The effect of storage period on calcium and magnesium level and antimicrobial activity of goat's milk kefir

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Abstract. The purpose of this reasearch was to determine the effect of storage period goat's milk kefir (GMK) on total LAB, total mineral levels included Ca and Mg and antimicrobial activity to pathogenic microbes. The materials used was GMK. This research was conducted using Completely Randomized Design with 4 treatments and 4 replications. The treatments were storage period that consist of D0 (0 day), D1 (7 days), D2 (14 days), and D3 (21 days). Total calculation of LAB used TPC (Total Plate Count). Samples were diluted from 10^{-1} to 10^{-8}, then incoculated PCA medium by using pour plate method. Petri dish was incubated at 37ºC for 48 hours. Calcium and magnesium levels were calculed base AAS method. The result of this study was analyzed using analysis of variance (ANOVA), then followed by Duncan's Multiple Range Test (DMRT). The storage time treatment has a significant effect on the total microbial count, mineral levels (Ca and Mg) and inhibition zone of *Staphylococcus aureus*, *Escherichia coli* and *Salmonella enterica serovar typhi* on goat's milk kefir. Average clear zone *Staphylococcus aureus* 2.60 – 3.18 mm. Average inhibition zone *Escherichia coli* 2.09 – 3.32 mm. Average clear zone *Salmonella enterica serovar typhi* 1.97 – 2.72 mm.

1 Introduction

Along with increasing human awareness of the importance of healthy living, there is also an increase in research and marketing of food and beverage products that have the potential to maintain a healthy body. Nutritious food and beverage products are better known as functional foods. One of the functional food products that are currently popular in the community is fermented milk [1]. Reported that kefir is a fermented milk beverage product by kefir grain that contain of lactic acid bacteria and yeast [2]. This lactic acid bacteria will cause a sour kefir taste. Kefir grains are a mass consisting of various kinds of bacteria and yeast arranged in a complex protein and carbohydrate matrix [3]. [4] argue that based on research in the world of health, kefir has many benefits, including inhibit tumor growth more effectively than yogurt, protect digestion from pathogenic bacteria attack, maintain

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metabolism and human immune function, and maintain cholesterol levels in the body blood. Kefir has long being considered beneficial to human health. For example, kefir enhance immune system, digestive health, treat the metabolic disorders, hypertension, ischemic heart disease, and allergies. However, it has antimicrobial, antitumor, antiviral, antimutagenic, antiinflammatory and antioxidant effects [5]

Kefir consists of 4% fat, 12% ash, 46% exopolysaccharide, 34% protein, vitamins B and K, tryptophan, several minerals such as Ca, P and Mg [6]. Minerals is nutrient that the body needs in small amounts, but has many benefits that are very important for health. Minerals can be found in dairy products, for example in kefir. Kefir is a good source of Ca, Co, Cu, Fe, Mg, Mo and Zn [6]. According to [7] the process of making kefir itself has an influence on the vitamin and mineral content in it. The content of several minerals such as phosphorus and selenium decreased significantly after the fermentation process [5]. Meanwhile, the manganese content increases after the fermentation process. This occurs because some minerals are important components for the growth and metabolic activity of most organisms, including Lactic Acid Bacteria (LAB) [8].

Kefir has a water content of 89.5%, 1.5% fat, 3.5% protein, 0.6% ash, 4.55% lactose, and a pH of 4.6% [9]. Fermentation is an aerobic and anaerobic process that produces various kinds of primary and secondary metabolites. Such as lactic acid bacteria which have the role of producing anti-microbial substances that can inhibit the growth of panthogens and extend the shelf life of products. Long storage period means extending the fermentation process at cold temperatures. Antimicrobial compounds are chemical or biological compounds that can inhibit the growth and activity of pathogenic microbes [11]. Many pathogenic microbes contaminate food and cause disease, pathogenic microbes also cause harm because they have the ability to infect, cause disease and damage the quality of foodstuffs [12]. Pathogenic microbes are one of the causes of disease in humans and other living things, for example *Staphylococcus aureus, Escherichia coli*, *Salmonella enterica serovar typhi* and others [13]

Microbial activity in the fermentation process and storage period can produce antimicrobial compounds [14]. Antimicrobial compounds are chemical or biological compounds that can inhibit the growth and activity of pathogenic bacteria [15]. Pathogenic bacteria that contaminate food and cause disease, for example *Staphylococcus aureus, Escherichia coli* and *Salmonella enterica serovar typhi.* There is limited research on the shelf life of fermented products, especially on antimicrobial activity. So that the purpose of this study was to determine the effect of storage period for goat milk kefir on the antimicrobial activity of pathogenic microbes.

2 Materials and Methods

This research was conducted at Livestock Product Technology Laboratory of Animal Science Faculty. Analysis of antimicrobial activity test results was conducted in Biomedical Laboratory, total microbial count was conducted in Laboratory of Animal Product Technology, Faculty of Animal Science and the mineral's level test was conducted in Chemical Laboratory of Brawijaya University. The material was fresh GMK from the farm of Mrs. Yuyun, Sumberejo, Batu. The equipment used in the manufacture of GMKwas container as fermentor, filter stirrers, measuring cups, analytical scales and spoons.

These study was a laboratory experiment using a completely randomized design (CRD) with 4 treatments and 4 replications. The treatments consisted of of D0 (0 day), D1 (7 days), D2 (14 days), and D3 (21 days). Data were analyzed using analysis of variance (ANOVA), followed by the Duncan Multiple Range Test (DMRT).

Analysis of mineral's level using AAS (Atomic Absorption Spectrophotometry), total microbial analysis using TPC (Total Plate Count) pour plate method, and analysis of antibacterial activity using the disc diffusion method. Antimicrobial activity testing was

carried out on Gram Positive Bacteria (*Staphylococcus aureus)* and Gram Negative Bacteria (*Escherichia coli* and *Salmonella enterica serovar typhi*.

2.1 Total microbial count test

A sample of 1 ml of kefir was prepared and then transferred with a sterile pipette into a solution of 9 ml of distilled water to obtain a 10^{-2} dilution. Do the same thing until the desired dilution is 10^{-8}. The media used was PCA (Plant Count Agar) media. A suspension (culture medium) was diluted and then inoculated on an empty petri dish. Pour the media so that it is still liquid then mix the media with the sample by turning the petri dish following the figure eight pattern [16]. The samples were then incubated at 37°C for 2 days. After that, the results of colony growth on agar media and the number of microbes can be calculated using a colony counter [17].

2.2 Test of mineral levels

Analysis of Ca and Mg using Atomic Absorption Spectrophotometry. Sample preparation was carried out by wet digestion method. In this digestion process, 8 mL of 65% HNO_3 and 2 mL of 30% H_2O_2 were used. The addition of HNO_3 functions as a destructor, H_2O_2 acts as an oxidizer to accelerate the oxidation process. After digestion, the sampe solution was added with 0.1 N HNO_3 then the sample solution was filtered using Whatman paper number 1 to produce a clear and free solution from particles that could interfere with the measurement process at AAS [18].

2.3 Test of antimicrobial on *Staphylococcus aureus*, *Escherichia coli* and *Salmonella enterica serovar typhi*

The antimicrobial test media used by Salmonella typhi is Media Mueller Hinton Agar (MHA). Put the microbial culture test into the petri dish. Inserted dipped disc paper in the sample. Incubated at 37°C for 24 hours. After 24 hours, observed areas of clear zones formed and measured in diameter.

2.4 Data analysis

In this study the data are presented as mean with standard deviation. Significance between the mean values was determined statistically by ANOVA in 4 treatments with 4 replications. Results are reported as mean ± standard deviation.

3 Results and discussion

3.1 Analysis of Total Microbial Count

The results of statistical analysis of total microbial showed that tthe storage period showed significant effect ($P < 0.01$) on the total value of kefir microbes. The results showed that the total number of microbes in D0 treatment (storage time on day 0) had the highest number of microbes, while D3 (storage period on day 21st) showed the lowest total number of microbes.

It is suspected because the ability of kefir microbes to survive has decreased due to the unavailability of substrates used for metabolism and survival by kefir microbes. [19] explains that the death phase occurs after the log phase and occurs quickly. This is because the nutrient

sources found in the growth media have run out. The death phase is caused because cell growth begins to stop and the bacteria have used up their reserve energy (ATP) for respiration, so that many bacterial cells die. There was a decrease in the amount of LAB at the incubation period of 48 to 72 hours, this was due to the competition between LAB and yeast for nutrition. Then the accumulation of alcohol produced by yeast causes damage to the bacterial cell wall [20]. Decreasing bacterial population in D3 treatment causes kefir pH to decrease and acidity increases, so that lactic acid bacteria that cannot tolerate too high acidity will die, so that the population of lactic acid bacteria in D3 treatment decreases [21].

Table 1. Mean of total microbial count

Storage period	Colony average (CFU/mL)	Log average (CFU/mL)
D0	$2.0 \times 10^{9\,a}$	8.89 ± 1.96
D1	$1.2 \times 10^{8\,ab}$	7.78 ± 0.47
D2	$1.7 \times 10^{7\,abc}$	7.12 ± 0.55
D3	$1.1 \times 10^{7\,c}$	6.78 ± 0.11

3.2 Analysis of Ca (Calcium) Levels

The results of statistical analysis of Calcium levels showed that the duration of the storage process had a significant effect ($P < 0.05$) to Calcium levels of kefir.

Table 2. Mean of Ca levels

Storage Period	Mean of Ca levels (mg/kg)
D0	191.62 ± 26.83^{a}
D1	$214.75 \pm 21.40a^{b}$
D2	$228.03 \pm 22.19a^{bc}$
D3	248.34 ± 13.86^{c}

It is suspected because Ca is one of the factors needed in cell metabolism. At the period of storage on Day 0 (D0) the total number of kefir microbes reaches the highest value, so the need for calcium is also a lot, so that a lot of calcium is used and possibly only a little left. Meanwhile, at D1 (7^{th} day of storage), the total kefir microbes had started to decline, but the amount of calcium increased slightly from the amount of D0. It is suspected that some of the dead microbes still have some residual calcium, so that when these microbes die, they still leave the remaining calcium in their bodies and accumulate so that they increase the amount of calcium levels. Quantitatively, the calcium increased 22.69% was compaired with control. Calcium and inorganic phosphate is the main components of the kefir grain culture wall as calciset cell wall component (mannose oligosaccharide, β-glucan), directly the composition of the cell did effect on kefir calcium, showed that during storage period the grain kefir culture synthesize calcium or calcium carbonate form as well as the culture growth.

Milk was the dietary source of (33.2%) calcium, (60.4%), vitamin D and (18.8 %) potassium [23]. The calcium was investigated as nutrition has been performed on elemental

calcium and relatively little on calcium as supplied by dairy food. Dairy products as a products that hight calcium and are responsible for about 76% of the calcium available in diet [24]. In this study milk provided 22.5% of calcium intake and there were a larger number of smaller contributors to calcium intake than seen in previous of studies, primarily due to fortification of various products (e.g., fruit juice, RTEC). [25].

3.3 Analysis of Mg (Magnesium) Levels

The results of statistical analysis of Magnesium levels showed that the duration of the storage process had significant effect ($P < 0.01$) to Magnesium levels of kefir. Magnesium kefir levels increase with the length of the storage process. The longer the kefir is stored in the refrigerator temperature, the higher the Mg content in the kefir. The results of the analysis of variance carried out on magnesium kefir levels showed that the storage period had a very significant effect ($P < 0.01$) on the levels of Magnesium kefir.

Table 3. Mean of Mg Levels

Storage Period	Mean of Mg Levels (mg/kg)
D0	11.68 ± 0.73^{a}
D1	$13.74 \pm 0.31a^{b}$
D2	15.44 ± 0.45^{c}
D3	16.62 ± 0.93^{d}

It is suspected because magnesium is one of the substances needed for cellular metabolism. This is because the function of the minerals themselves is for metabolism and for cell growth. This is in accordance with the opinion of [7] this occurs because some minerals are an important component for the growth and metabolic activity of most organisms including LAB. The bacteria in kefir also need magnesium to help in this metabolic process. Therefore, it is suspected that on the 0^{th} day of storage, the magnesium content in kefir has the lowest value because at that time the mineral, in this case magnesium, is needed by microbes for the overhaul of the substrate. In this case, the total number of microbes at the period of storage on the 0^{th} day also has the highest value, so that the magnesium needed is also a lot, eventually the levels are reduced a lot.

The result has accordance with the opinion of goats' milk contains a similar magnesium concentration to cows' milk (mean, 122 mg/L; range 110–144 mg/L [26]. Magnesium is essential micromineral of biosynthetic processes. In both kefir samples produced from the goat milk, the concentration of Mg was found to increase when compared with the levels of Mg in milk samples (Tables 2 and 3). Furthermore, the concentration of Mg in kefir produced from goat milk was higher than fresh milk [27]. [28] Reported that increasing magnesium, calcium and phosphorus contents by 7.2, 12.4 and 9.6% in line with a similar increase of milk protein content. Finally, the essential nutrients, calcium, magnesium, and phosphorus was higher acoordance with aging period, minerals value has improved in kefir.

3.4 Analysis of antimicrobial activity in *Staphylococcus aureus, Escherichia coli and Salmonella enteritica serovar typhi*

The results showed that the effect of storage time treatment gave very significant difference ($P < 0.01$) effect on the bland zones of *Staphylococcus aureus and* the effect of storage period

gave significant difference ($P < 0.05$) effect on the clear zones of *Escherichia coli* and *Salmonella enteritica serovar typhi*.

Table 4. Mean of inhibition zone for antimicrobial activity in inhibition *Staphylococcus aureus, Escherichia coli* and *Salmonella enteritica serovar typhi*

Treatments	Inhibition zone (mm) *Staphylococcus aureus*	Inhibition zone (mm) *Escherichia coli*	Inhibition zone (mm) *Salmonella enteritica serovar typhi*
D0	2.23 ± 1.87^{a}	3.32 ± 0.74^{b}	2.72 ± 0.21^{b}
D1	3.18 ± 0.33^{b}	2.70 ± 0.26^{ab}	2.37 ± 0.09^{ab}
D2	3.14 ± 0.42^{b}	2.37 ± 0.47^{a}	2.01 ± 0.14^{a}
D3	2.60 ± 0.10^{ab}	2.09 ± 0.19^{a}	1.97 ± 0.64^{a}

The results showed that the effect of storage period gave very significant difference ($P < 0.01$) effect on the inhibition zones of *Staphylococcus aureus.* Increase or decrease in the average of the inhibition zone along with the increase or decrease in the total lactic acid bacteria. D0 (0 days) lactic acid bacteria are still in the adaptation phase to the storage environment and cells are still able to carry out positive transport in the form of nutrients for growth. D1 (7 days) lactic acid bacteria enter the logarithmic phase which is the phase when bacteria divide rapidly indicated by the population of lactic acid bacteria begins to increase [29] and produce the bioactive compound as a metabolite such as peptide and organic. This compound have antibacterial activity which generated by utilizing lactose and protein of goat's milk. D1 (7 days) and D2 (14 days) enter the stationary phase of the growth of bacterial cell populations that tend to be constant. This is because in this phase (stationary) it is suspected that BAL produces the highest secondary metabolites for self-defense. D3 (21 days) there is a decrease in the average inhibition zone as the population of lactic acid bacteria enters the phase leading to the death of the growth curve. The phase to death is the phase when the medium nutrients and the number of bacterial populations decreases, which is followed by change the bioactive component.

The results showed that the effect of storage Period treatment gave significant difference ($P < 0.05$) effect on the clear zones of *Escherichia coli* and *Salmonella enteritica serovar typhi*. The decrease in the average inhibition zone in *Escherichia coli* and *Salmonella enteritica serovar typhi* is thought to be due to the long shelf life affecting antimicrobial activity, because the longer the shelf life, the more passive lactic acid bacteria and the fewer the number, so that it has the ability to break down the substrate the smaller. At a shelf life of 0 days has the ability to inhibit the growth of *Escherichia coli* and *Salmonella enteritica serovar typhi* highest, then decreased in shelf life 7 days, 14 days and 21 days. At a length of 21 days, it is suspected that the substrate has started to run out (Fardiaz, 1987). This is according to research [29], that antimicrobial activity is produced in the decay phase. According to [30], the criteria of antimicrobial power strength are divided into four groups, namely as follows: the diameter of the inhibition zone of 5 mm or less is categorized as weak, the 5 mminhibition zone is categorized as moderate, the 10 mm bland zone is categorized as strong, and the clear zone of 20 mm or more is categorized as very strong. The results of the study when compared to the standard fall into the category of weak antimicrobial activity.

4 Conclusion

Based on the research results, it can be concluded that the storage period treatment has a significant effect on the total microbial count, mineral levels and inhibition zone of *Staphylococcus aureu*, *Escherichia coli* and *Salmonella enteritica serovar typhi*. Storage period of 7 days and 14 days give the best results against the average inhibition zone in *Staphylococcus aureus*. Storage period of 0 days gives the best results to the average inhibition zone in *Escherichia coli* and *Salmonella enteritica serovar typhi* but the effect does not show any difference to the length in storage period of 7 days.

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