

The Effects of Different Fermentation Time on Functional Properties of Kefir Optima

Cow Milk and Goat Milk *In Vitro*

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Abstract. Kefir is a traditional fermented milk beverage with the addition of a starter grain kefir. Fermentation time is one of the critical points in the processing of kefir and the resulting compounds that have the potential for their functional properties. The purpose of the research was to study the effect of different types of milk and fermentation time on the potential functional properties of kefir optima cow milk and goat milk *in vitro*. This research was carried out with two factors, namely the type of milk (cow milk (K1)) and goat milk (K2)) and the fermentation time of kefir consist of 12 hours (F1), 24 hours (F2), 36 hours (F3), and 48 hours (F4), each treatment has three replicates. Variables observed were functional properties included potential antihypertensive, antibacterial, antidiabetic, and cholesterol assimilation. The data were statistically analyzed using the General Linear Model (GLM) test with a 95% confidence level ($p < 0.05$) and if there was a significant effect, the Duncan Multiple Range Test (DMRT) was further tested. The results showed that fermentation time in kefir optima cow milk and goat milk had an effect ($p < 0.05$) on the functional properties. It can be concluded that the fermentation time had a significant effect and the 48 hours of fermentation time had the potential to provide the best functional properties.

Keywords : kefir optima, antihypertensive, antibacterial, antidiabetic

1. Introduction

Kefir is a fermented milk product which has unique characteristics, namely sour taste and creamy due to the combination of alcoholic fermentation and lactic acid from lactose in milk. Kefir is categorized into three types, namely whey which comes from a clear layer (whey),

prime kefir which comes from a solid layer (curd), and optima kefir which is a unit or whole layer of clear and stirred solids. Kefir is generally produced using raw materials in the form of cow milk but can also be processed from sheep milk, camel milk, buffalo milk and goat milk. Cow milk and goat milk are types of milk that are easy to find.

Kefir has potential functional properties because it contains bioactive components, namely peptides and exopolysaccharides formed during fermentation so that they provide health benefits [1]. One of the critical points that need to be considered in the processing of kefir is the length of fermentation. Fermentation for too long can produce excessive lactic acid bacteria, while the lack of fermentation time causes a lack of nutritional needs which reduces the number of lactic acid bacteria so that fermentation fails [2]. Therefore, it is necessary to conduct research to determine the potential functional properties of kefir, such as antihypertensive, antibacterial, antidiabetic, and cholesterol assimilation *in vitro* with different fermentation time treatments on kefir optima of cow milk and goat milk.

2. Material and Methods

This research was carried out with a factorial design consisting of two factors, namely the type of milk (cow milk (K1) and goat milk (K2)) and the fermentation time of kefir 12 hours (F1), 24 hours (F2), 36 hours (F3), and 48 hours (F4). Each treatment has three replicates. The materials used in this research were goat milk, cow milk, starter kefir grains, bacterial cultures of *Escherichia coli* and *Staphylococcus aureus*, ampicillin, Angiotensin Converting Enzyme (ACE), captopril, Cholesterol Oxidase – Peroxidase Aminoantipyrin (CHOD-PAP) reagent kit, cholesterol 99% (Sigma), α -glucosidase enzyme 0.04 Unit/ml.

2.1. Preparation Optima Kefir

Kefir making from cow milk and goat milk refers to Bayu *et al.*, [3] with some modifications. The tools to be used are sterilized first. Cow milk and goat milk were pasteurized at 70°C for 15 seconds, then lowered the temperature to 20-22°C, then transferred to sterile jars. Pasteurized milk is added with 5% kefir grains of the total milk (w/v) then covered with plastic wrap and jar lids, then fermented at room temperature with various fermentation time (12 hours; 24 hours; 36 hours; and 48 hours), then, filtered through a sterile wire filter to separate the clear kefir from the solids (granules).

2.2. Antihypertensive Testing

The sample preparation used for antihypertensive testing, namely the kefir sample extraction process which refers to Wulandani *et al.*, [4] with a modification, namely the kefir pH is set at 4.0, then heated in a water bath at 45°C for 10 minutes and then centrifuged then bring it to a pH of 7.0 and centrifuged for testing. Antihypertensive testing using the ACE inhibition method Febrisiantosa *et al.*, [5]. First, 50 L of HHL 7.6 mM solution was added to 6 µL sample in tube, then vortexed and incubated at 37°C for 5 minutes. Then 20 µL of ACE enzyme 20 mU/ml was added and incubated for 5 minutes at 37°C, then 554 µL 0.1 M HCl was added, then 1.5 mL ethyl acetate was added, then centrifuged. Supernatant was taken into a test tube and oven it at 100°C for 45 minutes, then 1 ml of 1M NaCl was added, and then the absorbance was read at a wavelength of 228 nm. ACE inhibitory activity (%) was calculated using the formula:

$$\text{ACE Inhibitory activity (\%)} : (C-S) / (C-B) \times 100\%$$

Description :

- C : Absorbance control
- B : Absorbance blank
- S : Sample absorbance

2.3. Antibacterial Testing

Antibacterial testing using agar well diffusion method refers Chifiriuc *et al.*, [6] with a modification. 25 ml of Mueller Hinton Agar (MHA) medium was poured to petri dish and wait until solidify. 0.1 ml bacterial suspension was prepared on the surface of the MHA medium. Then, wells with a diameter of 6 mm were created by using a cork borer. Sample as much as 50 µl were poured into the wells, and petri dish were incubated at 37°C for 24-48 hours. After that, the sensitivity and resistance of each tested bacteria were determined by measuring the inhibition zone diameter around the wells.

2.4. Antidiabetic Testing

Preparation of samples used for antihypertensive testing, namely the kefir sample extraction process referring to Wihansah *et al.*, [7] with modifications. 6 ml of kefir was prepared and the pH was adjusted to pH 4 and then incubated at 45°C for 10 minutes. After that, the sample was centrifuged then

the supernatant was taken and the pH was then raised to 7.0 and centrifuged to take the supernatant for testing. Enzyme inhibition α -glucosidase refers to the research method of Kabir *et al.*, [8] modified. The 10 μ L sample was put into a microplate, then added 50 μ L phosphate buffer (pH 7) and mixed with 25 μ L of α -glucosidase enzyme (0.04 U/ml), then pre-incubated at 37°C for 5 minutes, Then p-nitrophenyl -D-glucopyranoside (pNPG) was added, then incubated at 37°C for 20 minutes, then stopped by adding 100 μ L Na₂CO₃ 0.2 M, then the absorbance of the solution was measured at a wavelength of 410 nm. The percentage of inhibition of α -glucosidase determined by the following formula :

$$\text{Inhibition of } \alpha\text{-glucosidase (\%)} = (C - (S - B)) / C \times 100\%$$

2.5. Cholesterol Assimilation Testing

Cholesterol assimilation testing method refers to the method of Widodo *et al.*, [9] with a modification. Cholesterol solution as much as 1% was added to the kefir sample and incubated for 24 hours at 37°C, then centrifuged to obtain the supernatant. The cholesterol concentration in the supernatant was determined using the cholesterol oxidase phenol 4-aminoantipyrine peroxide (CHOD-PAP) reagent. As much as 2 μ L of sample was added to 200 μ L of CHOD-PAP reagent in microplate, then incubated at 37°C for 10 minutes and the absorbance was measured at 500 nm using ELISA reader. Cholesterol levels were determined based on the standard cholesterol curve with absorbance values using standard cholesterol concentrations of 10, 20, 30, 40, 50 mg/dl. Determination of cholesterol levels was carried out using the regression equation $y = bx + a$ resulting from the standard concentration plot and its absorbance. Calculation of cholesterol assimilation is calculated by the following formula:

$$\text{Cholesterol Assimilation (mg/dl)} = \text{Cholesterol level sample} - \text{control}$$

2.6. Statistical Analysis

Data analysis was carried out statistically with the General Linear Model (GLM) test with a 95% confidence level ($p < 0.05$) and if there was a significant effect, the Duncan Multiple Range Test (DMRT) was further tested.

3. Results and Discussion

3.1. Antihypertensive

The results showed that fermentation time in kefir optima cow and goat milk had an effect ($p < 0.05$) on the ACE inhibition that can be seen in Table 1.

Table 1. Analysis Results of Antihypertensive Testing on Kefir Optima Cow and Goat Milk with Different Fermentation Time

Type of milk	Fermentation time				Average
	12 hours (F1)	24 hours(F2)	36 hours(F3)	48 hours(F4)	
Kefir Optima Cow Milk(K1)	38.24±1.61	41.95±1.35	72.71±0.31	78.82±1.25	57.93
Kefir Optima Goat Milk (K2)	40.95±0.97	64.85±0.46	68.23±0.60	72.41±0.53	61.61
Average	39.60 ^a	53.40 ^b	70.47 ^c	75.62 ^d	

Note:

^{a-d} Values with different lowercase superscripts indicate a significant difference ($p < 0.05$)

Based on the results showed that the type of milk used had an effect ($p < 0.05$) on the ACE inhibitory activity and showed that the kefir with the fermentation time used had a significant effect ($p < 0.05$) on the ACE inhibitory activity of kefir optima cow milk and goat milk. The highest ACE inhibition was achieved by K1 which has 48 hours fermentation. Based on the results obtained, it can be seen that the inhibition of ACE increases with the duration of fermentation because there are bioactive peptides that are thought to have the ability to act as ACE inhibitors. The ACE inhibitory activity of milk fermented with probiotic bacteria increased with fermentation time and reached the highest value after 48 hours of fermentation, which was 85% [10]. The content of bioactive peptides is produced during fermentation through the proteolysis process of milk. High proteolytic activity can increase the formation of bioactive peptides, but not all types of bioactive peptides have the ability to inhibit ACE [11].

The results of a study on milk fermentation with lactic acid bacteria and yeast showed that the peptide compound that had the ability to inhibit ACE was derived from β -casein derivatives Val-Pro-Pro and Ile-Pro-Pro as well as k-casein [12]. Kefir optima goat milk has a higher average inhibition, presumably because it contains more k-casein than cow milk. The β -casein content of goat milk is 50.8% while that of cow milk is only 34.0% [13].

The positive control used was captopril which had ACE inhibition of 89.90%. When compared with positive control, kefir with fermentation duration of 12, 24, 36 and 48 hours had lower ACE inhibition because captopril is one of the synthetic drugs that can lower blood pressure by ACE inhibition, while the peptides in kefir are more natural sources, safe so that captopril is higher in ACE inhibition [14]

3.2. Antibacterial

The results showed that fermentation time in kefir optima cow and goat milk had an effect ($p < 0.05$) on the antibacterial properties that can be seen in Table 2 and Table 3

Table 2. Inhibition Zones (diameter in mm) of Antimicrobial Activity Kefir Optima Cow and Goat Milk with Different Fermentation Time Against *Escherichia Coli*

Type of Milk	Fermentation Time				Average
	12 hours (F1)	24 hours(F2)	36 hours(F3)	48 hours(F4)	
Kefir Optima Cow Milk (K1)	7.85±0.20	9.30±0.39	9.97±0.17	10.87±0.26	9.49
Kefir Optima Goat Milk (K2)	9.09±0.28	9.83±0.24	10.52±0.60	11.70±0.52	10.28
Average	8.47 ^a	9.56 ^b	10.24 ^c	11.28 ^d	

Note:

^{a-d} Values with different lowercase superscripts indicate a significant difference (p<0.05)

Table 3. Inhibition Zones (diameter in mm) of Antimicrobial Activity Kefir Optima Cow and Goat Milk with Different Fermentation Time Against *Staphylococcus. aureus*

Type of Milk	Fermentation Time				Average
	12 hours(F1)	24 hours(F2)	36 hours(F3)	48 hours(F4)	
Kefir Optima Cow Milk (K1)	7.08±0.93	10.01±0.88	9.89±0.46	10.40±0.68	9.34
Kefir Optima Goat Milk (K2)	6.00±0.00	6.76±0.24	8.29±0.37	10.03±0.42	7.77
Average	6.54 ^a	8.38 ^b	9.09 ^b	10.21 ^c	

Note:

^{a-c} Values with different lowercase superscripts indicate a significant difference (p<0.05)

Based on the results showed that the type of milk used had an effect (p<0,05) on antibacterial potential against *E. coli* and *S. aureus*., Kefir with the fermentation time used had significant effect (p<0.05) on antibacterial potential potential against *E. coli* and *S. aureus*. The highest diameter of the inhibition zone was achieved by kefir which has 48 hours fermentation. The highest inhibition was achieved by kefir which had a 48 hours fermentation time against *E. coli* (15 mm) and *S. aureus* (14.67 mm) [15]. The diameter of the inhibition zone increased with the duration of fermentation time because there are antibacterial compounds formed during fermentation consist of organic acids, bacteriocins, and hydrogen peroxide [16].The increased in organic acids was related to the lower pH on kefir. Microbes in kefir grains can break down lactose into organic acids, such as lactic acid and acetic acid, so can lowering the pH value on kefir and created acidic conditions that can inhibited the growth of pathogenic bacteria [17]. Lactic acid kill *E. coli* bacteria at pH below 5.

Based on the results, kefir optima goat milk has a higher potential to inhibit *E. coli* bacteria than kefir optima cow milk. Goat milk was able to inhibit *E. coli* bacteria higher which is proved by inhibition diameter zone at 27.1 mm, while cow milk was 17.5 mm [18]. This is presumably because the lower pH is able to accumulate organic acids so that it can kill

pathogenic bacteria. The accumulation of organic acids make lower pH below the pH of bacterial growth, then enter into the cells so the pathogenic bacteria can die.

Based on the results, Inhibition zone diameter increases with the duration of fermentation time that make decrease in the pH of kefir so that *S. aureus* bacteria cannot grow optimally. *S. aureus* bacteria are pathogenic bacteria that cannot grow optimally below pH 4 [19]. The inhibition of *S. aureus* is also due to antibacterial compounds that increase with the duration of fermentation, such as bacteriocin and hydrogen peroxide. Bacteriocins are easier to interact with gram-positive bacteria, such as *S. aureus* because the cell walls of gram-positive bacteria contain specific receptors capable of binding to bacteriocins, namely lipoteichoic acid. Bacteriocins that bind with lipoteichoic acid can disrupt cell membranes so formed the hole in target cells that can give effect to bactericidal activity [20]. Hydrogen peroxide compounds produced by *Lactococci* group bacteria are also effective in killing *S. aureus* by damaging the microbial structure resulting in the release of intracellular components, then oxidized resulting in disruption of protein synthesis and energy production resulting in cell death [15].

The positive control used was ampicillin capable of inhibiting *E. coli* and against *S. aureus*. This indicates that ampicillin can inhibit gram-positive and gram-negative bacteria. Ampicillin is a broad-spectrum penicillin class of antibiotics because it is able to penetrate both gram-positive and negative bacteria and resistant to acidic conditions [21].

3.3. Antidiabetic

The results showed that fermentation time in kefir optima cow and goat milk had an effect ($p<0.05$) on the α -glucosidase enzyme inhibition that can be seen in Table 4.

Table 4. Analysis Results of Testing And diabetic Kefir Optima Cow and Goat Milk with Different Fermentation Time

Type of Milk	Fermentation Time				Average
	12 hours(F1)	24 hours(F2)	36 hours(F3)	48 hours(F4)	
Kefir Optima Cow Milk (K1)	41.49±0.57	50.79±0.20	58.15±0.86	63.01±0.94	53.36
Kefir Optima Goat Milk (K2)	45.56±0.60	54.04±0.78	61.76±0.72	75.49±0.36	59.21
Average	43.52 ^a	52.41 ^b	59.95 ^c	69.25 ^d	

Note:

a-d Values with different lowercase superscripts indicate a significant difference ($p<0.05$)

Based on the results show that the type of milk used has an effect ($p<0.05$) on the inhibition of the α -glucosidase and shows that kefir with fermentation time used had a

significant effect ($p<0.05$) on the inhibition of the enzyme α -glucosidase kefir optima in cow milk and goat milk. The highest inhibition of enzyme α -glucosidase was achieved by K2 which has 48 hours fermentation. Inhibition of the α -glucosidase increases with the duration of fermentation because there are bioactive peptides and exopolysaccharides that are thought to have the ability to inhibit the α -glucosidase [22]. Bioactive peptides are produced from proteolysis. Protein degradation increases by about 6-8% as the fermentation time increases due to proteolytic enzymes from lactic acid bacteria that degrade milk protein into peptides and amino acids [23]. The kefir content in kefir is able to inhibit the α -glucosidase enzyme because kefir is an exopolysaccharide compound that can bind to the active site of the enzyme.

Based on the results obtained, the inhibitory ability of the α -glucosidase enzyme in goat milk optima kefir is greater than that of cow milk optima kefir. This is presumably because the protein content. Protein in goat milk kefir (3.69%) is higher than cow milk kefir (3.24%) [24]. Therefore, it is suspected that the more protein content in goat milk kefir can increase the formation of bioactive peptides that have the ability to inhibit α -glucosidase enzyme.

3.4. Cholesterol Assimilation

The results showed that fermentation time in kefir optima cow and goat milk had an effect ($p<0.05$) on the cholesterol assimilation that can be seen in Table 5.

Table 5. Analysis Results of Testing Cholesterol Assimilation Kefir Optima Cow and Goat Milk with Different Fermentation Time

Type of Milk	Fermentation Time				Average
	12 hours(F1)	24 hours(F2)	36 hours(F3)	48 hours(F4)	
Kefir Optima Cow Milk (K1)	5.65±0.35	6.67±0.86	6.74±0.37	7.15±0.38	6.40
Kefir Optima Goat Milk (K2)	1.13±0.12	2.06±0.15	4.68±0.28	6.03±0.73	3.48
Average	3.39 ^a	4.07 ^b	5.71 ^c	6.59 ^d	

Note:

^{a-d} Values with different lowercase superscripts indicate a significant difference ($p<0.05$)

Based on the result showed that the type of milk used had an effect ($p<0.05$) on the cholesterol assimilation. Kefir with the fermentation time used had a significant effect ($p<0.05$) on the cholesterol assimilation optima kefir in cow milk and goat milk. The highest cholesterol assimilation was achieved by K1, which was 48 hours fermentation time.

Cholesterol assimilation increased with the duration of fermentation time due to lactic acid bacteria [25]. The types of lactic acid bacteria, namely *Lactobacillus fermentum* and *Lactobacillus acidophilus* isolated from danke which is fermented product, were able to reduce cholesterol by 5.6 mg/dl because lactic acid bacteria were able to assimilate cholesterol [26]. Cholesterol can be bound to the cell wall of lactic acid bacteria, then enter to cell membrane, so it can resistant to lysis [27]. The highest cholesterol reduction was achieved during the bacterial growth phase because more bacteria can bind cholesterol [28].

Based on the results obtained that kefir optima cow milk has the potential to assimilate higher cholesterol than kefir optima goat milk, because presumably high lactose content in cow milk can increases growth of more lactic acid bacteria that thought to make more cholesterol assimilated. The lactose content in cow's milk is 4.56 g while goat's milk is 4.23 g [29].

4. Conclusion

Based on the results of the research conducted, it can be concluded that the type of milk and the duration of fermentation affect the functional properties of kefir optima for cow milk and goat milk. Different fermentation time also had a significant effect and 48 hours of fermentation had the potential to provide the best functional properties.

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