Prabawati *et al*. 2009). This variety possesses the potential for development into prebiotics that can contribute to human health. However, studies of prebiotics in local bananas have been limited (Kahlon and Smith 2007; Gibson and Wang 1994b).

Herein, prebiotic activity of purified oligosaccharides from various local bananas: Tanduk, Uli, Raja Sereh, and Cavendish were studied. Tests were conducted to evaluate their capability to promote the growth of *Lactobacillus paracasei* through PAS value, which is a method to determine the ability of a given prebiotics to better promote the growth of probiotic microorganism relatively better than to other pathogenic microorganisms (Huebner *et al*. 2007); and *in vivo* study were carried out on Balb/c mice by feeding them with different types of local bananas. Prebiotic activities were determined through their ability to promote the growth of *Lactobacillus* sp. by measuring increase of the bacterial count in feces.

MATERIAL AND METHODS

Samples and Subjects. Approximately 1.5 - 2 kg local bananas: Tanduk (T), Uli (U), Raja Sereh (RS), and Cavendish (C) were used for oligosaccharides extraction, purification, and *in vitro* analysis.

Approximately 3 - 4 kg of each fresh local bananas (T, U, RS, and C), and a total of 30 male, twenty weeks old Balb/c mice were used in the present *in vivo* study. All animal study were performed followed the Guidelines for The Housing of Mice in Scientific Institutions, Animal Welfare Unit, New South Wales Department of Industry (Fawcett 2012).

Oligosaccharides Extraction and Glucose Removal from Samples. Oligosaccharides extraction was performed according to method previously described by Livingston (1990). Eight hundred forty gram of each type of bananas (T, U, RS, and C) were blended with 1.1 L of 80% ethanol. Blended banana ethanol mixtures were filtered with a cotton filter to remove the coarse particles. All collected filtrate were then heated at 70 °C for 15 min to inactivate potential oligosaccharide degrading enzymes, such as invertase. The resulting thick banana pulps were blended twice for about 5 min with 100 mL of distilled water and then agitated at 200 rpm at room temperature for 1 h, to homogenized. All extracts were then centrifuge at 900 g, for 20 min at 10 °C. Supernatant were then collected and evaporated to remove residual ethanol. The thick extracts were mixed with absolute ethanol (1:1 v/v) and stored at -2 °C for 24 h. The extracts were further centrifuged at 900 g for 15 min at room temperature. Glucose fraction in the supernatant was discarded. Absolute ethanol was re-added into the remaining pellet, mixed and centrifuged at 900 g for 15 min at room temperature and the supernatant were decanted as above. The step was repeated for thirty times until glucose was not detected anymore through thin layer chromatography (TLC) with n-butanol/1-propanol/acetic acid/water (3:1:1:1) as eluent. After glucose removal, a thick milky suspension containing pure oligosaccharides was then subjected to freeze drying. Dried oligosaccharide samples were then weighed. Pure oligosaccharides were verified by TLC with 90% formic acid as eluent.

Prebiotic Activity Score (PAS) from Purified Banana Oligosaccharides. PAS was performed according to the procedure as described by Huebner *et al.* (2007). PAS is defined as follow:

$$PAS = \left(\frac{P24 - P0}{G24 - G0}\right) - \left(\frac{P24 - P0}{G24 - G0}\right)$$

Whereby, p is defined as probiotics (*Lactobacillus paracasei*/Lp), e is defined as enteric bacteria (*E. coli*/Ec), P_{24} is total plate count of Lp culture, and Ec culture in prebiotic added medium after 24 h incubation, respectively, P_0 is total plate count of Lp culture, and Ec culture in prebiotic added medium before 24 hours incubation, respectively, G_{24} is total plate count of Lp culture, and Ec culture in glucose added medium after 24 h incubation, respectively, G_0 is total plate count of

Lp culture, and Ec culture in glucose added medium before 24 h incubation, respectively.

In the formula above, the probiotic *Lactobacillus paracasei* (Lp) was cultured in 3 mL modified de ManRogosa Sharpe broth without carbon source, agitated at 100 rpm at 37 °C, microaerophilic for 24 h. No prebiotic was added to negative control. 1% of inulin was added to positive control. 1% of purified oligosaccharide from banana T, U, RS, or C was added as tested samples.

Escherichia coli (Ec) was cultured in 3 mL M9 minimal medium broth without carbon source, agitated at 100 rpm at 37 °C for 24 h to serve as basal media colony plate count. No prebiotic was added to negative control. 1 % of inulin was added to positive control. 1% of purified oligosaccharide from banana T, U, RS, or C was added as tested samples. Additional control plate containing 1 % glucose on basal media was also prepared.

The above PAS plate count was performed as follow. A 50 µL of serial dilluted samples were spread on MRS agar for *L. paracasei* count, and on MacConkey agar for *E. coli* count. Serial dilution was performed to obtain countable CFU plate count (30 - 300 CFU plate^{-1}). All plates were incubated at 37 °C for 24 h under microaerophilic condition for *L. paracasei* count and aerobic condition for *E. coli* count. Colony count in each plate was expressed in log CFU mL^{-1}.

Quantitative Analysis of *Lactobacillus* sp. and *Enterobacteriaceae* in Mice Feces Treated with Bananas. Balb/c mice were grouped into negative control (4 mice, standard feed), positive control (6 mice, 15 mg of inulin g^{-1} day^{-1}), and samples (5 mice, 150 mg of T, U, RS, or C banana g^{-1} day^{-1}). All supplemented prebiotics were measured in dry weight.

Fecal samples were collected from each mice at day 0, 17, 20, 25, and 40. For bacteria count, samples were diluted in physiological salt solution (1:9 v/v) (Hartemink and Rombouts 1999), and then 50 µL of each diluted feces was spread on Rogosa agar for *Lactobacillus* sp. and MacConkey agar for *Enterobacteriaceae,* at 37 °C for 24 - 48 h (Adami and Cavazzoni 1996). Colony count in each plate (CFU mL^{-1}) was converted into log CFU g^{-1} sample. The difference percentage log CFU g^{-1} relative to day 0 (D0) was calculated.

RESULTS

Purified Oligosaccharides (POS) from Banana. The purification of banana from glucose after serial ethanol suspension and centrifugation produce excellent result (Fig 1). The inclusion of glucose in oligosaccharide samples will interfere PAS value because glucose will be used as carbon source by both bacteria, *L. paracasei* and *E. coli* (Date *et al*. 2014; Kunova 2011). The appearance of extracted POS from each bananas were different in color and texture (Fig 2).

***In Vitro* Analysis Prebiotic Activity.** To ensure glucose was completely eliminated for the TLC analyses, oligosaccharides purification was conducted. Each banana POS was further used to determine PAS value, as shown in (Tabel 1) in log CFU mL^{-1}.

Cavendish (C) banana display highest PAS value of 0.77, followed by Uli (U) banana sample with PAS value of 0.33, Raja Sereh (RS) with PAS value of 0.15 and lastly Tanduk (T) with PAS value of 0.15. The PAS value of Uli (U) is comparable to inulin PAS value (0.33). Negative control shows a negative PAS value (-0.11).

Effect of Feeding Bananas on *Lactobacillus* sp. Growth in Feces. Balb/c mice were used to study the effect of banana feeding on *Lactobacillus* sp. growth in gastrointestinal tract. Treated mice were given T, U, RS, or C at 150 mg of banana g^{-1} day^{-1} for 40 d. As positive control inulin was given to animal feed at 15 mg of inulin g^{-1} day^{-1} for 40 d. Feces of mice were collected as described in the methods.

Uli (U), Raja Sereh (RS), and Cavendish (C) banana fed on mice result in a higher of *Lactobacillus* sp. count than negative control. In contrast inulin gave an inconsistant result throughout time fed. Tanduk (T) banana did not support the growth of *Lactobacillus* sp. but *Enterobacteriaceae.* Other nutrient factors might play a part in promoting *Lactobacillus* sp. growth. Fig 4 showed that T and RS banana promote *Enterobacteriaceae* growth, whereas U and C, and inulin as positive control did not promote growth. Result displayed on day 40 reveal more disperse result on the effect of different banana sample to the percentage log CFU g^{-1} relative to D0. PAS value of U and C indicates that these bananas promoted the growth of *Lactobacillus* sp. and reduced the growth of *Enterobacteriaceae* during *in vivo* study.

DISCUSSION

Purified oligosaccharides from each different banana yield different mass after purification. According to Livingston (1990), centrifugation and precipitation process at 900 g should successfully eliminate monosaccharide (glucose) and other simple

Fig 1 TLC result showing glucose elimination after repeated extraction. Glucose detection was used n butanol: propanol: acetic acid: water with ratio 3: 1: 1: 1 as eluent. Then, was dried and added with visualization solution and was dried again in oven until spots appeared. A: inulin standard (0,02 mg); B: glucose standard (0.02 mg); C: extract from T (10 µL); D: extract from U (10 µL); E: extract from RS (10 µL); and F: extract from C (10 µL).

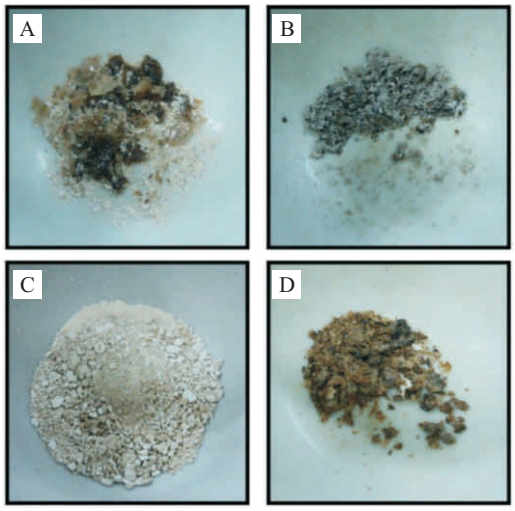

Fig 2 Purified oligosaccharides (POS) from banana samples. Purifified oligosaccharides after freeze drying A: oligosaccharides from T; B: oligosaccharides from U, C: oligosaccharides from RS, and D: oligosaccharidess from C.

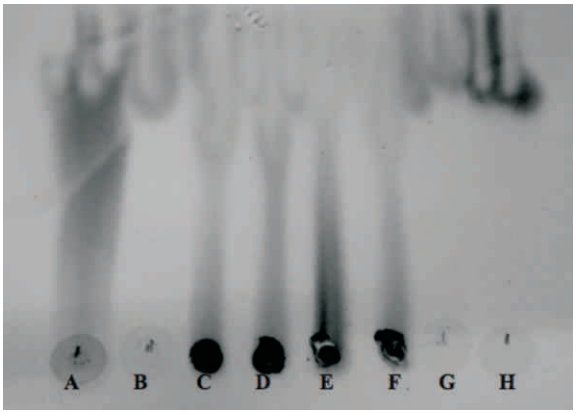

Fig 3 TLC result showing oligosaccharides detection after glucose elimination. Oligosaccharides detection from each various local bananas. Oligosaccharides detection was used 90 % of formic acid as eluent. Then, was dried and added with visualization solution and was dried again in oven until spots appeared. A: inulin standard (0,02 mg); B: glucose standard (0,02 mg); C: T oligosaccharides (20 µL); D: U oligosaccharides (20 µL); E: RS oligosaccharides (20 µL); F: C oligosaccharides (20 µL); G: maltose standard (0.02 mg); and H: raffinose standard (0.02 mg).

Table 1 Average results of PAS value from T, U, RS, and C

No	Samples	I				II				III		IV		V
		CFU mL^{-1} ($\times 10^5$)				log CFU mL^{-1}				Δ log CFU mL^{-1}		RGR		PAS value
		Lp		Ec		Lp		Ec						
		t0	t24	t0	t24	t0	t24	t0	t24	Lp	Ec	Lp	Ec	
1	Inulin	9.59	5058.25	22.49	8669.62	5.98	8.70	6.35	8.94	2.72	2.59	1.40	1.07	0.33
2	Negative control	13.00	128.82	26.18	831.76	6.11	7.11	6.42	7.92	1.00	1.50	0.51	0.62	-0.11
3	Glucose	17.30	1527.57	14.45	3767.04	6.24	8.18	6.16	8.58	1.95	2.42	-	-	-
4	T	89.81	3950.63	58.43	4786.30	6.95	8.60	6.77	8.68	1.64	1.91	0.84	0.79	0.05
5	U	28.71	3908.41	38.90	2766.94	6.46	8.59	6.59	8.44	2.13	1.85	1.10	0.77	0.33
6	RS	78.70	4813.93	57.28	4004.05	6.90	8.68	6.76	8.60	1.79	1.84	0.92	0.76	0.15
7	C	30.48	3265.88	35.48	164.44	6.48	8.51	6.55	7.22	2.03	0.67	1.04	0.28	0.77

This table shows results Average PAS value from Inulin, negative control, T, U, RS, and C. Columns I are CFU mL^{-1} ($x10^5$); Columns II are log from columns I; Columns III are are the difference (or Δlog CFU mL^{-1}) from column II, namely t_{24}-t_0 for Lp and Ec, respectively.Columns IV are relative growth ratio (RGR) obtained from column III, namely Δlog CFU mL^{-1} from Inulin, T, U, RS, and C for Lp and Ec, which divided by glucose Δlog CFU mL^{-1} for Lp and Ec, respectively. The last column V is PAS values which obtained from RGR (columns IV) Lp-Ec.

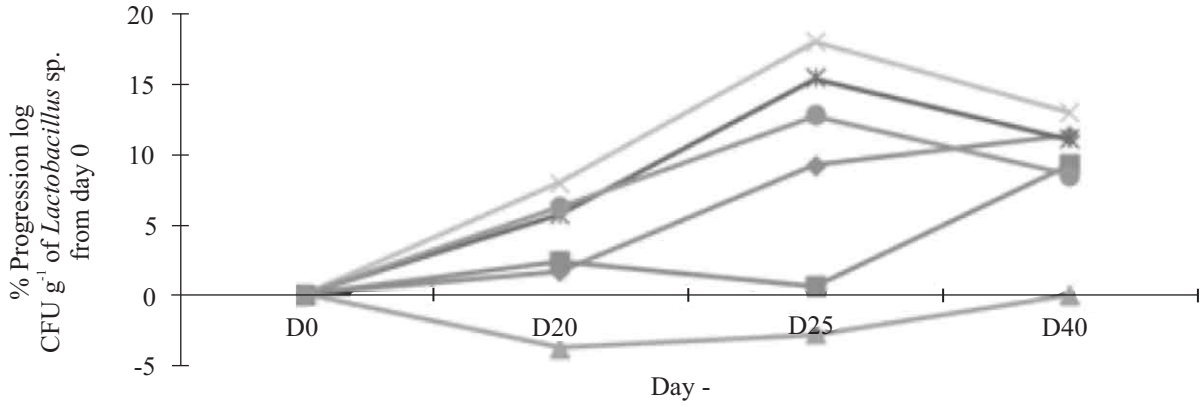

Fig 4 Progression of Log CFU percentage of *Lactobacillus* sp. per gram of fresh feces at day 20 (D20), day 25 (D25), and day 40 (D40) relative to day 0 (D0) in Balb/c mice fed by various local bananas. : negative control, : inulin, : tanduk, : Uli, : raja sereh, and : cavendish.

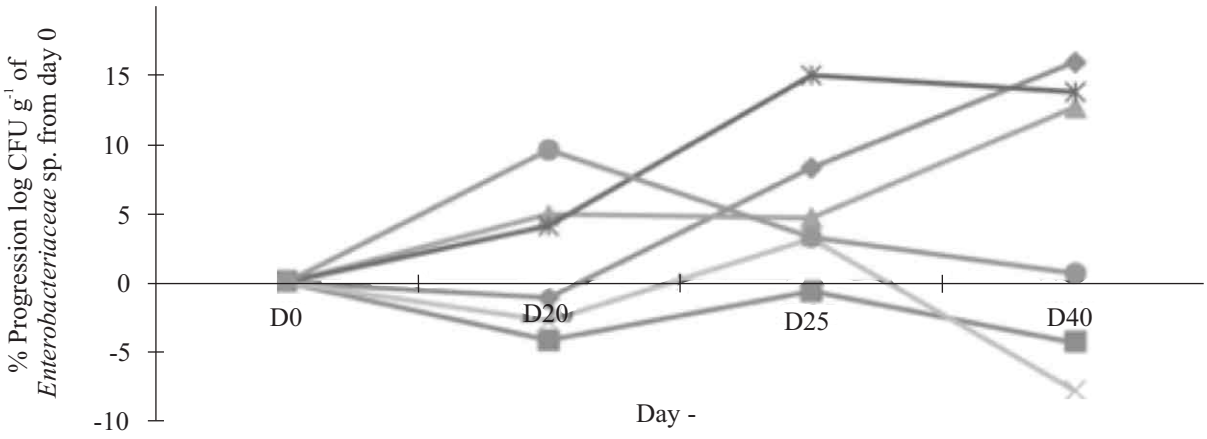

Fig 5 Progression of Log CFU percentage of *Enterobacteriaceae* per gram of fresh feces at day 20 (D20), day 25 (D25), and day 40 (D40) relative to day 0 (D0) in Balb/c mice fed by various local bananas. : negative control, : inulin, : tanduk, : Uli, : raja sereh, and : cavendish.

glucose that have low degree of polymerization range (DP 3 - 5) from the extract which. (Roberfroid 2007; Slavin 2013). Thus, considerable amount of oligosaccharides prebiotics were expected to be lost during purification. This is accountable to the minimum PAS value measurement. Banana POS from T, U, RS, and C were detected on TLC by using 90% of formic acid as eluent (Fig 3) which possesses very high polarity or strong acid. This suggest that POS with high DP might be undetected on the TLC performed (Holmes and O'Brien 1979; Zhang *et al*. 2007). Eluent of 100% acetic acid, and another of 1-butanol: formic acid: aquades, 4: 8: 1, did not produce good result.

Appearances of purified POS from each banana were different. RS purified POS obtained as white color granule or powder. Similar appearance was described by Oliveira *et al* (2011) with agglomerate texture and difficult to ravel due to the strong glycosidic bond between the polisaccharides. T, U, and C purified POS appeared as a mixture of white and dark color granule and powder (Oliveira *et al*. 2011).

The positive values of PAS for inulin, T, U, RS, and C, signifies that prebiotics or purified POS bananas are able to promote the growth of *L. paracasei* relatively better than *E. coli.* According to Rubel *et al*. (2014), the RGR value >1 means that tested samples (T, U, RS, and C), prebiotics/oligosaccharides addition as growth media could support the growth of probiotics such as *Lactobacillus* sp than using glucose as growth media (Rubel *et al.* 2014).

Data reflected in Table 1 showed average results of PAS from *in vitro* analysis prebiotic activity. RGR value of prebiotic inulin and purified POS of U and C are >1, while T and RS are < 1 which indicates that T and RS could not promote *L. paracasei* growth well.

As many types of oligosaccharides are present in fruits and plants, T and RS may have different oligosaccharide content than U and C (Roberfroid 2007). Other species of *Lactobacillus* should be studied to examine its capability to metabolize the oligoaccharides. C has much higher PAS value of 0.77 compare to inulin as control positive 0.33 (Tabel 1) which calls for further investigation.

The PAS values showed that only two purified banana oligosaccharides, U and C, were capable of promoting the growth of *L. paracasei* with RGR >1, while the other two purified banana oligosaccharides T and RS were inconclusive, RGR <1.

In vivo experiment was analyzed by using Balb/c mice to study the effect of banana feeding on *Lactobacillus* sp. growth in gastrointestinal tract. The microbiota of the feces reflects that of the distal colon. Many diseases, such as colon cancer, diverticular disease, ulcerative colitis, and inflammatory bowel disease originates from this organ (MacFarlane *et al*. 1992). Modulation in the microbiota of distal colon, such as higher count of *Lactobacillus* sp. and a lower count of *Enterobacteriaceae*, was able to prevent the onset of those diseases (Roberfroid *et al*. 2010).

CFU of *Lactobacillus* sp. and *Enterobacteriaceae* in the feces were quantified in selective media for minimizing the growth of contaminant microorganisms. *Lactobacillus* sp. was quantified in Rogosa medium which has been proven to be selective against *Lactobacillus* sp. according to Gram staining, catalase and endospora assays. Meanwhile, MacConkey medium was used to quantify *Enterobacteriaceae* and its selectivity against this group of bacteria has been tested with Gram staining method. This study was done to prove the possible positive health effect of fresh bananas on gastrointestinal tract by measuring the increasing number of *Lactobacillus* in feces in comparison to *Enterobacteriaceae*.

In feces of the treated Balb/c mice fed with the same bananas, the results showed that all bananas but T banana were able to promote *Lactobacillus* sp. growth, while T and RS, but not U or C, were able to promote growth *Enterobacteriaceae*. The contradicting result *in vivo* and *in vitro* could arise from different sample treatment. Glucose is not omitted in *in vivo* assay which could result in glucose interference towards colon bacteria yield. It is recommended that glucose is removed from all samples before administration to animal model. However, attention of the limitation on technique, that result in significant sample loss, used for purification of POS from banana sample used in this paper need to be consider.

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