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Research Article
Characterization and Antioxidant Activity of Fermented Milk Produced with a Starter Combination

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Abstract

Objective: This study aimed to evaluate the microbiological and chemical characteristics and antioxidant activity of fermented milk produced by different starter combinations. **Materials and Methods:** Nine combinations of starter produced fermented dairy products using single-starter Lactic Acid Bacteria (LAB), a combination of starter LAB and a combination of starter LAB and yeast. The starter combinations were as follows: *Lactobacillus plantarum* Dad 13, *L. plantarum* Dad 13+*Lactococcus lactis*, *L. plantarum* Dad 13+*Saccharomyces cerevisiae*, *L. plantarum* Dad 13+*Kluyveromyces marxianus*, *L. plantarum* Dad 13+*L. lactis*+*S. cerevisiae*, *L. plantarum* Dad 13+*L. lactis*+*K. marxianus*, *L. lactis*, *L. lactis*+*S. cerevisiae* and *L. lactis*+*K. marxianus*. Total LAB and yeast were analyzed using the Total Plate Count (TPC) method. Chemical characteristics were identified by the values of pH, titratable acidity and ethanol contents. Antioxidant activities were determined by calculating the percentage of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging. **Results:** Present study showed that the combination of starters did not differ significantly across total LAB and yeast. However, there were significantly differences in pH, acidity, alcohol, β-carotene and antioxidant activity. The fermented milk obtained from *L. plantarum* Dad 13+*L. lactis*+*K. marxianus* had the lowest pH and the highest acidity and alcohol content. The combination *L. plantarum* Dad 13+*L. lactis*+*S. cerevisiae* produced the maximum β-carotene content and the combination of *L. plantarum* Dad 13+*L. lactis*+*S. cerevisiae* had the best antioxidant activity. **Conclusion:** It is concluded that a combination of starter *L. plantarum* Dad 13, *L. lactis* and *S. cerevisiae* can be used to improve the chemical quality and antioxidant activity of fermented milk.

Key words: Microbiological characteristics, chemical characteristics, antioxidant activity, fermented milk, starter combination

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Lactobacillus plantarum Dad 13 is a local isolate from dadih, a traditional Indonesian fermented milk from West Sumatra. Generally, dadih is produced from buffalo milk and fermented in bamboo tubes. Pato *et al.*[1] and Nurliyani *et al.*[2] reported that *L. plantarum* Dad 13 and *L. lactis* subsp., *lactis* isolated from dadih can live in the gastric juices and are resistant to bile salt, indicating probiotic activity. According to Rahayu *et al.*[3], *L. plantarum* Dad 13 can inhibit the colonization of pathogenic bacteria, such as *Shigella dysenteriae* dky-4, *Escherichia coli* dky-1 and dky-2 and *Salmonella typhimurium* dky-3.

Milk naturally contains components that have antioxidant capacities, such as casein, lactoferrin, vitamins and some flavonoids. Milk casein (α-casein, β-casein and κ-casein) and lactoferrin are capable of inhibiting lipid peroxidation[4,5]. Vitamin E, vitamin C and carotenoids are non-enzymatic radical scavengers[6]. Some flavonoids act as antioxidants that play a role in radical scavenging and metal ion binding[7]. Milk processing into fermented products provides beneficial nutrients[8,9], increases antioxidant activity[10], inhibits oxidation and decreases lipid peroxidation, in addition to lowering the glutathione redox ratio[11]. During fermentation, milk may produce bioactive peptides that possess antioxidant activity[12]. Farvin *et al.*[13] found that yogurt is more oxidatively-stable than milk due to its proteolytic activity.

The role of yeast in dairy fermentation continues to show favorable progress. Yeast growth in fermented products provides flavor components[14], as result of the utilization of lactose or metabolites of Lactic Acid Bacteria (LAB)[15,16]. The interaction between LAB and yeast results in a decline in pH and maintains the stability of viable LAB[17]. Presence of yeast can also increase the antioxidant activity of milk peptides related to 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl radical scavenging[18]. In addition, β-carotene, produced as a result of yeast metabolism, can also act as an antioxidant. Yeast has been used as a co-culture in traditional fermented products in some regions, such as kefir, koumiss, amasi and dadih. However, the composition of natural starters is unknown and it is difficult to obtain the stability of fermentation products. Efforts are underway to utilize yeast as co-starter in the manufacturing of fermented products to obtain more stable product quality[19-21].

The ability of *L. plantarum* Dad 13 to produce fermented milk with the best microbiological, chemical and antioxidant characteristics have not yet been evaluated. Therefore, it is necessary to investigate the ability of *L. plantarum* Dad 13 individually or in combination with other lactic acid bacteria or a combination thereof with yeast to produce the most favorable fermented milk characteristics.

MATERIALS AND METHODS

Lactic acid bacteria and yeast: The starter culture of *L. plantarum* Dad 13, *L. lactis*, *S. cerevisiae* and *K. marxianus* was obtained from the Food and Nutrition Culture Collection (FNCC), Centre for Food and Nutrition Studies, at the Universitas Gadjah Mada in Yogyakarta, Indonesia. Skim milk (powder) was purchased from a departmental store in Yogyakarta and raw milk was obtained from the Dairy Processing Unit at the Universitas Gadjah Mada.

Preparation of culture stocks: The culture stock was made from 0.1 mL of a frozen culture that had been thawed. Culture stock was cultivated in 10 mL medium (MRS for LAB and YPG for yeast culture) and incubated at 37°C for 24 h. The culture stock was then reproduced using a similar procedure. The stocks were centrifuged at 3500 rpm for 10 min to obtain cell pellets. Approximately 2% (v/v) of glycerol and 10% (w/v) of skimmed milk was added to cell pellets to obtain a solution (1:1:1 ratio). Culture stock was stored in microtubes at -20°C.

Preparation of starter: As much as one inoculating loop from LAB and yeast culture was transferred into each tube containing 10 mL of medium (MRS for LAB, YPG for yeast), which had been sterilized by autoclaving at 121°C, 15 psi for 15 min. The LAB and yeast were incubated at 37°C for 24 h. Starter was prepared by inoculating 10% (v/v) of inoculum in 18% (w/v) skimmed milk, which had been sterilized at 110°C, 13 psi for 10 min, then incubated for 12-18 h to form the curd; these results are called the mother starter. As much as 10% (v/v) of mother starter was inoculated in 100 mL of 18% (w/v) sterilized skimmed milk followed by incubation for 12-18 h to produce a bulk starter. The starter was then inoculated into the milk to be fermented.

Preparation of fermented milk: Raw cow's milk was pasteurized at 85°C for 30 min, then cooled to 40°C. As much as 3% (v/v) of starter was inoculated followed by incubation at 37°C for 18 h. Starter inoculated in the milk represent the single starter of LAB, the combination of LAB starter (1:1 ratio), the combination of LAB and yeast starter (2:1 ratio) or the combination of two starters of LAB and yeast (1:1:1 ratio). Nine groups of fermented milk produced the following

combinations: *L. plantarum* Dad 13 (K1), *L. plantarum* Dad 13+*L. lactis* (K2), *L. plantarum* Dad 13+*S. cerevisiae* (K3), *L. plantarum* Dad 13+*K. marxianus* (K4), *L. plantarum* Dad 13+*L. lactis*+*S. cerevisiae* (K5), *L. plantarum* Dad 13+*L. lactis*+*K. marxianus* (K6), *L. lactis* (K7), *L. lactis*+*S. cerevisiae* (K8), *L. lactis*+*K. marxianus* (K9).

Viable microorganisms: *Lactobacillus plantarum* Dad 13 and *L. lactis* were enumerated (pour plate) on MRS agar and incubated at 30°C for 48 h. Yeast was enumerated (pour plate) on chloramphenicol (100 ppm) YPG agar and incubated at 30°C for 72 h.

Chemical analysis: Titratable acidity was determined by titration of a 10 g sample (3 drops of phenolphthalein were added) with 0.1 N of NaOH. Then, its value was calculated as equivalent percent (w/w) of lactic acid and expressed as a percent of lactic acid. The pH value of the samples was measured using a pH meter (Hana-HI 98103). Ethanol content was determined by gas chromatography. β-carotene was measured according to Cagampang and Rodriguez[22] with some modifications. Antioxidant activity was measured as the percentage of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity using the method performed by Son and Lewis[23].

Statistical analysis: The experimental results were expressed as the Mean±Standard Deviation. The statistical analysis was performed using IBM SPSS 22. The significance of differences between the samples were analyzed by the one-way ANOVA. Further analysis was conducted using the Duncan Multiple Range Test (DMRT) to determine significant differences between means at 5% significance level.

RESULTS

Effect on total LAB and yeast: The starter and starter combinations did not affect the LAB and yeast populations in the production of fermented milk ($p>0.05$) (Table 1).

Effect on pH: The starter and starter combinations significantly affected the pH value of fermented milk produced ($p<0.05$) (Table 2).

Effect on titratable acidity: The starter and starter combinations significantly affected the titratable acidity of fermented milk ($p<0.05$) (Table 2).

Ethanol content: The starter and starter combinations significantly affected the ethanol content of fermented milk ($p<0.05$) (Table 2).

β-carotene content: The starter and starter combinations significantly affected the content of β-carotene ($p<0.05$) (Table 2).

Table 1: Average lactic acid bacteria and yeast in fermented milk (log CFU mL^{-1})

Products	Population of LAB	Population of yeast
K1	8.15±0.72	-
K2	8.46±0.30	-
K3	8.28±0.17	4.68±0.03
K4	8.38±0.06	4.78±0.52
K5	8.21±0.25	4.57±0.10
K6	8.24±0.13	5.07±0.13
K7	8.17±0.03	-
K8	8.36±0.34	4.95±0.30
K9	8.34±0.07	5.05±0.15

K1: *L. plantarum* Dad 13, K2: *L. plantarum* Dad 13+*L. lactis*, K3: *L. plantarum* Dad 13+*S. cerevisiae*, K4: *L. plantarum* Dad 13+*K. marxianus*, K5: *L. plantarum* Dad 13+*L. lactis*+*S. cerevisiae*, K6: *L. plantarum* Dad 13+*L. lactis*+*K. marxianus*, K7: *L. lactis*, K8: *L. lactis*+*S. cerevisiae*, K9: *L. lactis*+*K. marxianus*

Table 2: pH, acidity, ethanol and β-carotene values of fermented milk based on the different combinations of starter

Products	pH	Titratable acidity (%)	Ethanol (mg mL^{-1})	β-carotene (ppm)
K1	$4.56±0.55^{f}$	$0.50±0.50^{a}$	-	$0.05±0.09^{a}$
K2	$3.87±0.14^{c}$	$1.09±0.01^{c}$	-	$0.05±0.01^{a}$
K3	$4.14±0.32^{de}$	$1.05±0.01^{c}$	$0.07±0.02^{abc}$	$0.10±0.02^{b}$
K4	$4.05±0.35^{d}$	$1.06±0.02^{c}$	$0.09±0.01^{bc}$	$0.05±0.08^{a}$
K5	$3.78±0.26^{ab}$	$1.11±0.05^{d}$	$0.03±0.02^{a}$	$0.13±0.05^{b}$
K6	$3.70±0.10^{a}$	$1.18±0.06^{d}$	$0.11±0.04^{c}$	$0.05±0.23^{a}$
K7	$4.16±0.02^{e}$	$0.89±0.01^{b}$	-	$0.04±0.42^{a}$
K8	$3.80±0.03^{bc}$	$1.06±0.03^{c}$	$0.04±0.01^{ab}$	$0.11±0.11^{b}$
K9	$3.73±0.14^{b}$	$1.07±0.04^{c}$	$0.07±0.03^{abc}$	$0.05±0.37^{a}$

K1: *L. plantarum* Dad 13, K2: *L. plantarum* Dad 13+*L. lactis*, K3: *L. plantarum* Dad 13+*S. cerevisiae*, K4: *L. plantarum* Dad 13+*K. marxianus*, K5: *L. plantarum* Dad 13+*L. lactis*+*S. cerevisiae*, K6: *L. plantarum* Dad 13+*L. lactis*+*K. marxianus*, K7: *L. lactis*, K8: *L. lactis*+*S. cerevisiae*, K9: *L. lactis*+*K. marxianus*, $^{a-f}$Values with different superscripts are significantly different in a column ($p<0.05$)

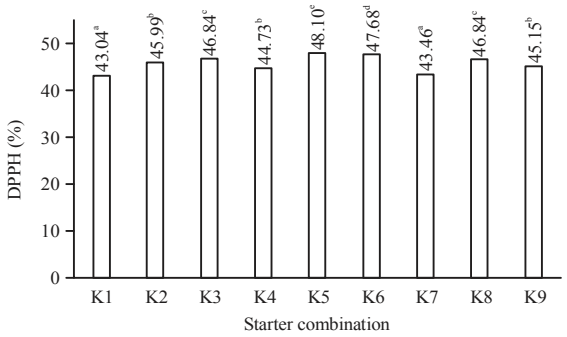

Fig. 1: DPPH radical scavenging activity (%) of fermented milk from different starter combinations

K1: *L. plantarum* Dad 13, K2: *L. plantarum* Dad 13+*L. lactis*, K3: *L. plantarum* Dad 13+*S. cerevisiae*, K4: *L. plantarum* Dad 13+*K. marxianus*, K5: *L. plantarum* Dad 13+*L. lactis*+*S. cerevisiae*, K6: *L. plantarum* Dad 13+*L. lactis*+*K. marxianus*, K7: *L. lactis*, K8: *L. lactis*+*S. cerevisiae*, K9: *L. lactis*+*K. marxianus*, $^{a-f}$Significant difference between combinations ($p<0.05$)

DPPH radical scavenging activity: The starter and starter combinations affected the DPPH radical scavenging activity of fermented milk (Fig. 1).

DISCUSSION

The results of this study showed a positive interaction between *L. plantarum* and *L. lactis*, as well as between LAB and yeast (*S. cerevisiae* or *K. marxianus*) in the production of fermented milk. The LAB population tended to be higher in combination compared to the single starter LAB (*L. plantarum* or *L. lactis* only), although there was no significant difference ($p>0.05$). The presence of yeast as a co-starter increased LAB population as result of the capability of yeast to stimulate the growth of LAB, perhaps due to the utilization of yeast metabolites in the form of vitamins, amino acids and peptides[14,24,25]. The yeast population tended to be higher if *K. marxianus* was used as a co-starter compared to *S. cerevisiae* (K4>K3, K6>K5, K8>K9), possibly due to the ability of *K. marxianus* to utilize lactose as a carbon source[15].

The pH of fermented milk depends on properties of the organic acids produced[26] and is also influenced by the ability of LAB to produce lactic acid, in addition to LAB population and the type of LAB used[27]. Lactic acid, as the main product of fermentation, can be dissociated to H^+ and $CH_3CHOHCOO^-$ ions. The accumulation of lactic acid increased the pH of the fermented medium due to an increase in the concentration of H^+ released[28]. The results showed that the combination of LAB (*L. plantarum*+*L. lactis*) or the combination between LAB and yeast significantly lowered the pH of fermented milk compared to a single starter (*L. plantarum* or *L. lactis* only). *Lactobacillus lactis* is homofermentative bacteria which can rapidly metabolize lactose into 90% of lactic acid[29]. The positive synergy between *L. plantarum* and *L. lactis* in dairy fermentation[30] could lead to an increased rate and amount of lactic acid[24], resulting in a decline in the pH value of the medium. The contribution of yeast as a co-starter in lowering pH due to the production of acid compounds is a result of its lipolytic and proteolytic activity. Titratable acidity and pH values were inversely related. The highest titratable acidity was obtained from the starter combination of *L. plantarum* Dad 13+*L. lactis*+*K. marxianus* (1.18±0.06 %); however, it did not differ significantly from the combination of *L. plantarum* Dad 13+*L. lactis*+*S. cerevisiae* ($p>0.05$).

The concentration of alcohol obtained from these experiments demonstrated the effect of yeast as a co-starter. During milk fermentation, ethanol, CO_2 and organic acids were produced as the result of yeast carbohydrate metabolism. Amino acids are also converted by yeast into alcohol through a series of decarboxylation and reduction[31]. The fermented milk using *K. marxianus* tended to produce higher alcohol content than the *S. cerevisiae* milk ($p<0.05$). The ability of *K. marxianus* to utilize lactose that was not produced by *S. cerevisiae* could explain this result[16].

The β-carotene is a component of milk that acts as an antioxidant and can be obtained from *S. cerevisiae*[32]. Cow's milk contains β-carotene entirely derived from the diet. The antioxidant capacity of fermented milk is enhanced by the utilization of *S. cerevisiae* as a co-starter through increasing levels of β-carotene. The results showed a significant increase in levels of carotenoids when using *S. cerevisiae* as a co-starter. The highest carotenoid content was obtained from the combination of *L. plantarum* starter Dad 13+*L. lactis S. cerevisiae* (0.13±0.05 ppm) but the content was not significantly different from the combination of *L. plantarum* Dad 13+*S. cerevisiae* or *L. lactis*+*S. cerevisiae*. The other combinations were not significantly different compared to β-carotene content in raw milk (0.05 ppm).

The antioxidative properties of fermented milk in this study were measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging (%). Raw milk had 22.17% of DPPH radical scavenging which increased to 23.11% after pasteurization. This result is likely due to the formation of Maillard Reaction Products (MRP), which have antioxidant activity during pasteurization[33]. The highest rate of DPPH radical scavenging in this study was obtained from the combination of *L. plantarum* Dad 13+*L. lactis*, *S. cerevisiae* (48.10%) (Fig. 1). This value is relatively higher than the value obtained by Fitrotin *et al.*[21] in the fermentation of sesame milk using *L. plantarum* Dad 13 (45.2% from the initial conditions,

19.3%). Other studies have reported 27% in the fermentation of whey from cow's milk using *L. lactis* subsp., *lactis* as the starter[34] and 22.2% in the manufacturing of Kunnu (traditional fermented milk from Nigeria) using *L. plantarum*[35]. Antioxidant activity of fermented milk increases due to proteolysis activity, which produces peptides[13,36] that act as an electron donor and react with free radicals to form more stable products[37]. Lactic acid as a metabolite may also serve as a chelating agent that plays a role in inhibiting free radicals[38].

Lactobacillus plantarum Dad 13 combined with *L. lactis* and *S. cerevisiae* can be used to produce the best product characteristics, particularly antioxidant activity. *Saccharomyces cerevisiae* in fermentation is essential to promote β-carotene and the use of *L. lactis* in fermentation increases the production of lactic acid, which can act as a scavenger of free radicals. The presence of *L. lactis* also plays a role in breaking down milk protein into bioactive peptides during fermentation to increase the antioxidant activity of the fermented milk.

CONCLUSION

It is concluded that the combination of LAB and yeast had no effect on microbiological characteristics, whereas the combination of starter *L. plantarum* Dad 13, *L. lactis* and *S. cerevisiae* improved the chemical quality and antioxidant activity of fermented milk.

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