Kefir Produced from Red Rice Milk by *Lactobacillus bulgaricus* and *Candida kefir* Starter

A R Sulistyaningtyas¹, A T Lunggani², E Kusdiyantini²

¹University of Muhammadiyah Semarang, Faculty of Nursing and Health Sciences, Medical Laboratory Technology Study Program, Street of Kedungmundu No.18, Semarang 50273, Indonesia
²Diponegoro University, Faculty of Biology, Street of Prof Soedarto No. 1, Semarang 50275, Indonesia

Corresponding author: ayurs@unimus.ac.id

**Abstract.** Kefir is a dairy product commonly fermented by bacteria and yeasts. One of the potential vegetable milk materials to be used in kefir production is milk from red rice. Studies of fermentation of milk from red rice to produce kefir are barely found. This study aimed to determine the nutrient content of red rice milk kefir fermented using mixed of microorganisms, *Lactobacillus bulgaricus* and *Candida kefir*, in various concentrations of 3%, 9%, and 15%, respectively. The content of carbohydrate, fat, protein, water, and ash was measured using proximate analysis, while their content of lactic acid and alcohol was assayed using titration method. The results showed that fat, water and ash content in all resulted kefir products met standard quality of kefir, whereas carbohydrates and protein content did not. Treatment using the set mixed starters at a concentration of 9% was found to be able to produce kefir with a lactic acid content of 2.19%. Increasing alcohol content of kefir was dependent by starters. Generally, all treatments were able to produce kefir with alcohol content of 1.5% on average. As conclusion, fermentation of red rice milk at concentration of mixed starters at 9% has nutrient content which meet standard quality of kefir.

**Keywords:** Kefir, red rice milk, *Lactobacillus bulgaricus*, *Candida kefir*

1. **Introduction**

Fermentation is a process that produces a variety of products both aerobically and anaerobically by involving the activity of microbes or their extracts in a controlled condition. Fermentation can lead to food diversity and produce products with distinctive flavor, aroma, and texture[1]. In addition, fermentation can increase biomass of products. Fermented milk is milk processed by involving microbes to produce various products such as cheese, yogurt and kefir [2].

Kefir is one of dairy products that have fermented with bacteria and yeast as starter. Kefir has made from milk which inoculated by kefir granules [3]. Usually, kefir starter consists of lactic acid bacteria and yeasts. Lactic acid bacteria (LAB) used as fermentation starter such as *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus lactis*, *Bifidobacteria sp* and *Lactobacillus*...
dextranicum [4]. In addition to LAB, yeasts that can be used in fermentation such as Saccharomyces cerevisiae and Candida kefir [5].

The raw material for making kefir can come from animal or vegetable milk [6]. The superiority of vegetable materials as raw materials because it has a lower fat content compared with materials derived from animals. Vegetable materials that have been used as raw material for the manufacture of kefir such as Phaseolus radiates, Vigna unguiculata, Arachis hypogea, Phaseolus vulgaris and Glycine max [6]. In addition, Poaceae family has potential as raw material for making kefir such as red rice (Oryza nivara L.).

Making kefir with raw material of red rice with starter L. bulgaricus and C. kefir has not been done. This is allegedly due to the low availability of red rice. Relatively, its price was expensive. The composition of nutrient content per 100 g of brown rice consists of 75.7 g of carbohydrates; 8.2 g protein; 1.9 fat; 16 mg of calcium; 243 mg of phosphorus and 0.21 g of vitamin B1[7]. In addition, brown rice contains phenol compounds and antioxidant activity is higher than white rice (Oryza sativa L.) [8]. Based on the nutritional content contained in red rice is quite high, then red rice milk has the potential as a raw material for making kefir. This study aimed to determine the nutrient content of red rice milk kefir fermented using mixed of microorganisms, Lactobacillus bulgaricus and Candida kefir, in various concentrations of 3%, 9%, and 15%, respectively.

2. Materials and Method
2.1. Materials
The material were used red rice (O. Nivara L.), bacteria L. bulgaricus (PAU Laboratory in Universitas Gadjah Mada), yeast C. kefir (Microbiology Laboratory in Medical Faculty Universitas Diponegoro), cow milk, distilled water, MRS agar, yeast extract, malt extract, nutrient agar, buffer pH 4 solution, buffer pH 7 solution, phenolphthaline, NaOH 1 N, 40% NaOH, absolute alcohol, anhydride acetic acid, chloroform, 0.9% methanol, 4% borax acid, H2O2, H2SO4, SeOCl2, and peptone.

2.2. Method
2.2.1. Culture breeding pure L. bulgaricus
L. bulgaricus was grown in MRS broth medium. The MRS broth medium is prepared by dissolving 55 g MRS into 1000 mL of distilled water. Sterilization at 121 °C for 15 minutes by using autoclave, then cooled in room temperature. After that, L. bulgaricus was taken one ose from a culture of pure culture then inoculated in MRS broth medium then incubated with a rotary shaker of 120 rpm for 24 hours [9,10].

2.2.2. Culture breeding pure C. kefir
C. kefir was cultured in yeast malt extract broth (YMEB). The YMEB medium was made by dissolving 1.5 g of yeast extract, 1.5 g malt extract, 5 g glucose, 2.5 pepton to 500 mL distilled water. Sterilization at 121 °C for 15 minutes by using autoclave. After that, C. kefir was taken one ose from a culture of pure culture then inoculated in YMEB Medium then incubated with rotary shaker 120 rpm for 24 hours [11].

2.2.3. Growth Curve
Making growth curve was conducted to evaluate the growth phase of bacteria and yeasts. The microorganism calculations were performed every 3 hours during 24 hours incubation using the total plate number (TPC) method. L. bulgaricus was poured in MRSA medium while C. kefir is in YMEA medium.

2.2.4. Preparation of red rice milk
One kilogram of red rice that has been pasted and then soaked in 3 L distilled water for 8 hours. The red rice is boiled for 20 minutes with low heat. Red rice was blended until smooth and then
added 5 L aquaest. Dilute porridge is filtered and then grab the filtrate. The filtrate as red rice milk is pasteurized at 85 °C for 30 minutes.

2.2.5. Preparation of starter kefir
Firstly, culture was inoculated in pre-pasteurized cow's milk medium at 85 °C for 30 minutes. The addition of pure culture L. bulgaricus and C. kefir with starter ratio of 1:1 (v/v) was done when both of them had reached exponential phase. Both of them was incubated at 43 °C for 6 hours. This phase happened when microorganism cells increase in the highest amount as 100% (v/v) starter concentration. The second stage, the starter was inoculated in a medium mixture of cow's milk and red rice milk with a medium ratio of 1:1 (v/v). The starter was incubated at 43 °C for 6 hours in the incubator [12,13]

2.2.6. Treatment of study
Rice pureed pasteurized milk at 85⁰C for 30 minutes, added glucose by 10% (w/v). Red rice milk was cooled at room temperature. Furthermore, a starter has containing L. bulgaricus and C. kefir was added 3%, 9% and 15% (v/v) in 100 mL of red rice milk. After that, red rice milk was incubated at 43 °C for 6 hours in the incubator [14,15,16].

2.2.7. Parameter Measurement

Proximate Analysis

The proximate analyses (moisture, fiber, ash, crude fats, proteins and carbohydrates) of all the samples were determined. Fiber content was estimated from the loss in weight of the crucible and its content on ignition. Carbohydrate was determined when the sum of the percentages of moisture, ash, crude protein, either extract or crude fiber were subtracted from 100. The nitrogen value which is the precursor for protein of a substance was determined by micro Kjeldahl method described by Pearson (1976), involving digestions, distillation and finally titration of the sample. The nitrogen value was converted to protein by multiplying a factor of 6.25. Carbohydrate was determined by difference method. All the proximate values were reported in percentage [17,18].

Determination of Lactic Acid Level

A total of 10 mL of sample was put into a 100 mL measuring flask distilled water up to 100 mL volume then homogenized and filtered. The solution is taken 10 mL and put into Erlenmeyer, then drop the 2 drops phenolftalin indicator, then titrated with Next titrated using 1 M NaOH until the solution color becomes pink [19].

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\text{Lactic Acid Level (\%) = \frac{(V_xN)aOHxBE\text{Lactic acid}}{V_{sample}(ml)} \times 100\%}
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Determination of Alcohol Levels

Making standard alcohol curve aimed to evaluate the regression equation used to determine the level of alcohol kefir samples. The standard alcohol curve expresses the relationship between the need for NaOH as the X axis and the alcohol content as Y axis. One mL of standard alcohol with a concentration of 0.1 to 1% is incorporated into Erlenmeyer and then added 1 mL acetic acid anhydride and 2 drops of phenolphthalaein. Further, it was titrated using 1 M NaOH until the color of the solution becomes pink. The same stage was performed for kefir samples. Alcohol levels can be determined by adding the required NaOH Volume into a known standard alcohol regression equation [20].

2.2.8. Data Analysis

This study design used a single-factor complete randomized design, i.e. starter concentration. Starter treatment 3% (v / v), 9% (v / v), 15% (v / v) carried out in 6 replications. The treatment effect of the number of kefir starter was tested ANOVA statistically if there is a real effect then proceed with a different test using the Duncan test at the level of 5%.
3. Result and Discussion

3.1. Proximate Analysis

Parameters proximate levels (carbohydrate, fat, protein, water, ash), lactic acid and alcohol levels have resulted of red rice kefir with starter concentration (*L. bulgaricus* and *C. kefir* are different.

| Table 1. Result of proximate analysis of red rice milk and cow's milk kefir (control) per 100 mL |
|---------------------------------------------------------------|
| **Starter concentration (%) v/v** | **Proksimat (%)** | **Water** | **Ash** | **Protein** | **Fat** | **Carbohydrate** |
| control | 88.76 | 0.23 | 3.58 | 3.48 | 3.95 |
| 3% | 81.48 | 0.08 | 1.13 | 1.13 | 16.98 |
| 9% | 81.75 | 0.1 | 1.28 | 1.28 | 16.75 |
| 15% | 82.09 | 0.18 | 1.64 | 1.64 | 16.06 |

Based on Table 1, carbohydrate levels of red rice milk kefir in all starter treatment 3%, 9%, and 15% have not met the quality requirements of kefir. Terms of quality kefir for carbohydrate levels is 4.5%. The results showed that carbohydrate levels were decreasing during fermentation due to the breakdown of carbohydrates into simpler compounds. The addition of glucose in red rice milk kefir aims to stimulate the activity of *L. bulgaricus* because the bacteria use monosaccharide compounds first for nutrients. *L. bulgaricus* bacteria was capable of fermenting glucose, lactose and galactose but not fermenting mannose and sucrose [21,22].

Fat content of kefir was decreased in higher concentration starter. Compared with cow's milk kefir, red rice milk kefir has a lower fat content. Red rice has a low-fat food which can potentially produce low-fat kefir. Lactic acid bacteria have secondary lipolytic activity that can break down milk fat into simpler compounds with the help of lipase enzymes [23].

The protein content of red rice milk kefir tended to increase with increasing inoculated starter concentration. Increasing protein content might be caused by starter protein [24]. One percent of the total protein of kefir comes from microorganisms [25].

Starter concentration is one of the most important factors causing increased water content. The inoculated starter concentration is increasing, feeding higher water content of kefir. This is because *C. kefir* will digest the subtract during kefir fermentation resulting in CO₂, H₂O and ATP [26].

Ash content was expressed percentage of mineral content contained in a product. Cow's milk kefir produces higher levels of ash than red rice milk kefir. Minerals content in red rice milk consists of calcium, phosphorus, selenium, magnesium, and iron while cow milk minerals consist of calcium, phosphorus, selenium, magnesium, iron, chlorine, copper, potassium, sodium and sulfur [27].

3.2. Lactic acid and Alcohol Content

*L. bulgaricus* and *C. kefir* have played role in the fermentation of red rice milk for organic acids formation. Total acid in red rice milk kefir was expressed as lactic acid. The results of the study of lactic acid with different starter concentrations can be seen in Table 2 below:
Table 2. Determination of Lactic Acid and Alcohol Level in Red Rice Milk Kefir with Various Starter Concentrations

| Starter Concentration (% v/v) | Lactic acid (%) | Alcohol (%) |
|-------------------------------|----------------|-------------|
| 3                             | 0.90           | 1.01        |
| 9                             | 2.19           | 1.29        |
| 15                            | 3.60           | 1.59        |

The results showed that the starter concentration had an effect on lactic acid level in red rice milk kefir. The quality requirement of alcohol in kefir is 2% so the 9% starter treatment was closest. The process of nutrient degradation will produce organic acids such as lactic acid and acetic acid [28][29] The level of formed lactic acid is influenced by carbohydrates in the milk of red rice. Most carbohydrates in the milk of red rice are starch, starch is a polysaccharide to be decomposed into monosaccharides in the form of glucose. The increased starter concentration will increase the polysaccharide degradation agent, resulting in lactic acid which is a breakdown product of glucose compound. L. bulgaricus is a homofermentative lactic acid bacteria capable of decomposing glucose into pyruvic acid which is subsequently converted to lactic acid via the Embden-Meyerhoff pathway [30].

Alcohol levels tend to increase with increasing number of starter. The more microorganisms inoculated into the fermentation medium, the faster the nutrient degradation process occurs [31] The minimum quality of alcohol in kefir is 1.5 %. Therefore, the alcohol content of starter treatment 15 % meets the quality requirements of kefir. L. bulgaricus bacteria will decompose the polysaccharide compound into monosaccharides whereas C. kefir will produce ethyl alcohol and CO₂ by fermentation [32].

4. Conclusion
In conclusion, fermentation of red rice milk using concentration of mixed starters at 9% has resulted product with nutrient content which meet standard quality of kefir.

References
[1] Gulitz, A., Stadie, J., Wenning, M., Ehrmann, M.A. and Vogel, R.F., 2011. The microbial diversity of water kefir. *International journal of food microbiology, 151*(3), pp.284-288.
[2] Leite, A.M., Mayo, B., Rachid, C.T., Peixoto, R.S., Silva, J.T., Paschoalin, V.M.F. and Delgado, S., 2012. Assessment of the microbial diversity of Brazilian kefir grains by PCR-DGGE and pyrosequencing analysis. *Food microbiology, 31*(2), pp.215-221.
[3] Alsayadi, M., Al Jawfi, Y., Belarbi, M. and Sabri, F.Z., 2013. Antioxidant potency of water kefir. *The Journal of Microbiology, Biotechnology and Food Sciences, 2*(6), p.2444.
[4] Diosma, G., Romanin, D.E., Rey-Burusco, M.F., Londero, A. and Garrote, G.L., 2014. Yeasts from kefir grains: isolation, identification, and probiotic characterization. *World Journal of Microbiology and Biotechnology, 30*(1), pp.43-53.
[5] Bourrie, B.C., Willing, B.P. and Cotter, P.D., 2016. The microbiota and health promoting characteristics of the fermented beverage kefir. *Frontiers in microbiology*, 7, p.647.
[6] Fratiwi, Yulneriwarni dan Noverita. 2008. Fermentasi Kefir Dari Susu Kacang-Kacangan. J Vis Vitalis 1(2):45-54.
[7] Sirirat, D. and Jelena, P., 2010. Bacterial inhibition and antioxidant activity of kefir produced from Thai jasmine rice milk. *Biotechnology, 9*(3), pp.332-337.
[8] Muntana, N. and Prasong, S., 2010. Study on total phenolic contents and their antioxidant activities of Thai white, red and black rice bran extracts. *Pakistan Journal of Biological Sciences, 13*(4), p.170.

[9] Guzel-Seydim, Z.B., Kok-Tas, T., Greene, A.K. and Seydim, A.C., 2011. Functional properties of kefir. *Critical reviews in food science and nutrition, 51*(3), pp.261-268.

[10] Leite, A.M., Mayo, B., Rachid, C.T., Peixoto, R.S., Silva, J.T., Paschoalin, V.M.F. and Delgado, S., 2012. Assessment of the microbial diversity of Brazilian kefir grains by PCR-DGGE and pyrosequencing analysis. *Food microbiology, 31*(2), pp.215-221.

[11] Miguel, M.G.C.P., Cardoso, P.G., Magalhães-Guedes, K.T. and Schwan, R.F., 2013. Identification and assessment of kefir yeast potential for sugar/ethanol-resistance. *Brazilian Journal of Microbiology, 44*(1), pp.113-118.

[12] Supriyono, T. 2008. Kandungan Beta Karoten, Polifenol Total dan Aktivitas “Merantas” Radikal Bebas Kefir Susu Kacang Hijau (Vigna radiata) oleh Pengaruh Jumlah Starter (Lactobacillus bulgaricus dan Candida kefir) dan Konsentrasi Glukosa. *Thesis*. Program Pascasarjana Universitas Diponegoro.

[13] Nielsen, B., Gürakan, G.C. and Ünlü, G., 2014. Kefir: a multifaceted fermented dairy product. *Probiotics and antimicrobial proteins, 6*(3-4), pp.123-135.

[14] Kunaepah, U. 2008. Pengaruh Lama Fermentasi dan Konsentrasi Glukosa Terhadap Aktivitas Antibakteri, Polifenol Total dan Mutu Kimia Kefir Susu Kacang Merah. *Thesis*. Program Pascasarjana Universitas Diponegoro.

[15] Kim, D.H., Chon, J.W., Kim, H., Kim, H.S., Choi, D., Hwang, D.G. and Seo, K.H., 2015. Detection and enumeration of lactic acid bacteria, acetic acid bacteria and yeast in kefir grain and milk using quantitative real-time PCR. *Journal of Food Safety, 35*(1), pp.102-107.

[16] Mainville, I. and Farnworth, E.R., 2003. Kefir: A Fermented Milk Product. In *Handbook of Fermented Functional Foods* (pp. 93-128). CRC Press.

[17] AOCS (American oil chemist society).2005. Washington, DC, USA.

[18] Kök-Taş, T., Seydim, A.C., Özver, B. and Guzel-Seydim, Z.B., 2013. Effects of different fermentation parameters on quality characteristics of kefir. *Journal of dairy science, 96*(2), pp.780-789.

[19] Prado, M.R., Blandón, L.M., Vandenbergh, L.P., Rodrigues, C., Castro, G.R., Thomaz-Soccol, V. and Soccol, C.R., 2015. Milk kefir: composition, microbial cultures, biological activities, and related products. *Frontiers in microbiology, 6*, p.1177.

[20] Magalhães, K.T., Pereira, G.V.D.M., Campos, C.R., Dragone, G. and Schwan, R.F., 2011. Brazilian kefir: structure, microbial communities and chemical composition. *Brazilian Journal of Microbiology, 42*(2), pp.693-702.

[21] Suriastih, K., Aryanta, W.R., Mahardika, G. and Astawa, N.M., 2012. Microbiological and chemical properties of Kefir made of Bali cattle milk. *Food Science and Quality Management, 6*, pp.2225-0557.

[22] Rosa, D.D., Dias, M.M., Grześkowiak, Ł.M., Reis, S.A., Conceição, L.L. and Maria do Carmo, G.P., 2017. Milk kefir: nutritional, microbiological and health benefits. *Nutrition research reviews, 30*(1), pp.82-96.

[23] Garofalo, C., Osimani, A., Milanović, V., Aquilanti, L., De Filippis, F., Stellato, G., Di Mauro, S., Turchetti, B., Buzzini, P., Ercolini, D. and Clementi, F., 2015. Bacteria and yeast microbiota in milk kefir grains from different Italian regions. *Food microbiology, 49*, pp.123-133.

[24] Zanirati, D.F., Abatemarco Jr, M., de Cicco Sandes, S.H., Nicoli, J.R., Nunes, Á.C. and Neumann, E., 2015. Selection of lactic acid bacteria from Brazilian kefir grains for potential use as starter or probiotic cultures. *Anaerobe, 32*, pp.70-76.
[25] Golowczyc, M.A., Silva, J., Teixeira, P., De Antoni, G.L. and Abraham, A.G., 2011. Cellular injuries of spray-dried Lactobacillus spp. isolated from kefir and their impact on probiotic properties. *International journal of food microbiology, 144*(3), pp.556-560.

[26] Bamforth, C.W., 2008. *Food, fermentation and micro-organisms*. John Wiley & Sons.

[27] Stadie, J., Gulitz, A., Ehrmann, M.A. and Vogel, R.F., 2013. Metabolic activity and symbiotic interactions of lactic acid bacteria and yeasts isolated from water kefir. *Food microbiology, 35*(2), pp.92-98.

[28] Farnworth, E.R., 2006. Kefir—a complex probiotic. *Food Science and Technology Bulletin: Fu, 2*(1), pp.1-17.

[29] Bolla, P.A., de los Angeles Serradell, M., de Urraza, P.J. and De Antoni, G.L., 2011. Effect of freeze-drying on viability and in vitro probiotic properties of a mixture of lactic acid bacteria and yeasts isolated from kefir. *Journal of Dairy research, 78*(1), pp.15-22.

[30] Corona, O., Randazzo, W., Miceli, A., Guarcello, R., Francesca, N., Erten, H., Moschetti, G. and Settanni, L., 2016. Characterization of kefir-like beverages produced from vegetable juices. *LWT-Food Science and Technology, 66*, pp.572-581.

[31] Kaewprasert, P. and Poosaran, N., 2010. Production of kefir like product from mixed cultures of Saccharomyces cerevisiae, Streptococcus cremoris and Streptococcus lactis. *Asian Journal of Food and Agro-Industry, 3*(1), pp.13-24.

[32] Vardjan, T., Lorbeg, P.M., Rogelj, I. and Majhenič, A.Č., 2013. Characterization and stability of lactobacilli and yeast microbiota in kefir grains. *Journal of dairy science, 96*(5), pp.2729-2736.