Antioxidant Activity of Fermented Goat's Milk with the Use of *Bifidobacterium longum*

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ABSTRACT

Fermented goat's milk with *Bifidobacterium longum* has the potential as a source of antioxidants to counteract free radicals. This study aims to determine the antioxidant activity of fermented goat's milk using *Bifidobacterium longum* as a starter which is thought to have high antioxidant activity. The design used was a completely randomized design (CRD) consisting of 4 (four) treatments, namely Control (pasteurized goat milk), 2.5% *Bifidobacterium longum*, 5% *Bifidobacterium longum,* and 7.5% *Bifidobacterium longum*, each treatment was repeated 4 times. The parameters in this study were total plate count, pH test, lactic acid test, and antioxidant activity. The results showed that the treatment of fermented goat's milk with the addition of starter *Bifidobacterium longum* with different percentages had a very significant effect (P<0.01) on the total number of lactic acid bacteria, pH values, and lactic acid levels. In addition, it also increases antioxidant activity with the lowest IC_{50} value at 7.5% *Bifidobacterium longum* which is classified as strong antioxidant activity. In conclusion, *Bifidobacterium longum* starter is potential enough for further research to be carried out.

Keywords: antioxidant, Bifidobacterium longum, goats milk.

1. INTRODUCTION

An Antioxidant is an electron-donating chemical compound that works by donating one of its electrons to a free radical to inhibit its radical activity [1]. According to [2], sources of antioxidants are divided into two groups, namely synthetic and natural antioxidants. Synthetic antioxidants include those obtained from the synthesis of chemical reactions, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate, and ethoxyquin [3]. This type of antioxidant is widely used to control oxidation, but its after-effects can be carcinogenic.

Natural antioxidants are those sourced from plants and animals [4]. One of the livestock commodities, milk, is said to contain several natural antioxidants such as lactoferrin, ascorbic acid, α-tocopherol, and carotenoids [5]. Natural antioxidants may play a role as a reducing agent, free radical scavenger, pro-oxidant metal chelator, and singlet oxygen quencher [6,7].

Fermented milk can be made of goat milk that has several benefits such as serving as an alternative to milk for those with lactose intolerance, providing probiotic bacteria for the digestive system, antioxidants, and acting as free radical scavengers [8]. Fermented goat milk contains bioactive peptides that consist of 16 amino acids that play an antioxidative role [9]. A study by [10] emphasized that antioxidant is one of the important benefits of fermented milk. Fermented goat milk has an increased content of active antioxidants [11] which is thought to originate from metabolites formed during the lactic acid fermentation process such as bioactive peptides.

The use of *Bifidobacterium longum* as a starter is quite potent in fermented milk. *Bifidobacterium longum* is a Gram-positive, catalase-negative, non-motile, rod-shaped bacterium [12]. This *Bifidobacterium* strain can produce a natural antibiotic called bifid. In addition to producing lactic acid, this bacterium can also produce acetate acid that can reduce gastrointestinal pH and is bactericidal, which means it can prevent the growth of

pathogenic bacteria in the intestine, prevent dysbiosis of the gut bacteria, and function as an antioxidant [13]. [14] added that the population of *Bifidobacterium longum* in the human ileum (intestinal absorption) reaches 10^6 cfu/g and 10^{10} cfu/g in the large intestine. The cell generally appears in pairs like the letter V or Y.

Lin and Chang demonstrated that *Bifidobacterium longum* has free-radical-preventing property. A study by [15] on fermented milk using *Bifidobacterium longum* BB536 starter showed high antioxidant activity. In this study, it is expected that the use of *Bifidobacterium longum* with different percentages can improve the antioxidant activity and quality of the fermented goat milk.

2. MATERIALS AND METHODS

The study was conducted in the Milk Processing Science Laboratory and Technology of Animal Husbandry Major of Faculty of Agriculture of Universitas Syiah Kuala, Darussalam, Banda Aceh. The analysis of antioxidant activity was conducted in the Food Analysis Laboratory of Agricultural Product Technology Major of Faculty of Agriculture of Universitas Syiah Kuala.

The instruments used in the study were a laminar air flow (Sanyo), autoclave (Sanyo), incubator (Sanyo), digital scale (Acadapter), hotplate (IKA), water bath, vortex (IKA), colony counter, burette, pH meter, spectrophotometer, micropipette, microtip, 500-ml beaker, test tube, denatured alcohol lamp, petri dish, 100-ml beaker, 200-ml sample bottle, 200-ml Erlenmeyer flask, and 500-ml Erlenmeyer flask. The materials used included goat milk, *Bifidobacterium longum* starter FNCC 02010, deMan Rogosa Sharpe Agar (Oxoid), aquades, 70% alcohol, NaOH 0,1 N, phenolphthalein solution, denatured alcohol, aluminum foil, a pair of gloves, masks, cottons, tissues papers, and paper labels.

This study was conducted using a Completely Randomized Design consisting of 4 treatments, namely P_0 = Control (pasteurized goat milk), P_1 = 2.5% *Bifidobacterium longum*, P_2 = 5% *Bifidobacterium longum*, and P_3 = 7.5% *Bifidobacterium longum*, with each treatment repeated 4 times resulting in 16 test units. The total plate count, pH values, and lactic acid levels obtained were then analyzed using Analysis of Variance (ANOVA), and if the results showed an effect on the treatment, then proceeded with the Duncan Multiple Range Test (DMRT) [16]. The antioxidant activity data obtained were analyzed with a descriptive analysis using SPSS software.

2.1. Procedure for Making Fermented Goat Milk

The production of fermented goat milk was based on the method by [17]. Raw goat milk was pasteurized at a temperature of 85°C for 15 seconds and then cooled to a temperature of 40°C. The pasteurized goat milk was put into sample bottles, inoculated with *Bifidobacterium* starter according to the predetermined treatments, and homogenized by stirring. The inoculated goat milk was incubated for 18 hours at 37°C. Then, pH tests, lactic acid level, and fermented goat milk antioxidant activity tests were carried out.

2.2. Starter Lactic Acid Bacteria Population Test

The lactic acid bacteria population test used a method by [18]. Total lactic acid bacteria were counted using the pour plate method. 1 ml starter was diluted starting from 10^{-1} dilution to 10^{-7} dilution. Total lactic acid bacteria were tested from the 10^{-7} dilution, then inoculated in a deMan Rogosa Sharpe Agar (MRSA) medium. Then, a petri dish was rotated clockwise to homogenize the bacterial suspension and the medium. Once the agar solidified, the petri dish was incubated in the incubator upside down at 37°C for 48 hours. The number of BAL colony growth was counted using a colony counter. BAL colonies in cfu/ml used the following formula.

$$CFU/ml = Number\ of\ Colony \times \frac{1}{diluent}$$

2.3. pH Value Test

The pH value test used a method by [19]. pH was measured using a pH meter. Before use, the pH meter was calibrated with buffer solution with pH 4 and 7. Then, the electrode was flushed with aquades and dried using tissue papers. Then, the sample's pH was measured by dipping the pH meter electrode into 10 ml of the sample and the number displayed on the pH meter was recorded. After that, the electrode must be flushed with aquades and dried using tissue papers before another sample was put in.

2.4. Lactic Acid Level Test

The lactic acid test was performed using a method by [20]. 18 ml of the sample was put into an Erlenmeyer flask, then 3 to 4 drops of phenolphthalein solution were added as an indicator. Then, the sample was titrated using NaOH 0.1 N solution until a stable pink color formed. The amount of the NaOH 0.1 N solution used for titration was recorded. Total lactic acid was calculated using the following formula:

$$Lactic\ Acid\ Level = \frac{ml\ NaOH \times N \times 90}{Sample\ weight\ (g) \times 1000} \times 100\%$$

2.5. Antioxidant Activity Test with the IC$_{50}$ Method

Each concentrate was tested for antioxidant activity using a method by [21]. 0.2 mM sample plus 0.1 mM

DPPH dissolved in 4 ml of ethanol until a purple color formed on the sample. Ethanol was used as a blank which was treated the same as the sample. Then, the sample was homogenized using a vortex, incubated in a dark room for 30 minutes, and treated to a spectrophotometer at a wavelength of 517 nm to produce an absorbance. Determination of antioxidant activity used the following formula. The value inhibition to determine the antioxidant activity value in the sample was determined using the following formula [22].

$$\% \, AA = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \, x \, 100\%$$

Description:

Sample absorbance	:	DPPH absorbance being reacted with the sample
Control absorbance	:	DPPH absorbance without the sample
DPPH	:	*1.1-diphenyl-2-picrylhydra-zyl*

Then, the IC_{50} value was calculated using a regression equation formula. IC_{50} was the concentrate needed to reduce DPPH by 50%. This was intended to obtain a more optimal concentration as an antioxidant in suppressing free radicals. IC_{50} was calculated using a linear regression method, with sample concentration as x axis and % inhibition as y axis.

From the equation $y = a + bx$, IC_{50} could be calculated using the following formula:

$$y = a + bx$$
$$50 = a + bx$$
$$(x) \, IC_{50} = \left(\frac{50 - a}{b}\right)$$

Information:
Y : % inhibition
a : Intercept (Y-line intersection)
b : Slope
X : Concentration

3. RESULTS AND DISCUSSION

3.1. Total Population of Lactic Acid Bacteria

Based on the analysis of variance, fermented goat milk treatments with different percentages of *Bifidobacterium longum* starter have a significant effect (P<0.01) on the total lactic acid bacteria value. The average total population of lactic acid bacteria can be seen in Figure 1.
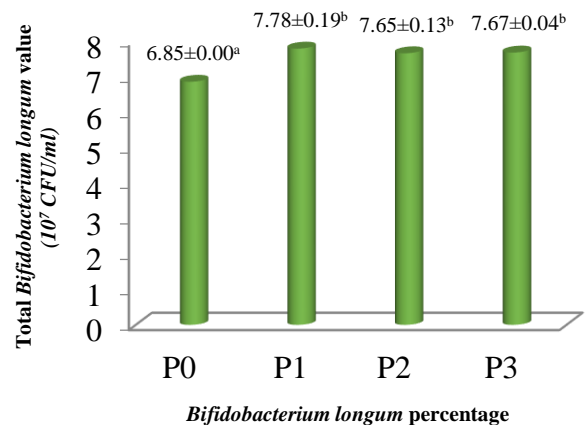
Note: a-b indicated highly significant different effect (P<0.01)

P0 = Control (pasteurized goat milk), P1 = 2.5% *Bifidobacterium longum*, P2 = 5% *Bifidobacterium longum*, and P3 = 7.5% *Bifidobacterium longum*

Figure 1 Total population of lactic acid bacteria

The analysis results of total *Bifidobacterium longum* ranged between 6.85×10^7 to 7.78×10^7 cfu/ml which meets the standards for fermented milk. It is in accordance with the [23] stating that the minimum requirement for the total number of good lactic acid bacteria is 10^6 cfu/ml. [24] stated that the minimum requirement for total lactic acid bacteria is 10^7 cfu/ml.

Further test with the Duncan Multiple Range Test shows a significant difference between control and other treatment groups, but not between P1, P2, and P3. Goat milk (P0) has high nutritional content and has the potential as a source of nutrition for the growth of *Bifidobacterium longum*. [25] explained that bacterial growth was influenced by several factors such as the chemical composition of milk, amount of inoculum, temperature, incubation time, and cooling time of milk. To produce quality fermented milk, starter culture, fermentation condition, and milk quality should be carefully chosen [26].

3.2. pH Value

The results of the analysis of variance show that the use of *Bifidobacterium longum* at different percentages has a significant effect (P<0.01) on pH values. The average pH value can be seen in Figure 2.

An average pH value in goat milk (P0) of 6.30 already meets the standard pH value according to the [27], namely pH 6.3 - pH 6.8. This helps optimal growth of *Bifidobacterium longum* at pH 6.0 - 7.0 [28]. The average pH value of fermented goat milk with *Bifidobacterium longum* at different starter percentages ranged from 3.92 to 4.03, which overall is in accordance with the standard according to [29], saying that the pH value of good fermented milk ranges from 3.5 to 4.5.

The low pH value is strongly related to the acid production during the milk fermentation process [30]. A Similar result was also obtained by [31] where the pH value of synbiotic drink added with skimmed milk using *Bifidobacterium longum* was 3.66.
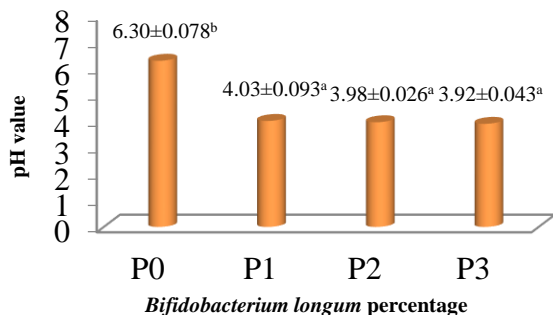
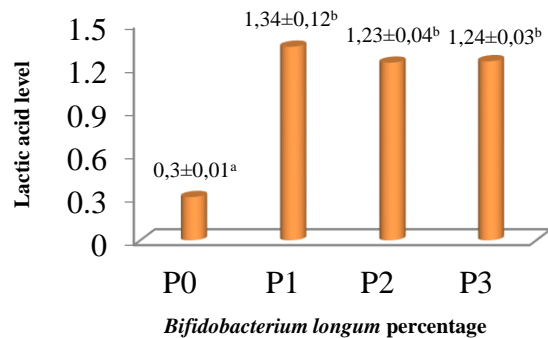Note: a-b indicated highly significant different effect (P<0.01)

P0 = Control (pasteurized goat milk), P1 = 2.5% *Bifidobacterium longum*, P2 = 5% *Bifidobacterium longum*, and P3 = 7.5% *Bifidobacterium longum*

Figure 2 Average pH value

Based on the Duncan Multiple Range Test, there was a significant difference between control and other treatment groups. In this study, *B. longum* works well in the three starter presentations, however, no difference was found between treatments that were added with starter (P1, P2, and P3). According to [32], during the fermentation process, lactic acid bacteria will remodel carbohydrates such as lactose to form lactic acid. The formation of lactic acid causes the pH value of the growth environment to decrease and causes the formation of coagulation. pH value is closely related to the amount of acid produced during the milk fermentation process [33]. This is because the type of lactic acid bacteria found in fermented milk plays an important role in the production of lactic acid thereby affecting pH [34]. During fermentation, lactic acid bacteria will produce lactic acid, citric acid, and acetic acid which will lead to a decrease in pH [35].

3.3. Lactic Acid Levels

Based on the analysis of variance, fermented goat milk treatments with different percentages of *Bifidobacterium longum* starter have a significant effect (P<0.01) on the lactic acid level. According to [36], the standard lactic acid for fermented milk is 0.5% to 2.0%. In our results, the lactic acid levels ranged from 1.23% to 1.34% which is still within the [36] range. The lactic acid level in the goat milk (P0) was 0.3%. The average lactic acid level can be seen in Figure 3.

Note: a-b indicated highly significant different effect (P<0.01)

P0 = Control (pasteurized goat milk), P1 = 2.5% *Bifidobacterium longum*, P2 = 5% *Bifidobacterium longum*, and P3 = 7.5% *Bifidobacterium longum*

Figure 3 Average of the lactic acid level

Bifidobacterium longum bacteria are classified as heterofermentative bacteria, meaning that they produce not only lactic acid but also acetic acid, ethanol, and CO_2. This type of bacteria takes longer to ferment carbohydrates than other types of BAL such as *Streptococcus* and *Lactobacillus* [37]. This study used starter percentages of 2.5%, 5%, and 7.5% which were incubated at 37ºC for 18 hours. According to [38], fermenting milk for 12 hours to 18 hours will produce fermented with high lactic acid and favorable physical quality. The amount of starter will affect the degree of acidity because carbohydrates will be converted into lactic acid during the fermentation process [30].

Based on the Duncan Multiple Range Test, there was a significant difference between control and other treatment groups (P1, P2, and P3). There was an increase in the lactic acid level due to the addition of *B. longum*. According to [39], The amount of lactic acid will increase along with the increase in the number of lactic acid bacteria. Lactic acid bacteria convert lactose into lactic acid [40], with enzyme activity produced by lactic acid bacteria and compounds contained in milk such as albumin, casein citrate, and phosphate [41]. Lactose in milk is a carbohydrate available for the formation of energy by lactic acid bacteria which have the enzyme lactate dehydrogenase for the formation of lactic acid [42].

3.4. Antioxidant Activity

Antioxidants, in food terms, are compounds in food that possess the property to slow down, inhibit, or prevent the oxidation process that causes food to spoil [43]. The samples of goat milk fermented using *Bifidobacterium longum* reacted with a solution of DPPH (*2,2-diphenyl-1-picrylhydrazyl*) changed the color of the solution from purple to yellow, then absorbed at a wavelength of 517 nm. [44] stated that the

presence of a compound that can donate its hydrogen atom causes the change in DPPH's color from purple to pale yellow. This wavelength will provide maximum absorbance.

Based on the study, a regression equation as seen in Table 4 was obtained. This equation can be used to calculate the IC_{50} value. The calculation used in determining the antioxidant activity was the IC_{50} value (50% Inhibitor Concentration). This value describes the concentration of the test compound that can capture free radicals by 50%. The IC_{50} value was obtained by using a linear regression equation which stated the relationship between the sample concentration as the abscissa (X axis) and the percent value of antioxidant inhibition as the ordinate (Y axis) [4]. The IC_{50} value was inversely proportional to the antioxidant activity, that is, the smaller the IC_{50} value, the higher the antioxidant activity.

As seen in Table 1, IC_{50} values from the lowest to the highest are 99.117 ppm (P3), 108.833 ppm (P2), 118.003 ppm (P0) and 123.463 ppm (P1). According to [44], based on IC_{50} values antioxidant activity can divided into 5 categories, namely very strong (IC_{50} < 50 ppm), strong (50 ppm < IC_{50} > 100 ppm), moderate (100 ppm < IC_{50} > 150 ppm), weak (150 ppm < IC_{50} > 200 ppm), and very weak (IC_{50} > 200 ppm).

Table 1 Antioxidant (IC_{50}) value

Sample	Equation	Value of Y	IC_{50}
P0	y = 0,2704x + 18,092	50	118,003
P1	y = 0,4671x + 7,6696	50	123,463
P2	y = 0,5064x - 5,1131	50	108,833
P3	y = 0,499x - 0,5408	50	99,117

P3 is in the strong antioxidant category (50 ppm < IC_{50} >100 ppm), while P0, P1, and P2 are in the moderate antioxidant category (100 ppm < IC_{50} > 150 ppm). This could be because the *Bifidobacterium longum* percentage of 7.5% in P3 was higher compared to other treatments. [15] also obtained similar results on commercial yogurt products with Bifidobacterium starter showing an IC_{50} value of 144.7%.

The high antioxidant activity of goat milk protein hydrolysate is caused by the total amount of hydrophobic and aromatic amino acids, one of which is tyrosine [45]. The increase in antioxidant activity was caused by the formation of lactic acid during the milk fermentation process. According to [46], lactic acid in fermented milk contains α-hydroxy acids (AHA) which function as antioxidants. Lactic acid produced by lactic acid bacteria acts as a hydrogen atom donor for free radicals.

Apart from lactic acid, the increase in antioxidant activity is caused by the activity of lactic acid bacteria, which in this study was *Bifidobacterium longum*.

Molecularly, lactic acid bacteria produce antioxidants that can reduce pro-oxidants so that lactic acid bacteria can survive in conditions of oxidative stress [47]. According to [48], *B. longum* has an antioxidative property since it can chelate metal ions. [49] explained that *Bifidobacterium* has a potent active antioxidant activity. Goat milk fermented using a combination of *Lactobacillus acidophilus* and *Bifidobacterium longum* in a ratio of 1:4 was able to increase the antioxidant content by 45.11% [50].

4. CONCLUSION

The results of this study show that goat milk fermentation treatment using 7.5% *Bifidobacterium longum* starter produces increased antioxidant activity, which is evidenced by the total amount of lactic acid bacteria, lactic acid levels, and decreased pH value.

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