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Preparation of Indigenous Lactic Acid Bacteria Starter Cultures for Large Scale Production of Fermented Milk

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Abstract

Lactobacillus plantarum Dad 13, an indigenous probiotic was examined its ability to be used as a single starter culture or mixed cultures with *Streptococcus thermophilus* Dad 11 for milk fermentation. Both cultures were isolated from *dadih*, a traditional fermented buffalo milk. The purposes of this study were to produce indigenous lactic acid bacteria starter cultures using halal growth medium and evaluate their application on large scale fermented milk production. The halal medium was developed using natural compounds such as sucrose, meat peptone, mung bean sprout extract, tomato extract, and young coconut water. Meat peptone was prepared by hydrolysis of halal meat using crude bromelain. Lactic acid bacteria were grown in the halal growth medium then harvested, frozen and freeze-dried. A single freeze-dried starter culture of *L. plantarum* Dad 13 and frozen mixed cultures of *L. plantarum* Dad 13 and *S. thermophilus* Dad 11 were prepared for production of fermented milk drink and yogurt respectively in industrial scale. The growth of these lactic acid bacteria in halal growth medium increased the viable cell to two log cycles (10^9 CFU/mL) for *L. plantarum* Dad 13 and one log cycle for *S. thermophilus* Dad 11 (10^8 CFU/mL), respectively. The viable cell of freeze-dried *L. plantarum* Dad 13 and *S. thermophilus* Dad 11 were 7.57 x 10^{10} CFU/g and 6.35 x 10^9 CFU/g, respectively. The number of viable cells and pH of both fermented milk drink and yogurt products was relatively stable to 10^7 CFU/mL and 10^8 CFU/mL, respectively during cold storage for four to six weeks. The sensory characteristics of the products were comparable to the ones using commercial starter cultures. It can be concluded that these indigenous starter cultures can be applied for the production of probiotic fermented milk.

Keywords

indigenous lactic acid bacteria, halal starter cultures, milk fermentation

1 Introduction

Lactic acid bacteria play an important role in many Indonesian fermented foods such as fermented fish [1,2], fermented meat [3], tempe production [4], and fermented buffalo milk [5]. The genus of *Lactobacillus* plays the most important role in Indonesian fermented foods, and *L. plantarum* has been found to be the most common species found in traditional Indonesian fermented food [6]. There are numerous application areas for the use of lactic acid bacteria in foods. The involvement of lactic acid bacteria in foods could be grouped into three different aspects, as starter cultures for producing fermented food, as bio-preservatives and as components for probiotic food. A combination of these three aspects is certainly possible, such as using probiotic strains that also have antimicrobial agents or as a starter culture. Lactic acid bacteria (LAB) are widely used as starter cultures in milk fermentation. There are various fermented milk products such as yogurt, Dahi, Bulgarian buttermilk, and "Caspian Sea yogurt" which require different starter cultures, either mixed cultures or a single culture [7,8].

There are many fermented dairy products that contain probiotics. Probiotics can be introduced into the product at the end of fermentation time or in the initial time with the starter culture. The majority of products containing probiotics are dairy-based, that include yogurt, fermented milk beverage, and cheese. It has been reported that many lactic acid bacteria isolated from traditional fermented food fulfilled the

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basic requirements as probiotics [9]. To be able to give beneficial effects, probiotics must be able to survive during food processing and have the resistance against gastric acid, bile salt, and digesting enzyme before reaching the small intestine. In Indonesia, the market for fermented milk products develops significantly, especially for yogurt and fermented milk drink. Although numerous fermented milk products can be found in the market, the starter cultures that be used for the production of fermented milk are still imported.

Lactobacillus plantarum Dad 13 is an indigenous lactic acid bacteria isolated from traditional fermented buffalo milk in West Sumatera, Indonesia. Our previous research showed that *L. plantarum* Dad 13 had some functional properties, such as antimicrobial activity against pathogenic bacteria [10], antioxidant activity [11], and also probiotic characteristics [12]. This indigenous probiotic candidate showed good performance when applied to milk fermentation either as a single culture or mixed cultures at a laboratory scale [13].

There are many challenges and opportunities for the development of indigenous lactic acid bacteria as starter cultures for the fermented milk industry. Lactic acid bacteria are fastidious microorganisms which need exogenous sources of carbon, nitrogen, vitamins, minerals, and growth factors for their growth. Laboratory scale medium for the growth of lactic acid bacteria such as MRS broth cannot be used to develop starter culture for industrial scale, because it is too expensive for large scale fermentation. In addition, with a very high Muslim population in Indonesia, food industries also concern about halal materials for halal food, including halal media for starter culture preparation in fermented food products. Therefore, the aim of this research was to evaluate the production of indigenous lactic acid bacteria using natural halal materials media, preparation of starter culture and application of starter culture for production of fermented milk drink and yogurt in industrial scale.

2 Materials and methods

2.1 Materials

Lactobacillus plantarum Dad 13 and *Streptococcus thermophilus* Dad 11 which were used as starter cultures are the collection of Food and Nutrition Culture Collection (FNCC), Center for Food and Nutrition, Universitas Gadjah Mada, Yogyakarta, Indonesia. The cultures were stored in a mixture of 10% skim milk and 1% sucrose under -20°C. Batu variety pineapple as a source of crude bromelain, halal mince beef, and young coconut water was purchased from a local market. Mung bean sprout, tomatoes and sugar (Gulaku) were purchased from a local supermarket.

2.2 Preparation of halal media

Halal media consisted of halal meat peptone, sucrose, mung bean sprout extract, tomato extract, and young coconut water. Unskinned pineapples were cleaned and cut into small pieces and extracted the juice using a juicer. Halal meat peptone was prepared by hydrolyzes of halal mince beef with liquid crude bromelain (1:4 w/v) at shaker water-bath at 50°C for 3 h. To stop the reaction, the mixture was boiled at ± 90°C for 20 minutes and stood at room temperature. The mixture then was filtered using a double-layered cheesecloth. The liquid meat peptone then was stored at -20°C until it is used.

Preparation of mung bean sprout extract was carried out by boiling of mung bean sprout with water for 2 h and then followed by filtration using a double-layered cheesecloth to get the mung bean sprout extract. Cleaned tomatoes were cut into small pieces and sterilized in an autoclave (Hiramaya, Japan) at 121°C for 20 minutes. The liquid released was collected and filtered using a double-layered cheesecloth. All the components were stored at -20°C. The halal medium was prepared by a mixture of meat peptone (25%), sucrose (3%), mung bean sprout extract (40%), tomato extract (2%) and fresh young coconut water (30%).

2.3 Preparation of starter cultures

Starter culture for production of fermented milk drink was *L. plantarum* Dad 13 while starter cultures for plain yogurt fermentation were *L. plantarum* Dad 13 and *S. thermophilus* Dad 11. The isolates were growth in halal media for 24 h at 30°C. Starter cultures were prepared as frozen and freeze-dried cultures. The culture suspension was centrifuged using a refrigerated centrifuge (Beckman J-6B, Germany) to harvest the cell. The cell pellets were re-suspension using the liquid of 10% skim milk and 1% sucrose mixture.

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Every pellet from one Liter growth media was resuspended into 50 mL of 10% skim milk and 1% sucrose mixture and then were stored at the frozen condition.

To produce freeze-dried starter culture, pellets that be re-suspended in 10% skim milk and 1% sucrose mixture were frozen for overnight (at -20°C) and followed by freeze-dried cultures for 3 h. The viable cells were enumerated using dilution and pour plate method in MRA agar with $CaCO_3$. The freeze-dried starter cultures were examined their ability to grow in 10% skim milk at 37°C for 24 h.

2.4 Fermentation of milk using indigenous starter cultures

Indigenous lactic acid bacteria were used as a starter culture for the production of fermented drink and plain yogurt. Freeze-dried single culture of *L. plantarum* Dad 13 was examined its performance as a starter culture for the production of fermented milk drink in industrial scale. The viability of freeze-dried cells was evaluated for 60 days stored at room temperature. Freeze-dried starter culture was prepared for 12 L growth media and then harvested, frozen and freeze-dried for 3 days to get starter culture powder as those described in the previous section. The starter culture powder was sent to PT Yummy Food Utama, a fermented milk industry, and used as a starter culture for production of fermented milk drink (Yofit) in 1000 L fermenter. Fermentation, product formulation, and packaging were done by the Industry. The products were examined the viable cell and pH during storage. Sensory evaluation was carried out using a hedonic test for fermented milk drink products (plain, strawberry-flavor, and blueberry flavor fermented milk drink products) prepared using indigenous starter culture and commercial starter cultures. Thirty-one panelists were asked to give score for overall performance of six fermented milk drink products with seven points hedonic scale: extremely like very much (1); like very much (2); like moderately (3); neutral (4); dislike moderately (5); dislike very much (6); extremely dislike (7).

For the production of plain yogurt, each of the frozen starter culture of *L. plantarum* Dad 13 and *S. thermophilus* Dad 11 was activated in 10 % skim milk and incubated at 30°C for 18 h. Starter cultures were prepared by inoculation of each culture (1%v/v) into 6 x 1 L 10% skim-milk and then incubated at 30°C for 18 h. These starter cultures were used for the production of plain yogurt in PT Yummy Food Utama. Plain yogurt products were tested for viable cells and pH for 6 weeks. The cell viability of total lactic acid bacteria and *L. plantarum* were examined using MRS agar and LPSM agar media respectively.

3 Results and discussion

3.1 Development of indigenous starter cultures

Lactobacillus plantarum Dad13 and *S. thermophilus* Dad 11 were grown in culture media to get enough amount of starter cultures for industrial scale. The population of *L. plantarum* Dad 13 and *S. thermophilus* Dad 11 during starter culture preparation were presented in **Table 1**. After incubation at 30°C for 24 h, the population of *L. plantarum* Dad 13 and *S. thermophilus* Dad 11 increased two and one log cycle respectively to 1.90×10^9 CFU/mL and 2.52×10^8 CFU/mL. The lower growth of *S. thermophilus* Dad 11 was due to the low temperature of incubation. The freeze-dried cultures obtained were 10.58 log CFU/g and 9.80 CFU/g for *L. plantarum* Dad 13 and *S. thermophilus* Dad 11 respectively.

Microorganisms should be cultured or grown in a suitable medium in order to get a sufficient number of viable cells. MRS is a suitable medium for lactic acid bacteria. Commercial MRS contains peptone, meat extract (Lab Lemco), and yeast extract as nitrogen sources [14]. Usually, the most expensive component of microbial growth media is the nitrogen source. Here, we developed halal meat peptone by hydrolyzed of halal meat using crude bromelain from pineapple juice. Based on the soluble nitrogen, the degree of hydrolysis of meat protein by crude bromelain was more than 95% after 3 h at 50°C [15]. Natural halal media were used for the production of lactic acid bacteria, which consisted of halal meat peptone, sucrose, mung bean sprout extract, tomato extract and young coconut water. Lactic acid bacteria are fastidious microorganisms, which have an absolute need for some amino acids. It has been reported that isoleucine, leucine, and valine were essential for the growth of *L. plantarum* NCFB 1752 and *L. plantarum* NC8 [16]. It has been found that besides those three amino acids, *L. plantarum* ATCC 8014 absolutely required L-glutamic acids, L-methionine, L-phenylalanine, and L-tryptophan [17]. Therefore, those amino acids should be provided in the growth media. Here, halal meat peptone produced by hydrolysis of minced beef by crude bromelain provided those amino acids. Vitamins, minerals, and growth factors were provided by

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germinated mung bean extract, young coconut water and tomato extract. Coconut water also contains glucose and sucrose as carbon sources for lactic acid bacteria [18]. Tomato extract contains some growth factors for lactic acid bacteria [19].

Starter cultures should be healthy, active and sufficient amount before introducing to the fermentation medium. Initial starter culture of 6.88 log CFU/mL was considered to be enough for fermentation of skim milk in laboratory-scale which increased the cell population to about one log cycle (**Table 2**). It has been reported that the optimum temperatures for cell growth of *L. plantarum* Dad 13 and acid production were 30°C and 37°C respectively [13]. Therefore, incubation temperature for cell propagation was 30°C, meanwhile fermentation temperature was 37°C.

Table 1 The growth of L. plantarum Dad 13 and S. thermophilus Dad 11 in halal media and their viability during the preparation of freeze-dried cultures

Step	*L. Yummy* Dad 13	*S. thermophilus* Dad 11
Initial (CFU/mL)	7.34 ± 6.92	7.26 ± 5.98
After incubation (CFU/mL)	9.28 ± 8.92	8.40 ± 7.34
After harvesting and re-suspension in cryo-protectant (CFU/mL)	10.58 ± 10.22	9.70 ± 8.64
After freezing (CFU/mL)	10.57 ± 10.24	8.69 ± 7.89
After freeze drying (CFU/g)	10.57 ± 9.85	9.80 ± 9.34

Table 2 The growth of freeze-dried starter cultures in skim-milk at 37°C for 24 hours

Fermentation Time (h)	Cell amount (log CFU/mL)	
	L. plantarum Dad 13	*S. thermophilus* Dad 11
0	6.88 ± 0.04	6.89 ± 0.02
4	6.91 ± 0.05	7.15 ± 0.11
8	7.30 ± 0.24	7.32± 0.01
12	7.42 ± 0.12	7.41 ± 0.03
16	7.52 ± 0.04	7.75 ± 0.10
20	7.62 ± 0.09	7.93 ± 0.01
24	7.81 ± 0.13	8.05 ± 0.03

3.2 Application of indigenous starter cultures for production of the large scale of fermented milk drink and yogurt

Freeze-dried powder of *L. plantarum* Dad 13 was used as a starter culture for the production of fermented milk drink. Production of starter culture was carried in 12 L growth media. After incubation at 30°C for 24 hours, the viable cells were 2.28 x 10^9 CFU/ml or a total of 2.4 x 10^{13} CFU. After freeze-drying, the freeze-dried powder obtained was 72.3 g with the total viable cells of 5.60 x 10^{12} CFU. The reduction of the viable cells during freezing and freeze-drying was less than one log cycle. Freezing and freeze-drying can significantly affect cell viability. Here we used a mixture of 10% skim milk and 1% sucrose. All of these freeze-dried starter cultures were immediately sent to the fermented milk industry and applied for the production of fermented milk drink. The freeze-dried starter culture of *L. plantarum* Dad 13 was quite stable during storage at vacuum-sealed aluminum foil at room temperature (**Table 3**). For the application of dairy probiotic, skim milk is commonly used as a cryoprotectant. Disaccharides such as sucrose, lactose, and trehalose also can be used as cryoprotectants. It has been reported that among sucrose, trehalose, and sorbitol, sucrose at 5% and 1% were the best protection to the cell of *L. plantarum* NCIMB 8826 whereas sorbitol was the least [20].

Table 3 Cell viability of freeze-dried L. plantarum Dad 13 during storage at room temperature

Storage time	Viable cell (Log CFU/g)
Initial	10.88 ± 9.86
30 days	10.48 ± 9.98
60 days	10.16 ± 9.72

Fermented milk drink was produced using a freeze-dried starter culture of *L. plantarum* Dad 13 with three different flavors namely plain, strawberry and blueberry fermented milk drink. The viable cells of *L.*

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plantarum Dad 13 in the fermented milk drink were quite stable during storage in the range of 1.47 x 10^7 CFU/mL to 5.80 x 10^7 CFU/mL with pH ranged from 4.05 – 4.14 (**Table 4**). These values were similar to the pH of the product produced by commercial starter cultures (4.05-4.09). Since the starter culture is a probiotic candidate, the amount of the viable cells in the product is very important. Yakult contains 6.5 x 10^9 CFU *L.casei* Shirot strain per bottle. Consumption of *L. casei* Shirota strain fermented milk by 26 healthy Indonesian people showed markedly increase the population of *L. casei* Shirota strain in the feces to 6-7log CFU/g [21]. The volume of this fermented milk drink per bottle was 180 mL. Therefore, the viable cell of probiotic in one bottle is 1.04 x 10^{10} CFU. Consumption of *L. plantarum* Dad 13 containing fermented milk drink by 30 healthy Indonesian people showed a significant increase in the population of *L. plantarum* in the feces from about 3log CFU/g to 7log CFU/g [12]. It means that this indigenous probiotic not only can be used as a starter culture for fermented milk but also survive during storage in still alive in the gastrointestinal tract.

Table 4 pH and viable cell of fermented milk drink using indigenous starter culture during storage

Storage time at 4°C	Plain fermented milk drink		Strawberry fermented milk drink		Blueberry fermented milk drink	
	pH	Cell CFU/mL	pH	Cell CFU/ml	pH	Cell CFU/ml
2 weeks	4.05 ± 0.07	7.17 ± 6.65	4.05 ± 0.05	7.74 ± 6.60	4.07 ± 0.08	7.61 ± 6.76
3 weeks	4.09 ± 0.07	7.25 ± 6.57	4.08 ± 0.06	7.76 ± 6.82	4.09 ± 0.05	7.71 ± 6.51
4 weeks	4.11 ± 0.05	7.56 ± 6.40	4.11 ± 0.07	7.59 ± 6.58	4.14 ± 0.05	7.60 ± 6.79

Hedonic test for fermented milk drink with three different flavors using *L. plantarum* Dad 13 and commercial starter cultures is presented in **Table 5**. All fermented milk drink produced either using indigenous or commercial starter cultures were packed in the same plastic bottle as the one produced commercially in the market. All the panelists were the students who familiar with fermented milk products. Based on the hedonic test, panelists gave the value in the range from "like very much" to "neutral" with the average like moderately. There was not any different preference for the flavor of fermented milk drink. It means that fermented milk drink produced using the culture of *L. plantarum* Dad 13 was comparable to the one produced using the imported commercial starter culture. The home-use test was carried out in Yogyakarta involving 100 family members to get the family perception of fermented milk drink produced using our indigenous lactic acid bacteria [22]. The overall perception of panelists to peach-flavored fermented milk drink and strawberry-flavored fermented milk drink was neutral.

Table 5 Hedonic test of fermented milk drink using commercial starter cultures and indigenous starter culture

Fermented milk drink	Starter culture	
	Commercials	*L. plantarum* Dad 13
Plain fermented milk drink	2.94 ± 1.09	2.90 ± 1.11
Strawberry fermented milk drink	3.03 ± 1.02	2.84 ± 1.13
Blueberry fermented milk drink	2.90 ± 1.22	2.74 ± 1.12

Plain set yogurt was produced using liquid starter cultures of 6 L of *L. plantarum* Dad 13 and 6 L *S. thermophilus* Dad 11 in the industry using 1000 L fermenter. The plain yogurt consisted of only milk and starter cultures without the addition of any ingredients. The product was smooth with compact curd. The industry sets the self-life of 6 weeks for the product. The viable cell and pH of the plain yogurt were monitored until the end of the self-life time (**Table 6**). Total lactic acid bacteria and *L. plantarum* were 10^8 CFU/mL and 10^7 CFU/mL respectively. It seems that that *S. thermophilus* Dad 11 grew faster than *L. plantarum* Dad 13. Physically yogurt produced using indigenous starter cultures was quite stable with good curd formation and syneresis only about 3% at the end of self-life time. During yogurt fermentation, lactic acid bacteria consumed carbon source for their growth and metabolic activity. The pH gradually decreased as the acid increased. When the pH drops to the casein isoelectric points, the colloidal dispersion of casein micelles collapse, and then acid casein precipitates forming curd [23]. The acidification process results in the formation of a three-dimensional network consisting of clusters and casein chains [24]. It could be that the production of acid by these two starter cultures was high enough resulted in the high three-dimensional network formation and a quite strong curd formation.

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Table 6 Cell viability and pH of plain set yogurt during storage at 4°C

Storage time (week)	Total lactic acid bacteria (CFU/mL)	*L. plantarum* (CFU/mL)	pH
2	8.80 ± 7.39	7.52 ± 6.63	4.30 ± 0.08
3	8.71 ± 7.51	7.53 ± 6.49	4.44 ± 0.10
4	8.36 ± 7.69	7.13 ± 6.77	4.22 ± 0.04
5	8.19 ± 7.79	7.17 ± 6.63	4.06 ±0.05
6	8.24 ± 7.65	7.14 ± 6.54	4.02 ± 0.05

4 Conclusions

Preparation of freeze-dried lactic acid bacteria starter cultures has been developed for milk fermentation. Freeze-dried *L. plantarum* Dad 13 and *S. thermophilus* Dad 11 could grow well in 10% skim-milk. The indigenous probiotic of *L. plantarum* Dad 13 can be used as a single starter culture or mixed cultures with *S. thermophilus* Dad 11 for large scale production of fermented milk. The pH and viable cells in fermented milk produced by *L. plantarum* Dad 13 starter culture were quite stable in the range of 4.05-4.11 and 10^7 CFU/mL respectively. The pH and viable cells of plain yogurt produced using *L. plantarum* Dad 13 and *S. thermophilus* Dad11 were also quite stable until the self-lifetime of 6 weeks with total lactic acid bacteria and *L. plantarum* of 10^8 CFU/mL and 10^7 CFU/mL respectively. Based on the hedonic test, fermented milk produced using indigenous culture was comparable to the one using commercial starter cultures.

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