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Growth of *Lactobacillus paracasei* SNP-2 in Peanut Milk and Its Survival in Fermented Peanut Milk Drink During Storage

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Abstract

Fermentation of peanut milk added with various sucrose concentrations using candidate probiotic strain of *Lactobacillus paracasei* SNP-2 was investigated, and the lactic acid bacteria survival during storage of the fermented peanut milk drinks were also studied. Peanut milk fermentations were carried out at 37°C for 18 h. It was found that peanut milk without addition of sucrose could support the growth of *L. paracasei* SNP-2, but not the production of lactic acid. Fermentation of peanut milk with addition of 2-10% sucrose significantly increased the production of lactic acid. The numbers of lactic acid bacteria showed no marked reduction in the fermented peanut milk drinks during storage at 4°C for 21 days, still sufficiently high to exert beneficial probiotic effects in the host. Fermented peanut milk drink using *L. paracasei* SNP-2 can be used as a non-dairy probiotic product.

***Keywords**:* Lactic acid bacteria, viability, probiotic, fermented peanut milk

Introduction

Probiotic products have become an important choice for consumers, primarily due to the potential health benefits attributed to their consumption. The probiotic beneficial effects on human health are including inhibition of pathogenic microorganism, stimulation of immune system, cholesterol lowering effect, and prevention of gastrointestinal disorder (Hopzapel et al., 1998; Ouwehand and Salminen, 1998).

Lactic acid bacteria are the most important group of microorganisms used in the fermented milk and much of them are considered probiotics (Sieber and Dietz, 1998). With the growing interest of consumers in health-related food, the market for fermented products containing probiotic lactic acid bacteria would have a promising future. Dairy products have been used as vehicle for the probiotic bacteria and marketed in the form of fermented milks, however, there are consumer demand for non-dairy-based probiotic products too (Prado et al., 2001) due to lactose intolerance, cholesterol content, and also demand for vegetarian probiotic products. The application of probiotic cultures in non-dairy products is a great challenge.

Peanut (*Arachis hypogaea* L.) is an important food crop with many health benefits. Peanuts were found to be a major source of edible oil and protein meal, and a good source of antioxidant, such as *p*-coumaric acid, that may be contributing factors to potential health benefits of their consumption (Talcot et al*.,* 2005; Duncan et al., 2006). Peanut milk is the water extract of peanut that is an inexpensive source of protein and calories for human consumption. Just like, it is seen as a low-cost substitute for dairy milk for the developing countries. Being free of cholesterol,

gluten and lactose, peanut milk is also a suitable food for lactose-intolerant consumers, vegetarians and milk-allergy patients. Like fermented soy products, peanut milk fermented with lactic acid bacteria may be suitable as a probiotic carrier to the host.

Several researchers have studied the fermentation of peanut milk by lactic acid bacteria (Beuchat and Nail, 1978; Bucker et al., 1979; Lee et al., 1991; Sunny-Roberts et al., 2004; and Isanga and Zhang, 2009). The growth of lactic acid bacteria in peanut milk depends on a number of factors, such as strain of lactic acid bacteria, availability of nutrition, and fermentation temperature and time. *Lactobacillus paracasei* SNP-2 which was previously identified as *L. Acidophilus* SNP-2 is found to be resistant to bile salt and low pH, and could be used as probiotic agent (Purwandhani and Rahayu, 2004). Consumption of *tape ketan* (fermented glutinous rice) supplemented with *L. acidophilus* SNP-2 improved of fecal-volunteer's microbiota (Rahayu and Purwandhani, 2004). In this study, the effect of addition of various sugar concentrations on the growth of *L. paracasei* SNP-2 and acid production during peanut milk fermentation were tested. The survival of this microorganism on fermented peanut milk drink subsequent storage at refrigerated temperature (4°C) was also evaluated.

Materials and Methods

Microorganism

The starter culture of *L. Paracasei* SNP-2 which was previously identified as *L. acidophilus* SNP-2 was obtained from Food and Nutrition Culture Collection, Center for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia. The culture was stored in a mixture of 10% glycerol and 10% skimmed milk (1:1) at -20°C, and maintained by sub-culturing into sterilized MRS media and stored at 4°C.

Preparation of Peanut Milk

The raw peanuts were purchased from a farmer in Bantul, Yogyakarta, Indonesia. Care was taken to ensure that good quality seeds were selected. Whole peanuts were first washed and soaked in water for seven hours at room temperature. After decanting the soaking water, the peanuts were ground and extracted with 20 times weight of water for 10 min. The resultant slurry was filtered using centrifugal separator with a double-layered cheesecloth to obtain peanut milk. Peanut milk was then heated to 90°C for 15 min. The moisture, ash, fat, proteins, and carbohydrates contents in peanut milk were determined (AOAC, 1990).

Fermentation of Peanut Milk and Storage

Each of peanut milk added with various concentration of sucrose (2-10% w/v) was innoculated with 1% (v/v) of 18 h of the single culture of *L. paracasei* SNP-2 in MRS broth. Fermented peanut milk without addition of sucrose served as the control. Peanut milk containing *L. paracasei* SNP-2 was incubated at 37°C for period of 18 h. The population of the culture, pH and acid content were determined in the initial and end of fermentation periods.

The fermented peanut milk drinks were held at 4°C for 21 days. During storage period, the pH, titratable acidity (expressed as % lactic acid), and viable counts of lactic acid bacteria of the fermented peanut milk drinks were determined.

Analytical Methods

Proximate composition analisis were done on peanut milk. Moisture, ash, fat, and protein, were determined according AOAC method (AOAC, 1990). Titratable acidity was determined by titration with 0.1 N NaOH solutions, and expressed as percent lactic acid (AOAC, 1990). The pH values of the samples were measured using pH meter (ToA/Jenway). MRS agar (Oxoid) with 1% $CaCO_3$ was used for the enumeration of lactic acid bacteria. One milliliter of appropriate serial dilutions of each sample was pour-plated onto MRS agar media. After 48 h of incubation at 37°C, the colonies with the clear zone that appeared on the plates were counted and calculated as CFU/ml.

Statistical Analysis

The mean values and the standard deviations were calculated from the data obtained with triplicate trials. Multiple comparisons of means were carried out by Duncan multiple range test.

Results and Discusion

Peanut Milk Fermentation

The proximate composition of peanut milk is presented in **Table 1**. The moisture content of peanut milk was 96.21% (w/w). The ash, fat, protein and carbohydrate (by different) contents in peanut milk are 0.09%; 1.41%; 1.48%; and 0.81% respectively. In this study, the purpose of fermentation of peanut milk was to produce fermented peanut milk drink so that the total solids were quite low, not to be confused with peanut milk yogurt that have higher solid materials. Previous researchers reported much higher solid materials, protein content of peanut milk reported by Isanga and Zhang (2009) and Bucker et al., (1979) were 3.71% and 2.8% respectively. Other workers also reported lower moisture content of peanut milk i.e., 87.15% (Isanga and Zhang, 2009), and 90.6% (Bucker et al., 1979). These differences were due to different water proportion on peanut milk preparation procedure, which were 1:20, while Isanga and Zhang (2009), and Bucker et al., (1979) used the peanut and water ratio of 1:5 and 1:9 respectively. In general, protein content of yoghurt is about 3.12% (Tamime and Robinson, 1985), therefore protein content of yoghurt drink is approximately 1.6% which is close to 1.41% of protein content of peanut milk.

Table 2 shows the growth of *L. paracasei* SNP-2 in peanut milk with various concentration of added sucrose. The initial populations of lactic acid bacteria in peanut milk were adjusted to about 10^7CFU/ml. After incubation at 37°C for 18 h, the population of lactic acid bacteria reached the levels of 4.8 x 10^8 CFU/ml for peanut milk without addition of sucrose to 1.3 x 10^9 CFU/ml for peanut milk with addition of 10% sucrose. This result showed that *Lactobacillus paracasei* SNP-2 needs sucrose in the peanut milk, and its growth was more rapidly by the present of sucrose. Higher concentration of sucrose resulted in higher level of viable cell counts, however the differences in viable cell count of fermented peanut milk between no added sucrose and 10% added sucrose was less than 1 log cycles. It means that in the case of no added sucrose, *L. paracasei* SNP-2 can utilize carbohydrate in peanut milk for its growth. Sucrose was the major fermentable carbohydrate in the peanut milk (Bucker et al., 1979). Beuchat and Nail (1978) reported that the growth of *L. acidophilus* B-2092 was not significantly affected by the addition of 2% glucose, sucrose or lactose, however, *L. acidophilus* B-1910 was stimulated by glucose and lactose. Wang et al., (2003) reported that during 24 h of fermentation of soymilk by *L. acidophilus* CCRC 14079, sucrose, raffinose, and stachyose contents decreased, and fructose content increased. Capability of lactic acid bacteria to utilize sucrose, the main disaccharide in peanut milk and soymilk varied. *Lactobacillus paracasei* SNP-2 can utilize sucrose in the peanut milk, and its growth was affected by the present of sucrose.

Table 1. Proximate composition of peanut milk

Constituent	% of constituent in peanut milk
Moisture	96.21
Ash	0.09
Protein	1.41
Fat	1.48
Carbohydrate (by different)	0.81

Table 2. The growth of *Lactobacillus paracasei* SNP-2 at 37°C in peanut milk with various concentration of sucrose

Addition of sucrose (%)	Viable cell (cfu/ml)	
	Fermentation time (hours)	
	0	18
0	1.1×10^7	4.8×10^8
2	1.1×10^7	6.0×10^8
4	1.1×10^7	7.3×10^8
6	1.2×10^7	7.9×10^8
8	1.3×10^7	1.0×10^9
10	1.4×10^7	1.3×10^9

The initial pH and titratable acidity of peanut milk were about 6.4 and 0.03%, respectively. Changes in pH and titratable acidity during fermentation of peanut milk inoculated with *L. paracasei* SNP-2 with various concentration of added sucrose are summarized in **Table 3**. In general titrarable acidity increased and the pH decreased after 18 h of fermentation. The lowest acid production was found in the fermented peanut milk without addition of sucrose which was seen that the titratable acidity increased from an initial level of 0.03% to only 0.14% after 18 h, and the final pH was only 4.83. This result indicated that although *L. paracasei* SNP-2 could grow in Peanut milk without added sucrose, but the titratable acidity was low. Sucrose at 0.60% (w/v) was the major fermentable carbohydrate present in peanut milk (Bucker et al., 1979). Although the lactic acid bacteria can utilize sucrose in the peanut milk for

their growth, however, the metabolism activity was very low indicated by the very low acid production. Addition of 2% sucrose significantly increased the acid production and reduced the pH, but further addition of sucrose (4-10%) resulted in relatively similar titratable acidity and pH of the fermented peanut milk. Thus addition of 2% of sucrose is sufficient for the cell growth and acid production during the 18 h lactic acid fermentation of peanut milk.

Table 3. pH and titratable acidity of peanut milk before and after fermentation at 37°C by *L.paracasei* SNP-2 with various concentration of sucrose

Addition of sucrose (%)	Fermentation time (hours)			
	0		18	
	pH	TA* (%)	pH	TA (%)
0	6.27[a]	0.03[a]	4.83[a]	0.14[a]
2	6.29[a]	0.03[a]	4.03[b]	0.29[b]
4	6.26[a]	0.04[a]	4.02[b]	0.29[b]
6	6.38[ab]	0.03[a]	4.00[b]	0.33[b]
8	6.52[b]	0.03[a]	3.99[b]	0.34[b]
10	6.45[ab]	0.03[a]	3.97[b]	0.33[b]

*) Titratable acidity expressed as percent of lactic acid
**) Values in the same column with different superscripts are significantly different (P<0.05)

Survival of Probiotic Bacteria on Fermented Peanut Milk Drink During Storage

Lactobacillus paracasei SNP-2 attained high cell population of 4.8 x 10^8- 1.3 x 10^9 CFU/ml in peanut milk with various concentration of added sugar after 18 h of fermentation. During storage at 4°C for 21 days, there were no marked changes in the cell number of *L. paracasei* SNP-2 in fermented peanut milk drink with no addition of sugar and with addition of 2% sugar (**Fig. 1**). In contrast, the numbers of *L. paracasei* SNP-2 in fermented peanut milk drink with higher addition of sucrose (4-10%) were decreased at the end of storage time. The viable cell counts of *L. paracasei* SNP-2 in the fermented peanut milk drink added with 8% sucrose decreased from 1.0 x 10^9CFU/ml on first day of storage to 6.2 x 10^8CFU/ml on day 21 whereas that of in the fermented peanut milk drink added with 10% sucrose decreased from 1.3 x 10^9CFU/ml on the first day of storage to 1.5 x 10^8CFU/ml on day 21.

Cell population of fermented peanut milk drinks were in the range of 4.8 x 10^8- 1.3 x 10^9 CFU/ml, which were sufficient amount for probiotic product. To exert their beneficial effects on the host, it is essential that lactic acid bacteria be alive in sufficient numbers in the products at the time of consumption. The number of probiotic bacteria in the food should be at least 10^7CFU per ml or per g at the time of consumption in order to exert beneficial effects in the host (Ouwehand and Salminen, 1998). During storage at 4°C for 21 days, there were no marked changes in the cell number of *L. paracasei* SNP-2 in fermented peanut milk drink with no addition of sugar and with addition of 2% sugar, but there some decreases of cell population fermented peanut milk drink with higher addition of sucrose (4-10%). Overall, all fermented peanut milk drink contained greater than 10^7CFU/ml of viable *L. paracasei* during 21 day storage at in yogurt 4°C. Many studied showed low viability of probiotics in yogurt (Dave and Shah, 1977, and Lourens-Hattingh and Viljoen, 2001). Our study found, however, that *L. paracasei* SNP-2 retained a satisfactory level of viable cells throughout the storage of fermented peanut milk drink. This is in agreement with study on lactic acid bacteria in fermented soymilk drinks (Wang et al., 2002). The numbers of *L. acidophilus* in fermented soymilk was relatively stable during 10 day storage at 5°C, but they were much reduced during storage at 25°C. The reduction of *L. paracasei* SNP-2 counts in the fermented peanut milk drink, especially the ones supplemented with sucrose, may be related to the post-acidification of fermented peanut milk drink during storage.

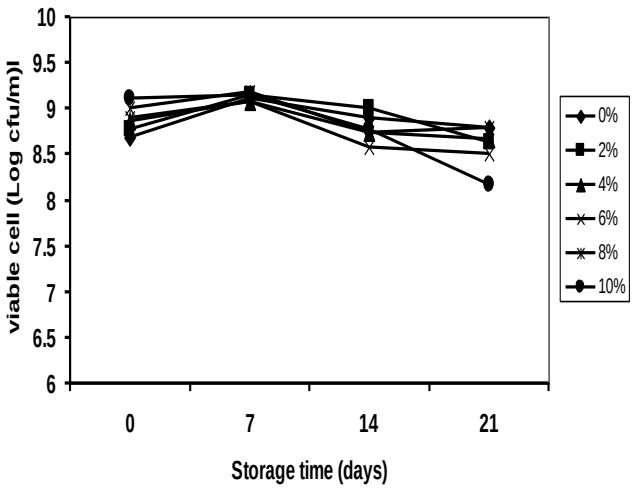

Fig. 1 Viability of *Lactobacillus paracasei* SNP-2 in fermented peanut milk drinkadded with various concentration of sucrose during storage at 4°C

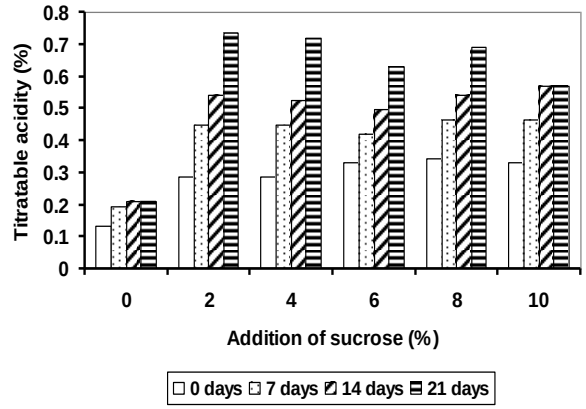

Fig. 2 Titratable acidity of fermented peanut milk drink with various concentration of sucrose during storage at 4°C

The initial pH of fermented peanut milk drink without and with addition of sugar, were in the range of 4.83 to 3.97 and after 21 days storage at 4°C, the pH were still in the range of 4.67 to 3.99. Thus, there were relatively no changes in the pH of fermented peanut milk drink during storage. In contrast the titratable acidities continued to increase during storage especially the ones with addition of sucrose (**Fig. 2**). After 21 day storage at 4°C, the titratable acidities of fermented peanut milk drink with addition of sugar were in the ranges of 0.29 to 0.33 %. As comparison, the titratable acidity of fermented peanut milk drink without addition of sugar after 21 day storage at 4°C was only 0.14 %. According to Bucker et al., (1979), the acidity change rather than pH change was the more sensitive method to follow fermentation since the titratable acidity continued to increase after the pH no longer decline. This study showed that the correlation between post-storage acid production in fermented peanut milk drinks and the viability of *L. paracasei* SNP-2 is affected by the addition of sucrose. Wang et al., (2002) reported that the addition of sucrose to fermented soymilk drinks increased the rate of decline of the pH and the viable lactic acid bacteria during storage at 25°C. Thus, further increase in acid production during cold storage may be attributed to greater metabolic activities of *L. paracasei* SNP-2 at 4°C in the peanut milk with sucrose addition. *Lactobacillus paracasei* SNP-2 could grow to a high cell population in peanut milk and had a high viable count during the storage time (8 log CFU/ml), indicating that fermented peanut milk drink would be an excellent vehicle for probiotic. Fermented peanut milk drink using *L. paracasei* SNP-2 can be used as a non-dairy probiotic product.

Conclusion

Lactobacillus paracasei SNP-2 showed good growth in peanut milk. The addition of 2% sucrose greatly enhanced the production of acid. Further increased in sucrose concentration did not significantly increased the acid production. During storage at 4°C the acid productions were still continued especially for those with sucrose addition. After 21 day storage at 4°C, all of the fermented peanut milk drinks retained in recommended levels of lactic acid bacteria. *Lactobacillus paracasei* SNP-2 could grow to a high cell population in peanut milk and had a high viable count during the storage time (8 log CFU/ml), indicating that fermented peanut milk drink suitable to be used as an excellent non-dairy probiotic product.

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