Effect of temperature and time storage towards alcohol level in cow milk kefir

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Abstract. Kefir is an acidic-alcoholic fermented milk product with little acidic taste and creamy consistency and has a distinctive yeasty aroma. Bacteria in the grain produce lactic acid and flavor components, carbon dioxide, and alcohol. Increased alcohol levels can occur with longer storage. The optimal temperature for the growth of alcohol-producing microorganisms can be at room temperature, whereas low temperatures can inhibit microbial growth and biochemical processes. This study aimed to determine the effect of temperature and storage time on the alcohol content of cow milk kefir. The research method used Randomized Block Design Factorial with two factors and three levels. The first factor was the storage time that consists of 7, 14, and 21 days. While, the second factor was storage temperature that consists of freezing storage (-10°C), refrigerator storage (15°C), and room storage (20°C). Data were analysed using ANOVA, then the further test DMRT with a 95% confidence interval. The best treatment result was tested using Zeleny. The best treatment result was attributed to freezing storage (day 7) at -10°C, with physical parameters include pH of 4.47 and total soluble solids of 10.00% Brix. Then, chemical parameters are total sugar (3.07%), total acid (0.26%), and alcohol content (0.04%). Lastly, microbiological parameters include the total of lactic acid bacteria (5.91 Log CFU/ml) and the total of yeast (6.56 Log CFU/ml). Kefir with the best treatment could decrease the alcohol level and safe for consumption.

1. Introduction
Milk contains several important substances needed by humans, such as carbohydrate (lactose), protein, fat, vitamin and mineral. Nevertheless, fresh milk was highly easy to get contaminated by decomposing bacteria. Since fresh milk has a short shelf life, it requires further processing. Fermentation could be applied as a way to preserve milk for a long period and one of the most common fermented milk is kefir [1].

Kefir is a complex probiotic fermented milk beverage produced by kefir grain on carbohydrates milk [2]. It was originated from central Asia between Caucasus mountain and Mongolia [3]. Kefir characteristics are acidic, viscous, slightly alcoholic rather effervescent milk beverages because of the bacteria and yeast inside the polysaccharide matrix [4]. Beside the cow's milk that has been used for kefir preparation traditionally, nowadays soy milk or milk from various mammals such as sheep, buffalo and goat can be used for kefir preparation as well [5]. Throughout the time of fermentation,
lactic acid bacteria produces lactic acid and breakdown lactose, moreover yeast produces alcohol in the form of ethanol and carbon dioxide from lactose [6]. Increased ethanol concentration could occur with longer storage time. In addition, the storage time of kefir also has the ability to inhibit the contamination of pathogenic bacteria from the environment [7].

Fermented food produced by natural fermentation and less than 1% ethanol is considered as safe preserving agent and halal. The ethanol concentration in kefir would increase uncontrollably by the inappropriate storing way and storage time of kefir. If the amount of ethanol produced from fermentation is higher than its allowed to, it could have an intoxicating effect and therefore not allowed for consumption by Muslims [8]. The objective of this research was to investigate the effect of temperature and storage time on physicochemical-microbiology properties as safe and halal kefir products.

2. Materials and Methods

2.1 Preparation of kefir milk
Fresh milk was bought from Balai Besar Pelatihan Peternakan Batu, Indonesia. Fresh milk was pasteurized at 90°C for 10 minutes using stove while stirred. The milk was transported in cold condition (25°C) and added 3% kefir grain. The milk that had been inoculated was fermented at room temperature for 24 hours. Throughout fermentation formed two layers; curd and whey. Milk kefir was separated from kefir grains by using a fine plastic strainer. This process was suggested by Triwibowo and Wicaksono [1].

This research used a randomized block design with 2 factor and 3 levels. The first factor was storage time (7, 14 and 21 days) and the second factor was the storage temperature (-10°C; 15°C; and 20°C). Each treatment was replicated 3 times. Kefir was analysed for physical, chemical and microbiology properties.

2.2 Evaluation of physical properties
Physical properties of kefir milk evaluated by total soluble solid using hand refractometer. The refractometer was calibrated with distilled water before it was used to measure. Dripped about 1 placing into prism refractometer and put it toward the light [9].

2.3 Evaluation of chemical properties
Chemical properties of kefir milk were evaluated by pH, total sugar, total acid and alcohol content. pH analysis was measured using a pH meter that had been calibrated with standard buffer 4.00 dan 7.00 and 9.00. pH meter was dipped into a 10 ml sample solution [10]. Total sugar analysis used the UV-Vis Spectrophotometry method with some modification. About 1 ml sample was dissolved in 100 ml aquadest, then added with CaCO3 until netral. It was then heated for about 20 minutes then filtered with filter paper. About 1 ml sample was put into the tube, 5 ml anthrone reagent was added. Sample was heated with reagent using water bath 100°C for about 10 minutes. Last, the sample was cooled down, and analyzed using spectrophotometry with wavelength 630 nm [11]. Total acid was determined by the visual titration method. First, put 10 ml sample that was added 2 drops phenolphthalein, then titration using NaOH [12]. The alcohol content was measured using GC-FID with modification. Sample solution 0.5 ml was dispensed into an l-ml capped sample vial, 5 mL of 1% internal standard solution was added. Last, after the mixing process, 0.1 µL of the sample solution was injected directly into a GC with a syringe [13].

2.4 Evaluation of microbiology properties
Microbiology properties of kefir milk evaluated by total lactic acid bacteria and total yeast. Total lactic acid bacteria were measured with number of colonies, counted that platted with MRSA (de Man Rogosan and Sharper Agar) medium. The platted sample was incubated at 37°C about 48 hours beforehand [14]. Total lactic acid bacteria were measured with a number of colonies, counted that
platted with Potato Dextrose Agar (PDA) medium. The platted sample was incubated at 30°C about 48 hours beforehand [15].

2.5 Statistical analysis
The data analysis was obtained by using Minitab statistics version 16 for Windows. The method used is Analysis of Variance (ANOVA) with a 5% confidence interval, and variance. If the results of the tests that have been carried out indicate an influence, it can be carried out for further tests, namely BNT (Least Significant Difference) or DMRT (Duncan Multiple Range Test) using a 5% confidence interval.

3. Results and Discussion

3.1 Characteristic of pasteurized milk
The raw material has been used for making kefir was pasteurized milk. The raw material was analyzed to identify the condition before and after treatment was done. Table 1 showed that no alcohol content was detected in raw material or it could be said that the results are negative. The result shows <LOQ, LOQ is the Limit of Quantification.

| Parameters                     | Result          |
|--------------------------------|-----------------|
| Total Acid (%)                 | 0.17 ± 0.009    |
| pH                             | 6.27 ± 0.057    |
| Total Sugar (%)                | 4.39 ± 0.081    |
| Total Soluble Solid (%Brix)    | 9.33 ± 0.057    |
| Total Lactic Acid Bacteria (Log cfu/ml) | 5.51 ± 0.026 |
| Total Yeast (Log cfu/ml)       | 6.58 ± 0.044    |
| Alcohol Content (%)            | <LOQ            |

3.2 Physical properties of kefir milk
Total soluble solids were dissolved materials, amount of material could be dissolved in water, including total sugars, organic acids, pectins and proteins. The result of total soluble solid showed that due to the variance of temperature and storage effect, total soluble solid decreased. Loss of nutritional components in freezing, breaking of carbohydrates becomes simpler as a source of nutrition for microorganisms which causes a decrease in total soluble solids [16]. The increase in the concentration of reducing sugars (glucose and fructose) correlated with the decrease of sucrose and total soluble solids (°Brix). The hydrolysis of sucrose by yeast invertase increased glucose and fructose levels [21].

3.3 Chemical properties of kefir milk
The result of decreased pH due to variance of temperature and storage effect, therefore it affected the lactic acid bacteria activity and yeast from kefir grains. Low temperatures storage could inhibit the growth of bacterial and the occurrence of biochemical-physical changes in food products [17]. The total acid analysis shows increased due to the variance of temperature and storage effect. According to Sawitri, the increase of total acid was caused by the low pH could make a suitable environment for lactic acid bacteria grown and formed lactic acid metabolites [7]. The low pH value after 24 fermentation causes the disappearance of the lactococci and the presence of the lactobacilli as the major bacterial species at times. Then, the titratable acidity increased progressively cause the presence of the minor organic acids in kefir which are also titrated by NaOH when determining the titratable acidity [22].
**Figure 1.** Physical properties - Total soluble solid.

**Figure 2.** Chemical properties – pH.
Figure 3. Chemical properties - Total sugar graphs.

Figure 4. Chemical properties – Total acid.
Another analysis result was showed in Table 3, total produced sugar decreased due to the variance of temperature and storage effect. According to Jeanette, sugar is a nutritional component in products that are also used by lactic acid bacteria to produce metabolites or another microorganism's life cycle. Longer storage and more nutrients could carry out metabolic processes. Alcohol content increased due to the variance of temperature and storage effect caused by the growth of yeast. Higher temperature also can make alcohol content increases because yeast activity will be increased too. Room temperature is the optimum temperature for alcohol content to increase [7,8].

3.4 Microbiology properties of kefir milk
Lactic acid bacteria are gram-positive with a coccus shape. These bacteria also namely asiduric bacteria because of the relatively low pH in their life cycle, about 4.6-5.4 [18]. Total lactic acid bacteria in kefir decreased due to the variance of temperature and storage effect. Lactic acid bacteria can not stay with a very acidic condition, they could lysis and decrease their metabolites [19]. The following species of lactic acid bacteria (83–90%) of the microbial count in the kefir grains were identified: *Lactococcus lactis* subsp. lactis, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. Bulgaricus, etc [23]
Yeast is a unicellular organism with a spore asexual reproductive system. In dairy products, the yeast responsible for providing and enhancing nutrients such as amino acids, vitamins, and pH control. These nutrients are available because there was a previous aerobic fermentation process that happened because yeast broke down the glucose into alcohol and carbon dioxide. The result was showed in Table 4, that total yeast increased due to the variance of temperature and storage effect. The increased caused optimum condition for yeast to break down lactose into ethanol. Kefir optimum temperature at 25-30°C [6,7]. The following species of yeasts (10–17%) identified were Kluyveromyces marxianus var. lactis, Saccharomyces cerevisiae, Candida inconspicua and Candida maris [23].
3.5 The best treatment for kefir milk
The best treatment of this kefir milk product with the variance of temperature and storage effect were determined using multiple attributes [20]. The multiple attribute method specifies how to attribute information is to be processed in order to arrive at a choice. These methods require both inter- and intra-attribute comparisons and involve appropriate explicit tradeoffs [24]. Based on the calculation of attribute, the best result was obtained with 7 days storage at -10°C (freezer storage).

4. Conclusions
This study aimed to determine the alcohol content in kefir with time and temperature storage effect on physicochemical-microbiology properties as safe and halal kefir products. There is an interaction between time and temperature storage of milk kefir, because p-value less than (α = 0.05), there was a significant effect for all parameters: total sugar, total acid, pH, total yeast, total lactic acid bacteria, alcohol content and total soluble solids. The best treatment was obtained at freezer storage temperature with 7 days of storage. The characteristics were pH of 4.47, total soluble solids of 10.00 %Brix, total sugar of 3.07%, total acid of 0.26%, the alcohol content of 0.04%, total lactic acid bacteria of 5.91 Log CFU /ml and a total yeast of 6.56 Log CFU /ml. This product is safe for consumption, also halal because alcohol content in the best treatment was <1%.

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