

Development of Fermented Etawa Goat Milk Containing Gamma-Aminobutyric Acid (GABA) by Indigenous *Lactiplantibacillus plantarum* subsp. *plantarum* T-3

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Abstract

This research presents the use of an indigenous *Lactiplantibacillus plantarum* subsp. *plantarum* T-3 strain as a starter culture to produce fermented goat milk containing gamma-aminobutyric acid (GABA). The fermented goat milk drink was produced by inoculating Etawa goat milk with a 2 % (w/v) skim and 3 sugar treatments: No added sugar, 8 % (w/v) sucrose, and 8 % (w/v) glucose. The inoculum was a 1 % (v/v) culture of *L. plantarum* T-3 at a concentration of 10^7 colony-forming units (CFU) per milliliter. The concentration of GABA, the pH, titratable acidity, physicochemical properties of the fermented milk, including syneresis, water-holding capacity (WHC), viscosity, and sensory properties, were also evaluated. The product was stored for 5 weeks to ascertain its stability. The concentration of GABA increased significantly during the 24-h fermentation period. At the completion of the process, the treatment involving glucose exhibited the highest GABA concentration, reaching 164.95 mg/L, while the treatment without added sugar demonstrated the lowest concentration, at 132.68 mg/L. The organoleptic test revealed that the panelists preferred the fermented goat milk drink with added sucrose for color, aroma, viscosity, sour taste, and sweet taste. After a period of 5 weeks at a temperature of 4 °C resulted in a notable decline in GABA levels across all treatment groups. The concentration of GABA in the treatment with no added sugar was 105.34 mg/L, followed by the sucrose treatment (77.39 mg/L) and the glucose treatment, which exhibited the lowest concentration (71.33 mg/L). The total lactic acid bacteria (LAB) remained stable at a level of 10^9 CFU/mL, which continues to comply with the requirements of SNI 2981:2009 for yogurt quality, which stipulates a minimum of 10^7 CFU/mL. To further enhance sensory acceptance and GABA concentration, a more comprehensive study is necessary.

Keywords: *Lactiplantibacillus*, Fermentation, Goat milk, Gamma-aminobutyric acid

Introduction

LAB are highly effective fermenters and are used extensively in the food industry to improve the organoleptic, rheological, and nutritional properties of

fermented products [1]. The *Lactobacillus* genus is considered safe for use in food products in the USA due to its extensive involvement in food fermentation [2].

Some LAB are classified as probiotics, living bacteria that can provide health benefits for their hosts when consumed in sufficient quantities [3]. Probiotics also have the potential to produce metabolites known as postbiotics, including GABA [4]. GABA plays a pivotal player in the prevention and management of numerous diseases. It has antihypertensive effects by inhibiting norepinephrine mechanisms in hypertensive patients [5], inhibiting glucagon secretion, inhibiting diabetics [6], and inhibiting cancer cell proliferation [4].

Numerous GABA-producing LAB have been identified in fermented foods [7-10]. Furthermore, some local LABs were successfully isolated from fermented food and had potential as probiotics, including *L. plantarum* Dad-13 isolated from fermented buffalo milk, *L. plantarum* Mut-7 isolated from *gatot* (a cassava-based fermented food), and *L. plantarum* T-3 isolated from *growol* (a cassava-based fermented food) [11]. To further expand the function of local LAB, it is necessary to investigate their potential to produce GABA in fermented milk. The genotype analysis was also carried out using *in silico* analysis to determine the presence of genes encoding enzymes involved in GABA biosynthesis and to determine the residues that play an essential role in its activity.

This study used crossbred Etawa goat milk, a crucial Genetic Resource Asset of Local Livestock in Indonesia that must be preserved and developed [12]. Goat milk is rich in protein, calcium, antioxidants, and antibacterial factors, meeting all the requirements for human nutrition and health [13]. All other amino acids are higher quantities in goat milk than in cow milk, with the exception for tyrosine and serine [14]. Hence, in this study, we used underutilized local isolate *L. plantarum* T-3 as a starter culture for developing fermented goat milk containing GABA and evaluating the product stability during storage at low temperatures. It is anticipated that the utilisation of goat milk as the fundamental ingredient in this study will result in the generation of elevated levels of GABA. This is due to the fact that goat milk contains a higher concentration of glutamic acid, which will undergo conversion by the glutamate decarboxylase (GAD) enzyme into GABA.

Materials and methods

The materials for making fermented milk were fresh raw Etawa goat milk with the following

composition: Fat 5.45 %, protein 4.53 %, total solids 15.61 %, and pH 6.74 (CV Prenjak Ganter, Yogyakarta, Indonesia), skim milk (PT Mirota KSM, Indonesia), and sugar (PT Sweet Indolampung Sugar Groups Companies, Indonesia). The *L. plantarum* T-3 was obtained from the Food Nutrition Culture Collection (FNCC), Centre for Food and Nutrition Studies, Universitas Gadjah Mada. The isolate was propagated in de Man, Rogosa, and Sharpe (MRS) broth (Merck, Darmstadt, Germany) and incubated at 37 °C for 24 h. It was then stored at 4 °C as a working culture. All chemical reagents were from Merck (Darmstadt, Germany) unless otherwise stated.

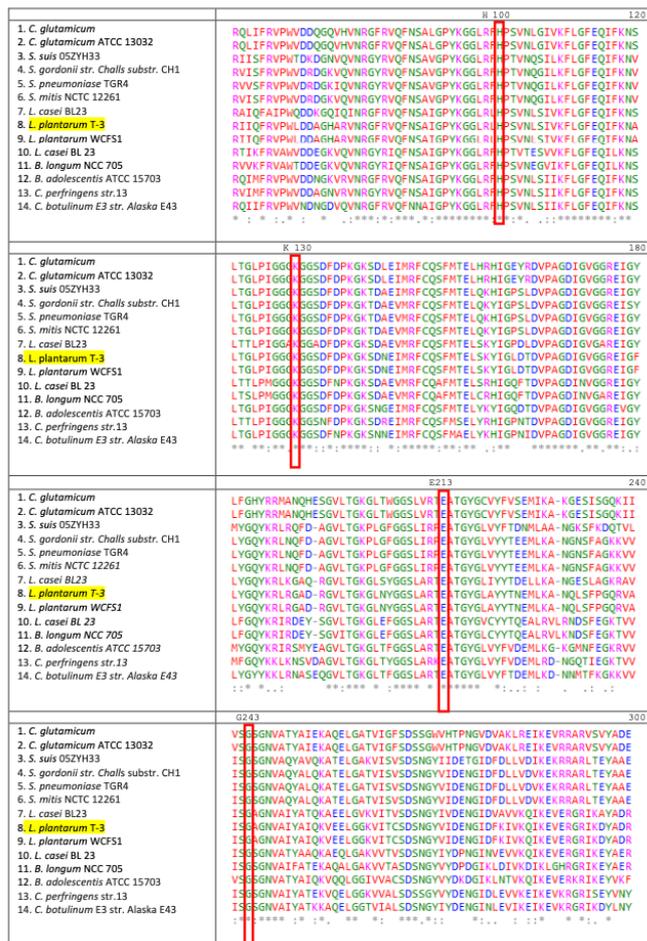
In silico analysis of GABA biosynthetic genes

Annotated genome sequences were identified using the Rapid Annotation Subsystem Technology (RAST) online program (<https://rast.nmpdr.org>) to investigate the presence of enzymes involved in the biosynthesis of GABA. Subsequently, the amino acid sequence of the biosynthetic enzyme was aligned with enzymes from GABA-producing microbes using the MEGA 11 application. This was followed by a comparison with other microbes using Multiple Sequence Comparison by Log-Expectation (MUSCLE) (<https://www.ebi.ac.uk/Tools/msa/muscle/>). Finally, the active side of the enzyme was determined using the Protein Data Bank (<https://www.rcsb.org>).

Fermentation of goat milk

Two percent (w/v) skim milk and an appropriate amount of sugar (depending on the treatment: No added sugar, 8 % (w/v) sucrose, and 8 % (w/v) glucose) were added to fresh goat milk, which was then homogenized at a pressure of 400 bar at 45 °C and then heated in a water bath at 80 °C for 30 min. After the temperature of the milk reached approximately 37 °C, it was inoculated with 1 % (v/v) starter culture at a population of 10⁷ CFU/mL. The milk was then incubated at 37 °C for 24 h. Samples for analysis were taken every 6 h at 0, 6, 12, 18 and 24 h. The analysis included measurement of GABA content, cell viability, evaluation of physicochemical properties (pH, titratable acidity, syneresis, water holding capacity/WHC, viscosity) and sensory evaluation.

A



B

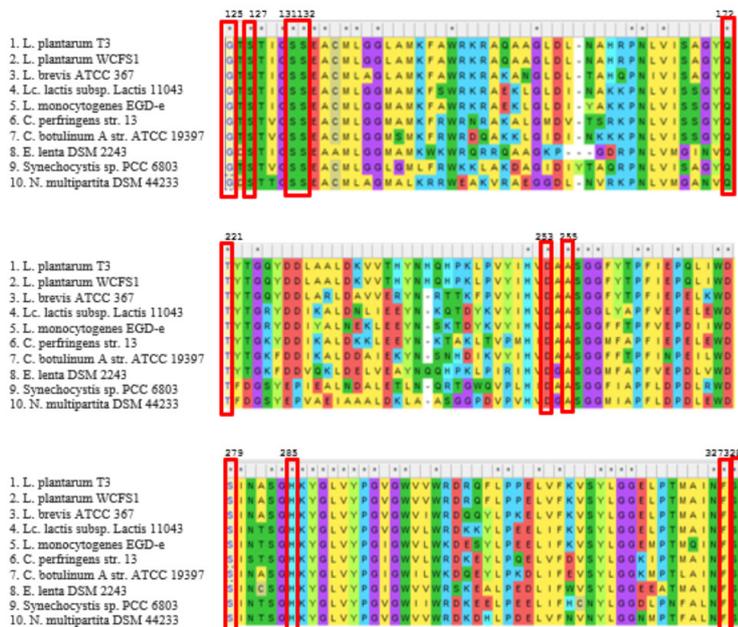


Figure 1 Results Alignment and conservation of amino acid sequence residues from glutamate dehydrogenase (GDH) (A); and glutamate decarboxylase (GAD) (B) in *Lactiplantibacillus plantarum* subsp. *plantarum* T-3 and other bacteria. Catalytic residue are shown in red boxes. Star emblem (*): a fully conserved residue with identical properties; colon (:): quite conserved with high similarity of traits; point (.): only slightly conserved with low resemblance of trait; unblematic (): not conserved with different properties; gap (-): the sequence has no associated nucleotides.

Storage of fermented goat milk

The fermented goat milk was stored in a refrigerator at a temperature of 4 °C for period of 5 weeks. The analysis was conducted on weekly basis at 0, 1, 2, 3, 4 and 5 weeks. The analysis conducted at this juncture encompasses an examination of the GABA content, cell viability, and physicochemical characteristics, including pH, titratable acidity, syneresis, water holding capacity/WHC, and viscosity.

Analytical method

GABA content analysis

A sample of 1.5 mL was transferred to an Eppendorf tube and centrifuged at 8,000 rpm for 10 min. The resulting supernatant was then separated from the cells and filtered through a 0.45 µm syringe filter. The cell-free supernatant was subjected to an assay for GABA using the pre-column derivatization method with o-phthaldialdehyde (OPA), and the sample mixture was injected into the high-pressure liquid chromatography (HPLC) system (Shimadzu LC-10AD VP Liquid Chromatography, Kyoto, Japan) equipped with a fluorescence detector (Shimadzu RF-10A XL, Kyoto, Japan), at wavelength λ_{ex} : 300 nm and λ_{em} : 500 nm using LiChrospher 100 RP-18 column (4.6 mm×150 mm×5µm), with a stepwise gradient of methanol: 50 mM sodium acetate: Tetrahydrofuran (THF) (2:96:2) and methanol: Double- distilled water (13:7). GABA was identified by comparison of the retention time of the GABA standard and the concentration of GABA was then calculated by comparison with the standard curve.

Cell viability

Cell viability was enumerated using the pour-plate method [15]; 1 mL of the sample was diluted with 9.0 mL of sterile distilled water containing 0.85 % NaCl. A series of tenfold dilutions was made and 0.1 mL of the diluted sample was plated onto on MRS agar and incubated at 37 °C for 48 h to allow cell growth. The colonies appearing on the plates were then counted, multiplied by the dilution factor, and expressed as log colony forming units per mL (log CFU/mL).

pH and titratable acidity analysis

The pH and titratable acidity analysis in this study was conducted in accordance with methodology

outlined by AOAC [16]. The pH value was determined using pH meter (OHAUS Starter 5000, New Jersey, United States). The titratable acid value was measured by transferring 5 mL of the sample to a 50 mL Erlenmeyer flask, adding 2 - 3 drops of phenolphthalein indicator, and titrating with a standardized 0,1 N NaOH solution. The titration was performed until the color changed to pink. The volume required to reach the equivalence point and calculate the equivalence point using the following formula:

$$\% \text{ Titratable Acidity} = \frac{\text{NaOH volume (mL)} \times \text{NaOH} \times \text{lactic acid molecular weight}}{\text{sample volume (mL)} \times 1000} \times 100 \% \quad (1)$$

Syneresis and water holding capacity (WHC) analysis

Syneresis and WHC analysis in this study was based on Oraç and Akin [17] with modifications. Fermented goat milk sample of 5 g (W1) was centrifuged at 4,500 rpm for 30 min at 10 °C, the supernatant or whey was collected and weighed (W2), and the syneresis and WHC (%) were calculated as follows:

$$\text{Syneresis (\%)} = \frac{W2 (g)}{W1 (g)} \times 100 \%$$

$$\text{WHC (\%)} = \frac{W1 - W2 (g)}{W1 (g)} \times 100 \% \quad (2)$$

Viscosity

Viscosity was measured using a Brookfield viscometer (model DV2T, Brookfield Engineering Laboratories Inc., Middleboro, MA, USA) with spindle numbers 61 at 60 rpm. Viscosity readings were taken after 30 s, and the sample was conditioned at 4 °C prior to measurement.

Sensory test

In this study, 70 untrained panelists were selected based on the Raosoft website (<http://www.raosoft.com/samplesize.html>) to represent the total population of the Yogyakarta Special Province community. The criteria for participation in this study were as follows: The panelists were required to be between the ages of 18 and 40, to be residents of Yogyakarta, to be in good health, and to be willing to participate in the sensory test.

The sensory test was approved by the Medical and Health Research Ethics Committee (MHREC) of the Faculty of Public Health Medicine and Nursing, Gadjah Mada University, with the approval number KE/0792/05/2023. The hedonic test method was employed, with a rating scale of 1 - 7. The rating scale employed is as follows: 1 (very much dislike), 2 (dislike), 3 (somewhat dislike), 4 (neutral), 5 (somewhat like), 6 (like) and 7 (very much like). The test was conducted by the aforementioned panelists, who provided a rating in the form of a score on the level of preference for the sample attributes, including aroma, color, taste, texture, and overall impression.

Statistical analysis

The mean of the analytical results from each replicate was used to describe the analytical results obtained. The mean SD deviation of the data analysis results was presented. To determine significance, data analysis was performed using the 1-way ANOVA

method, and Duncan post hoc tests were performed at 5 % using IBM SPSS Statistics software version 20.

Result and discussion

In silico analysis of GABA biosynthesis

The results of RAST showed that the *L. plantarum* subsp. *plantarum* T-3 strain possesses genes involved in the biosynthesis of GABA, specifically glutamate dehydrogenase (GDH) and GAD. **Table 1** shows that the GDH gene is situated on a different frame and length from the gene encoding GAD. GDH (EC 1.4.1.4) is involved in the conversion of α -ketoglutarate to glutamate, whereas GAD (EC 4.1.1.15) catalyzes the decarboxylation reaction of glutamate to GABA. The alignment of the amino acid sequences GDH and GAD in the *L. plantarum* T-3 isolate with 9 other GABA-producing strains is shown in **Figure 1**. The *L. plantarum* T-3 isolate and the other GABA-producing strains have conserved active catalytic residues in GDH and GAD in the form of glycine, serine, glutamine, threonine, aspartic acid, alanine, histidine, and phenylalanine.

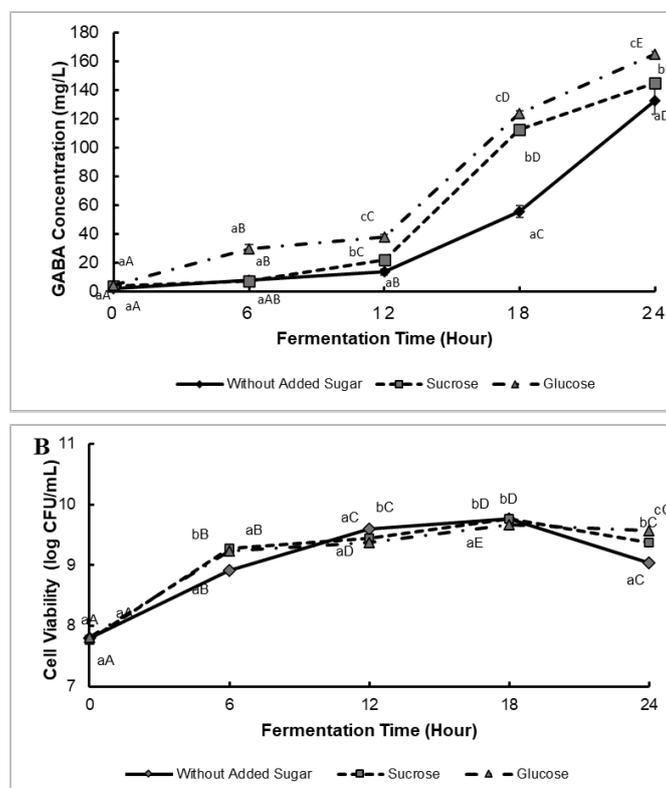


Figure 2 GABA concentration (A); Growth of *Lactiplantibacillus plantarum* subsp. *plantarum* T-3 (B) during fermentation of goat milk. Different letters showed significant differences ($p < 0.05$) based on the treatment of the type of sugar at the same fermentation time (lowercase letters) and based on the fermentation time on the treatment of the same type of sugar (uppercase letters).

Table 1 The Glutamate Dehydrogenase (GDH) and Glutamate Decarboxylase (GAD) encoding genes in *L. plantarum* T-3 based on RAST.

Encoding Gene	Contig	Start	Stop	Length
GDH (EC 1.4.1.4)	Scaffold16	6338	4992	1347
GAD (EC 4.1.1.15)	Scaffold6	76718	78127	1410

The amino acid sequence of GDH from *L. plantarum* T-3 exhibited a 99.55 % similarity to that of GDH from *L. plantarum* WCFS1. Furthermore, it exhibited 60.45 % similarity to the GDH from *Corynebacterium glutamicum* and *C. glutamicum* ATCC 1302, which are also glutamate-producing bacteria. In these isolates, the catalytic residues, histidine at position 100 (H100) and lysine at position 130 (K130) were identified as substrate binders. Similarly, the NAP binding site is formed by glutamate at position 213 (E213) and glycine at position 243 (G243) (Figure 1A). These residues are also conserved in *C. glutamicum* ATCC 1302, a glutamate-producing bacterium (21). In comparison, the amino acid sequence GAD from *L. plantarum* T-3 exhibited 99.57 % similarity to that of *L. plantarum* WCFS1. This finding is consistent with the amino acid sequence results, which demonstrated that *L. plantarum* Dad-13, Mut-7, Mut-3, and Kita-3 exhibited 99 % similarity to GDH (accession number CCC78558.1) and *gadB* (accession number CCC80401.1) of *L. plantarum* WCFS1 [18].

The amino acid sequence of GAD from *L. plantarum* T-3 exhibited 83.12 % similarity to amino acid sequence of GAD from *Lactobacillus brevis* ATCC 367. In *L. plantarum* T-3 and other reference strains, the catalytic residues were identified as glycine at position 125 (G125), serine at positions 127, 131, 132, and 328 (S127, S131, S132, S328), and glutamine at position 1. In addition, the amino acid sequence revealed the presence of glutamine at position 72 (Q172) and threonine at position 221 (Q221), aspartic acid at position 255 (A255), alanine at position 279 (S279), histidine at position 285 (H285) and phenylalanine at position 327 (F327) (Figures 1B). In *L. brevis* CGMCC 1306, threonine (T215) and aspartate (D286) have been identified as residues involved in the decarboxylation process [19]. Arginine (R422), histidine (H278), and lysine (K279) are residues involved in electrostatic interactions with glutamate as a substrate [8]. *L. brevis* is a GABA-producing lactic acid bacterium that is

generally capable of producing higher concentrations of GABA than other strains. This is likely due to the presence of 2 homologous genes encoding GAD, *gadA* and *gadB* [8]. In contrast, *L. plantarum* generally has only 1 gene encoding the GAD, *gadB*.

The biosynthesis of GABA begins with the formation of glutamate from α -ketoglutarate through a transamination reaction catalyzed by GDH. This is followed by the decarboxylation of glutamate to GABA, which is catalyzed by GAD [20]. The process of glutamate decarboxylation, catalyzed by GAD and pyridoxal-5'-phosphate (PLP) as cofactors, results in the formation of GABA and the release of carbon dioxide as a by-product. GAD is a pivotal enzyme in the GABA bioconversion process; thus, strains with genes encoding GAD enzymes are capable of synthesizing GABA [7]. GAD is an intracellular enzyme used by LAB to mitigate acid pressure by reducing the proton concentration in the cytoplasm in the presence of L-glutamate. This system is referred to as the glutamate-dependent acid resistance (GDAR) system. The GDAR system consists of 2 homologous genes, *gadA*, and *gadB*, and the glutamate/GABA antiporter, *gadC* [8].

GABA content of fermented goat milk beverage

Figure 2(A) demonstrates a notable elevation in GABA concentrations over time during fermentation, a finding that aligns with the observations documented by Sahab *et al.* [5]. The 12-h fermentation period exhibited a rapid increase in GABA content. This finding correlates with the pH of the fermented goat's milk, where the pH of Etawa goat milk treated with no added sugar, sucrose, and glucose during the 12-h fermentation period were 4.85, 5.16, and 5.25, respectively (Figure 2(A)). The synthesis of GABA is catalyzed by the enzyme glutamic acid decarboxylase, which is active in an acidic environment and exhibit optimal activity at pH 4.5 [5]. This enzymatic activity results in a significant increase in GABA levels though conversion of L-

glutamate via decarboxylation process with PLP as a cofactor [21].

The final product of fermented goat milk with glucose had the highest GABA concentration, at 164.95 mg/L, while the lowest GABA concentration was observed in fermented milk without sugar, at 132.68 mg/L (**Figure 2(A)**). These results aligns with those of Hussin *et al.* [15] who observed that the addition of glucose resulted the highest GABA production during yogurt fermentation, rather than the treatment of sugar and probiotics. The addition of simple sugars has been shown to enhance GABA production, as they serve as a substrate for the bacteria engaged in the fermentation process. The utilization of glucose is more efficacious in the production of GABA, as the glucose-6-phosphate and phosphoketolase pathways are formed directly. In contrast, sucrose must undergo an additional conversion step in the phosphotransferase system, which is dependent on phosphoenolpyruvate, before it can be converted to pyruvate, which then forms GABA [23].

The concentration of GABA in fermented Etawa goat milk with the addition of glucose produced in this study (164.95 mg/L) was found to be lower than that observed in fermented milk with *L. lactis* MK L84 with the addition of 5 mmol of MSG (249.31 ± 33.83 mg/L) and *L. lactis* MK L37 with the addition of 5 mmol MSG (113.41 ± 22.34 mg/L) [24], in pigeonpea milk with *L. plantarum* Dad-13 starter with the addition of MSG (5.6 g/L) [25], and in fermented milk with *L. plantarum* NDC75017 starter with the addition of 80 mM MSG (314.56 mg/100 g) [26]. The lower concentration in observed in this study may be attributed to the absence of the additional substrates such as glutamate or MSG which can be utilized by bacteria to increase GABA production. The production of GABA through fermentation by *L. plantarum* was found to increase with the addition of MSG [27] or glutamate [28]. Our findings indicated that the concentration of GABA in fermented goat milk with T-3 was higher than that observed in fermented milk with *Lactococcus lactis* MK L37 as the starter culture, without the addition of MSG (49.92 ± 11.45 mg/L). *L. lactis* MK L84 without MSG

(58.35 ± 5.14 mg/L) [24]. The results of this study indicate that fermented Etawa goat milk with the addition of glucose and using *L. plantarum* T-3 culture as a single starter without MSG can produce high levels of GABA, which presents an opportunity to create new functional foods that do not impart the characteristic umami flavour associated with MSG.

Cell growth of *L. plantarum* T-3 during fermentation of goat milk

Figure 2(B) illustrates the process of cell growth during fermentation. At the initial time point, the amount of BAL in Etawa goat milk without added sugar, with the addition of sucrose, and with the addition of glucose showed values of 5.87×10^7 , 6.20×10^7 , and 6.53×10^7 CFU/mL, respectively. These values were not statistically significantly different ($p > 0.05$). After 6 h of fermentation, the values increased significantly to 8.20×10^8 , 1.70×10^9 , and 1.90×10^9 CFU/mL, respectively. The total number of LAB continued to increase significantly in the 12 h of fermentation, of 2.35×10^9 , 2.77×10^9 , and 3.93×10^9 CFU/mL, respectively. The growth of LAB entered the stationary phase after 12 h. This result is consistent with the research by [29] that the growth of local LAB *L. plantarum* Dad-13, *L. plantarum* Mut-7, *L. plantarum* T-3, and *S. thermophilus* Dad-11 were increased, and the number of cells at the end of the milk fermentation reached 10^8 CFU/mL.

After 24 h incubation, fermented goat milk with added glucose had the highest cell viability, followed by fermented goat milk with added sucrose and no added sugar. The number of cells was 3.67×10^9 , 2.39×10^9 , and 1.1×10^9 CFU/mL, respectively. Shan *et al.* [26] also stated that fermented goat milk with added glucose has the highest cell viability because glucose has a higher utilization efficiency by the LAB strain. Glucose is easily phosphorylated to glucose-6-phosphate for bacterial cell growth, unlike galactose and fructose, which require an additional step in the phosphoenolpyruvate-dependent phosphotransferase system before being converted to pyruvate, which can be used by bacteria for glycolytic pathway growth [30].

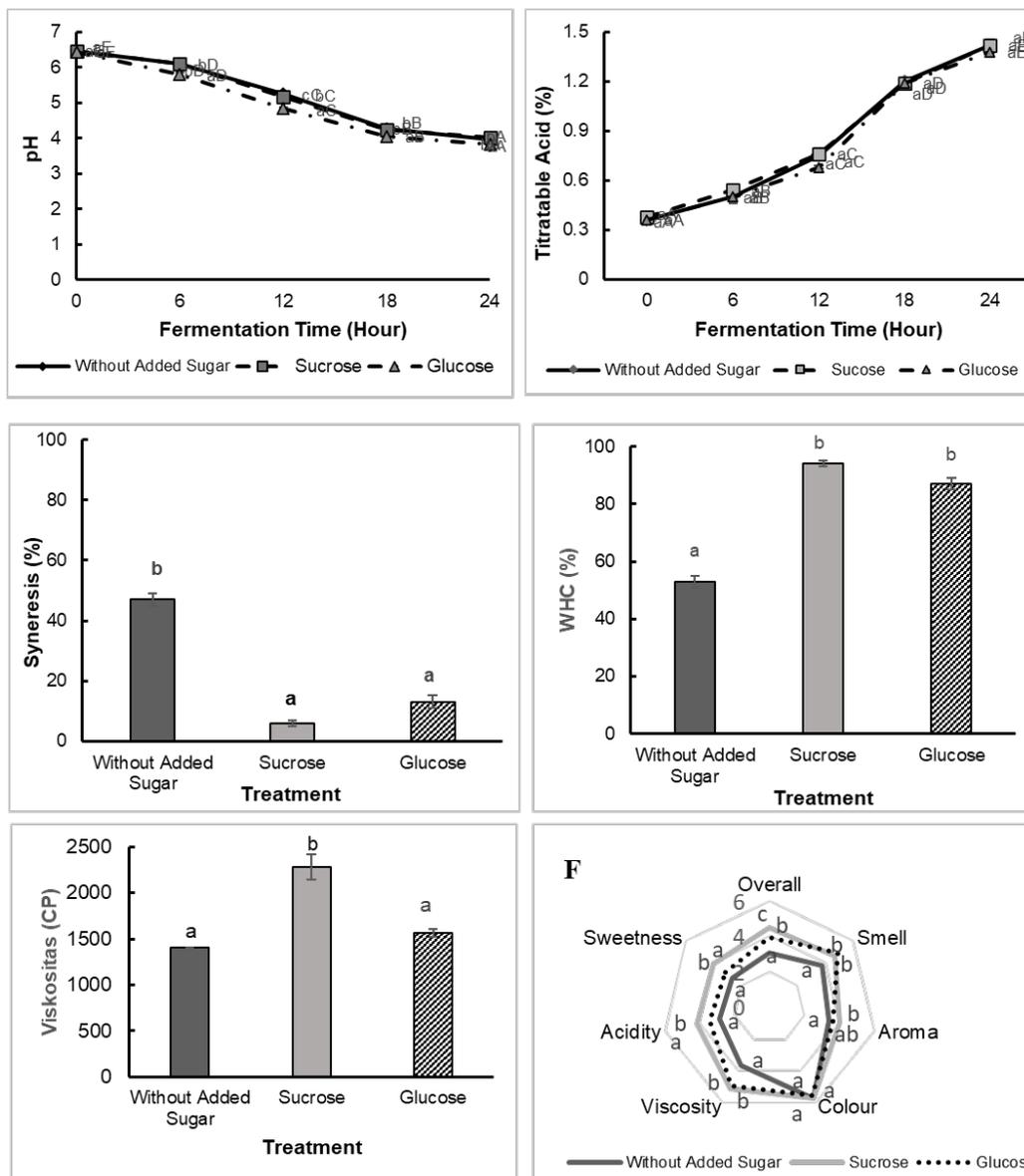


Figure 3 Physicochemical properties of fermented goat milk: pH (A); total titratable acid (B); Syneresis (C); water holding capacity (WHC) (D); Viskositas (E); organoleptic characteristics of fermented goat milk (F) Different letters showed significant differences ($p < 0.05$) based on the treatment of the type of sugar at the same fermentation time (lowercase letters) and based on the fermentation time on the treatment of the same type of sugar (uppercase letters).

pH and titratable acidity during fermentation

Figure 3(A) depicts the pH progression of fermented goat milk over the 24-h fermentation period for all 3 sample variants. The pH of the fermented goat’s milk exhibited a pronounced decline, with the lowest pH observed in the glucose-containing sample at the conclusion of the fermentation period. The presence of sugar serves to provide a nutrient source for LAB, which can influence the activity of these bacteria during the fermentation process, producing lactic acid and resulting in a pH reduction [31].

Figure 3(B) illustrates a notable increase in total acidity throughout the fermentation process of Etawa goat milk in all 3 samples. In the treatment with no added sugar, the total acidity increased from 0.36 ± 0.01 % (0 h) to 1.38 ± 0.04 % (24 h). In the treatment with added sucrose, the total acid increased from 0.36 ± 0.01 % at the initial time point to 1.42 ± 0.04 % at the final time point. In contrast, the treatment with added glucose, demonstrated a notable increase in the total acidity increased from 0.38 ± 0.02 % (0 h) to 1.42 ± 0.01 % (24 h). Moreover, the type of sugar used did not result

in a statistically significant difference in total acidity at the same fermentation time point. The total acidity value of the 3 treatments after 24 h of fermentation remains within the standard range for fermented milk drinks, as recommended by the FAO [32], which is at least 0.6 %. The increase in total acidity during the fermentation of Etawa goat milk was found to be inversely proportional to the decline in pH, a finding that consistent is with previous research on the fermentation of cow's milk using local probiotics [29].

Syneresis and WHC of fermented goat milk

This study indicates that there were notable discrepancies in syneresis and WHC between fermented goat milk with added sucrose, glucose and samples with no added sugar (**Figures 3(C) and 3(D)**). The highest percentage of syneresis was observed in fermented goat milk with no added sugar, followed by that with added glucose and sucrose. Conversely, fermented goat milk with added sucrose exhibits the highest percentage of WHC, followed by fermented goat milk with added glucose and no added sugar. The syneresis and WHC of fermented goat milk with added sugar for 24 h were found to be similar to those observed in previous research, where sucrose gave lower syneresis and higher WHC than glucose [33]. The increased syneresis observed in the fermented milk product without sugar was also documented in a previous study. The incorporation of sugar into yogurt resulted in an increased total solids content, which subsequently increased the WHC [34]. This result also provides an explanation for the observed phenomenon that fermented goat milk with no added sugar had the highest syneresis and the lowest WHC when compared to fermented goat's milk with added sucrose and glucose. The syneresis of a fermented milk product can be reduced by increasing its WHC [35]. Consequently, a reduction in WHC results in an increase in syneresis. Syneresis in fermented milk is a defect that results in an undesired texture and sensory character, which in turn leads to a reduction in consumer acceptability. The formation of gel in fermented milk is a consequence of the acidification process that occurs during fermentation. This process causes casein to form a gel and trap the whey within it through the formation of hydrogen bonds [35]. The occurrence of syneresis can be attributed to a reduction in the protein-water

interaction within the product, which subsequently resulted in the formation of curd and whey separation [36].

Viscosity of fermented goat milk

The addition of sucrose to fermented goat's milk resulted in the highest viscosity value, which was significantly different from that of fermented goat's milk with the addition of glucose and without the addition of sugar (**Figure 3(E)**). This can be attributed to the more hygroscopic properties of sucrose, which is stronger than other sugars and thus binds water more strongly [37]. Changes in viscosity values can be attributed to the activity of LAB which have the ability to produce extracellular polysaccharides during fermentation [38].

Sensory of fermented goat milk drink

The results of the sourness and sweetness liking test indicated a preference for the fermented goat milk with sucrose over fermented goat milk without sugar and with glucose (**Figure 3(F)**). This is attributed to the higher sweetness level of sucrose (100 %) in comparison to glucose (74 %), as documented in reference [39]. Nevertheless, no significant difference was observed in the total titratable acid among the treatments (**Figure 3(B)**). While the aroma of fermented milk without added sugar is significantly different from fermented milk with the addition of sucrose, no significant difference was observed between the 2 when glucose was added. The panelists' assessment of color attributes did not yield statistically significant results (**Figure 3(F)**).

Similarly, as previously reported by Li *et al.* [40], sugar plays a role in the formation of aldehyde compounds, which are compounds that play an important role in connecting the entire metabolism of flavor compounds. Glucose is converted to pyruvate through glycolysis, and then pyruvate forms acetyl-CoA under the action of pyruvate dehydrogenase or pyruvate formate lyase. Finally, acetyl-CoA acetyl aldehyde forms through aldehyde dehydrogenase. In general, fermented milk with the addition of sucrose is preferred to fermented milk with added glucose and without added sugar (**Figure 3(F)**). This result is consistent with prior research indicating that yogurt with no addition of sugar has the lowest acceptance rate among the samples tested [34].

Based on these sensory results, the development of GABA-rich fermented goat milk products as a healthy beverage with diverse GABA functions is a viable avenue, particularly given the absence of GABA-rich foods or beverages currently available on the market. A

search of the term “GABA” reveals that it is most commonly found in the context of supplements or pharmaceuticals. In the United States, GABA is marketed as a dietary supplement [41], it is utilized as an antidepressant medication.

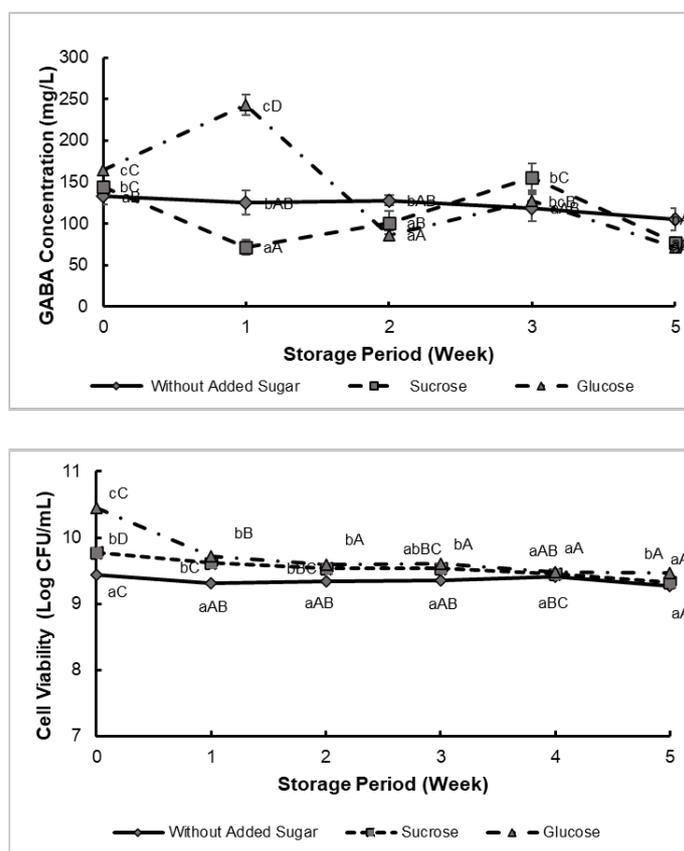


Figure 4 GABA concentration (A); Cell viability of *Lactiplantibacillus plantarum* subsp. *plantarum* T-3, (B) during storage of fermented goat milk. Different letters showed significant differences ($p < 0.05$) based on the treatment of the type of sugar at the same storage period (lowercase letters) and based on the storage period on the treatment of the same type of sugar (uppercase letters).

GABA concentration fermented goat milk beverage during storage

A reduction in GABA content was observed in all fermented goat milk treatments at 5 weeks (Figure 4 (A)). In fermented goat milk without addition of sugar, a linear decrease in GABA content was observed from 132.68 to 105 mg/L during storage period. The GABA content in fermented goat milk with sucrose exhibited a notable decline from 144.72 to 71.91 mg/L following a 1-week storage period, followed by an increase to 101.06 mg/L in weeks 2 and 3, reaching 155.83 mg/L. Subsequently, a decline was observed, with final concentration of 77.39 mg/L at 5 weeks of storage. The addition of glucose to the fermented goat milk resulted in an initial increase in GABA content, from 164.95 to

242.41 mg/L, after 1 week of storage. Subsequently, a further decline was noted, with a final concentration of 71.32 mg/L observed in weeks 2 to 5 of storage.

Similarly reported by Hussin *et al.* [15], there was a notable increased in GABA levels over the course of a 21-day storage period at temperatures ranging from 2 to 4 °C. This finding is consistent with the results obtained in the treatment of fermented goat’s milk with the addition of sucrose, which demonstrated an increase in GABA content during the 3rd week of storage. The observed increase in GABA content during storage reflects the continuous formation of GABA by the *L. plantarum* T-3 strain. The higher GABA content during storage is consistent with other research, which demonstrated that the cold storage of yogurt inoculated

with starter cultures and GABA-producing strains of *Lactobacillus cerevisiae* O-114, *Lactobacillus lactis* O-114, *Lactobacillus helveticus* Lh-B 02 and *Lactobacillus rhamnosus* B-1445, the GABA concentration increased from 5.71 mg/100 g (day 1) to 10.33 mg/100 g (day 14) [42].

A reduction in GABA levels may be attributed to several factors, including extracellular degradation of GABA, uptake of degraded GABA by GABA transaminase, and conversion of the latter to semialdehyde succinate, which subsequently becomes integrated into the citric acid cycle. GABA-transaminase and semialdehyde dehydrogenase are subsequently converted to succinate, which is then taken up by the Krebs cycle and used as a carbon and/or nitrogen source [43]. Despite a reduction in GABA content at week 5 of storage across all treatments, the GABA content remained higher than that observed for

the fermentation by *L. plantarum* C48 after 30 days of storage, which was 63 mg/L [45].

Cell viability of *L. plantarum* T-3 storage

The highest cell viability was observed in fermented goat milk with added glucose, followed by fermented milk with added sucrose and no added sugar (Figure 4(B)). Probiotic cell counts of 10⁷ CFU/mL are regarded as probiotic foods, provided that they are capable of maintaining a cell count of 10⁷⁻⁹ CFU/mL [46]. The maintenance of viability is a critical requirement for the storage of probiotic strains. A decline in the viability of the probiotic cells, *L. plantarum* T-3, was observed from week 3 to week 5 of storage. This may be attributed to a reduction in metabolic activity resulting from prolonged storage. A decrease in LAB viability was also observed in yogurt samples stored for 14 days [54] and in fermented goat milk samples stored for after 28 days [48].

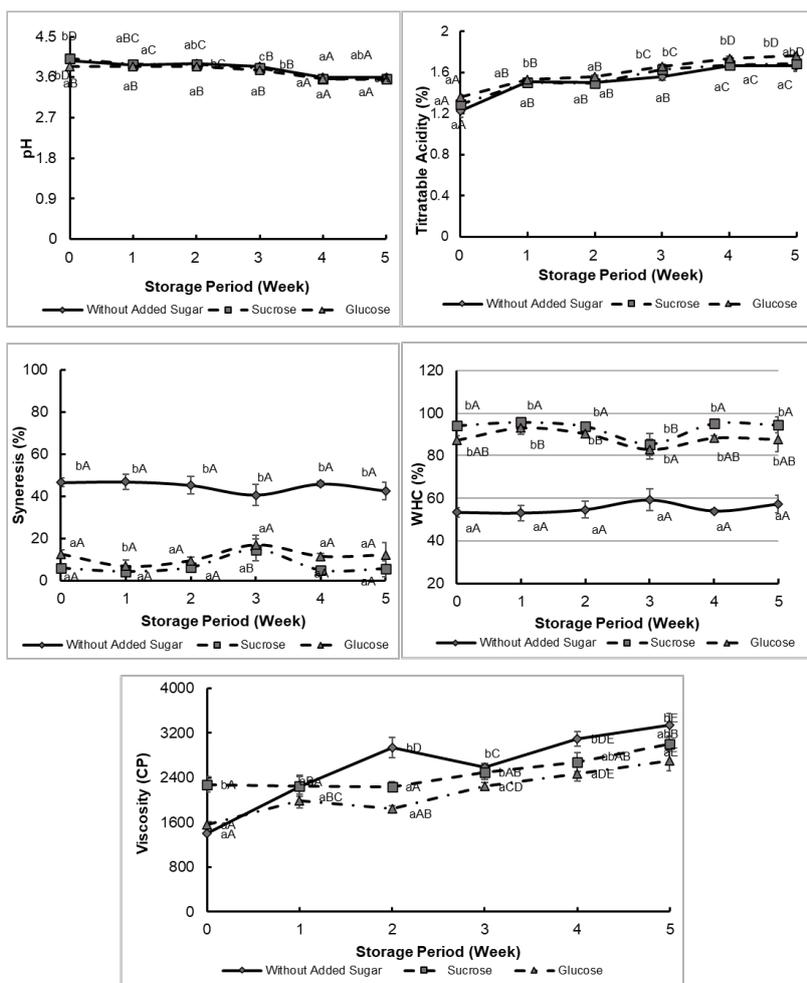


Figure 5 Physicochemical properties of fermented goat milk during a five-week storage period: pH (A); total titratable acid (B); Syneresis (C); water holding capacity (WHC) (D); Viscosity (E). Different letters showed significant differences ($p < 0.05$) based on the treatment of the type of sugar at the same storage period (lowercase letters) and based on the storage period on the treatment of the same type of sugar (uppercase letters).

pH and acidity of fermented goat milk drink during storage

During storage period, a decrease in the pH was observed in all treatments, while the total titratable acid showed an inverse trend compared to the pH (**Figures 5(A) - 5(B)**). The pH decreased during the 5-week storage period, with values ranging from 3.96 to 3.59 for the fermented goat milk drink without added sugar, from 4.02 to 3.57 for the fermented goat milk drink with added sucrose, and from 3.84 to 3.55 for the fermented goat milk drink with added glucose. Similarly, the titratable acidity of fermented milk increased with the length of refrigerated storage, resulting in a decrease in pH value after 5 weeks of storage time [45,46].

The percentage of total titratable acidity (TAT) demonstrated a notable increase over the 5-week storage period, with values ranging from 1.2 to 1.7 (**Figure 5(B)**). The percentage of TAT value falls within the range specified by the Indonesian National Standard (SNI 2981:2009), which outlines the quality requirements for yogurt, namely 0.5 - 2.0 % acidity (calculated as lactic acid). These results are consistent with those previous research, which demonstrated that the percentage TAT in the yogurt samples containing the probiotics *L. rhamnosus* ATCC 53103, *L. casei* ATCC 393, *L. plantarum* ATCC 14917 and a control yogurt increased over the 21-day storage period [50].

Syneresis and WHC of fermented goat milk drink during 5 weeks of storage

The syneresis and WHC of fermented goat milk drinks did not exhibit significantly different across all treatments during the 5-week storage period (**Figures 5(C) - 5(D)**). The addition of sugar to fermented goat milk products has been demonstrated to result in a reduction in syneresis, thereby enhancing the product's stability over an extended period of time, as evidenced by previous studies [51].

The occurrence of syneresis during storage in fermented goat milk drink products with the addition of glucose ranges from 16 - 24 %, while in fermented goat milk drink products with the addition of sucrose 11 - 23 %. These findings diverge somewhat from those reported by Husin *et al.* [15], in their study of GABA-rich yogurt, in which the syneresis was 11.70 - 15.03 % and 20.79 - 21.63 % in standard yogurt. The acidic

environment and effective trapping of water molecules in protein tissues can increase the capacity of gel tissues to resist syneresis during storage [52].

Viscosity of fermented goat milk drink during storage

The viscosity of fermented goat's milk drink products exhibited a notable increase across all treatments during the 5-week storage period (**Figure 5(E)**). The highest viscosity value was observed in fermented goat milk beverage products without added sugar, followed by those containing sucrose and glucose. Similarly, as reported by Lim *et al.* [53], an inverse relationship exists between the loss of water content in fermented milk products and the viscosity of the associated products. From the 1st week to the 5th week, fermented goat milk without sugar exhibited a higher percentage of syneresis than the other 2 samples, which could potentially impact the viscosity of each product. The viscosity of the product is influenced by the acidic and pH-decreasing conditions that occur during the storage period. During the fermentation process, casein present in milk may undergo coagulation due to the acidification that occurs, resulting in a change in the viscosity of the product [54]. As the pH value declines, the proteins present in milk will undergo aggregation and form a gel-like structure, which is characteristic of yogurt. A reduction in pH can result in the reduction of solubility for casein, which in turn causes hydrophobic interactions between casein micelles to form a structure and consistency that results in fermented milk drinks becoming thicker, thus increasing viscosity.

Conclusions

The research indicated that the addition of sucrose was the preferable option in terms of organoleptic properties when developing a fermented goat milk product containing GABA. The storage of the product for a period of 5 weeks at a temperature of 4 °C resulted in a reduction in the concentration of GABA across all treatments. However, the total cell count of LAB remained stable, within the range of 10⁹ CFU/mL, which still met the minimum requirements set per in SNI 2981:2009 for yogurt quality, namely 10⁷ CFU/mL. It is recommended that a more comprehensive study be

conducted in the future to improve the sensory acceptance of the product and to enhance the concentration of GABA.

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