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Characteristics, Antioxidant and Antihypertensive Properties of Buffalo Milk Dangke with the Addition of *Lactiplantibacillus plantarum subsp. plantarum* IIA-1A5 as Probiotics Stored at Cold Temperature

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ABSTRACT

Dangke is a traditional food from South Sulawesi-Indonesia especially Enrekang Regency, made from buffalo or cow milk coagulated with papaya fruit sap papain enzyme. The development of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 as a probiotic in various food products has been shown to increase the content of bioactive compounds, such as antioxidants, antihypertensive agents, and antibacterials. This study aims to evaluate the physical, microbiological, functional quality of dangke in the form of antioxidant and antihypertensive properties with the addition of *Lactiplantibacillua plantarum subsp. plantarum* strain IIA-1A5 at the level of 0% and 10% during storage for 0 days, 5 days and 10 days. The physical test results found significant differences in dangke with the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 as much as 10% in the parameters of pH, TAT and texture. The addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 10% in dangke was able to inhibit and reduce the growth of pathogenic bacteria such as *E. coli* and *S aureus* and was able to increase the value of antioxidant and antihypertensive activity by 83.45% and 45.60%. Based on the electrophoresis results, casein was found at a molecular weight of 22-29 kDa, and smaller peptides of hydrolyzed casein protein presented at a low molecular weight of 16-18 kDa.

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1. Introduction

In this era of awareness the importance of a healthy lifestyle, the demand for functional food products is increasing around the world including Indonesia. People are increasingly realizing the importance of foods that not only provide basic nutrition, but also have the potential to improve body health and well-being. Buffalo milk is one of the food material that contain high essential nutrients needed by the body. Bioactive components in buffalo milk are also associated with antioxidant potential that can help protect the body from oxidative stress. Buffalo milk can be processed into various functional food products, such as yogurt and cheese that contain bioactive compounds and probiotics [1]. Most Indonesians use buffalo milk as raw material for processed foods such as dangke. Buffalo milk dangke is made from the clotting of milk protein by the enzyme papain. According to Rahman dangke components consist of water (45.75%), fat (32.81%), protein (17.20%), and minerals (2.31%) [2]. Buffalo milk dangke has a chewy texture and a distinctive cheese-like flavor because the buffalo milk used is able to produce higher total solids (37.4%) than cow's milk (11.75%) so that it is classified into soft cheese products without fermentation [3]. Previous research has shown that dangke products can increase calcium and phosphate levels in saliva and can reduce the risk of dental caries [4]. Dangke naturally contains indigenous lactic acid bacteria (LAB) [5]. Based on previous research, Lactic acid bacteria (LAB) species successfully isolated from buffalo milk dangke include *Lactobacillus plantarum* and *Lactobacillus fermentum* species [6].

Lactobacillus plantarum is one of the probiotic microorganisms as a potential source of bioactive compounds. *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 is a LAB isolated from Peranakan Ongole beef and has been confirmed to have probiotic properties [7,8,9,10]. The development of *Lactiplantibacillus Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 as a probiotic in several food products has been tested to increase the content of bioactive compounds such as antioxidants, antihypertensive, and antibacterial. With previous research revealing the potential of dangke from buffalo milk as a functional food and the positive effects of *Lactobacillus plantarum* as a source of bioactive compounds, it is necessary to conduct further research to explore the physical characteristics, antioxidant and antihypertensive activities of dangke from buffalo milk with the addition of a specific strain *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 with different storage lengths. Thus, the consumption of buffalo milk dangke enriched with *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 is expected to provide additional benefits for health and disease prevention, as well as provide new opportunities for the food industry in producing innovative and value-added products.

2.MATERIALS AND METHODS

Refreshment of Starter Culture

Culture refreshment is done by taking culture stock *isolate Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 (GenBank: OR473281.1). Then, grow the bacterial isolate on 9 mL MRSB media as a purification stage and incubated for 24 hours at 37°C. The refreshment process continues until the culture adapts to live in the media and shows sufficient growth, characterized by media turbidity. The refreshed culture was then inoculated as 2% into a 10% sterile skim milk solution. Next, the culture was

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incubated at 37°C for 48 hours, and then pipetted into MRSA media to determine its initial population [11,12].

Preparation of Dangke

Buffalo milk was heated to 50°C and then 3 ml papain enzyme was added and stirred evenly until temperature of 115°C for 3 minutes, then the milk temperature was reduced to 40-45°C. Then, *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 was added with the treatment level, I. Control (without the addition of probotic *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5); II: Dangke with the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 by 10% (minimum level of LAB addition). Furthermore, it was waited until the clotting was perfect and the milk casein clot was then inserted into a mold made of split coconut shell or plastic and left for 2 hours until the texture of the clot hardened. Dangke was stored at refrigerator with temperature 10^0C for 10 days, and analyzed in the 0,5 and 10 days of cold storage.

Analysis of Physical Properties

pH value [13]

The pH value was tested using a Schott pH meter (Schott Instruments GmbH, Hertfordshire, UK). The electrode at the tip of the device was calibrated using buffer at pH 4 and then pH 7. Then, rinsed using distilled water. The sample was prepared as much as 10 mL and the pH meter was dipped in the sample and until the number on the pH meter was stable.

Total Titratable Acid Value [14]

This test is carried out by diluting a sample of 10 mL of sample with distilled water and a ratio of 1: 1 and then adding three drops of phenolphthalein (PP) as an indicator and then titrating with NaOH (0.1 N) until a pink color appears. Furthermore, the value obtained will be converted into a percentage of lactic acid with the following formula:

Total acid (%) : $\frac{NaoH\ volume \times NaOH\ normality \times BM\ of\ organic\ acid \times 100}{weight\ of\ material\ (mg)}$

Description :
N = Normality of Titrant (mol/L)V1
V1 = Titrant Volume (ml)
V2 = Sample Volmue (ml)
Eq.Wt = Acid equivalent weight (lactic acid = 90.8)

Aw value [15]

Water activity is the free water needed by organisms to develop. The water activity of a food product can be determined using an aw meter. Before use, the aw meter must be calibrated first using a saturated NaCl solution with an aw of about 0.7509. The next step is to place a number of samples into the aw meter. Once the device is ready, the start button is pressed and the aw value will be read if the device is in a completed state.

Texture Value with Texture Analyzer [16]

Dangke texture was analyzed using the Perten TVT 6700 Texture Analyzer instrument. Dangke samples were uniformly diced and pressed using the probe 2 times. The probe speed used was 2 mm/s with the sample pressed to 30% of the initial height. The parameter seen is the sprngness value that appears on the Textur Analyzer Perten TVT 6700 software.

Analysis of Microbiological Properties

Total Lactic Acid Bacteria [7]

Dangke samples weighed as much as 25 mg and then inserted into an erlenmeyer containing 225 ml of BPW solution so as to obtain a dilution of 10-1. Furthermore, dilutions up to 10-8 were carried out by mixing 1 ml of the sample from the previous dilution into 9 ml of BPW solution. Then, the calculation of bacterial colonies was carried out by taking a sample of 1 ml at a dilution of 10-6 to 10-8 and inserted into a Petri dish and mixed with MRSA as much as 15 ml. then the

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cup was incubated in an inverted position with a temperature of 37°C for 48 hours.

Total Pathogenic Bacteria *Escherichia coli [12]*

A total of 1 mL of dangke was reacted with 9 mL of sterile 0.1% buffer peptone water (BPW) solution in a sterile container aseptically for dilution 10-1. Then repeated up to 10-3. Furthermore, each dilution was taken 1 mL by pour plate method on eosin methylene blue agar (EMBA) media. Then, incubated at 35°C for 24 hours. *E.coli* (+) colonies are metallic green with a black dot in the center. Observations were made of *E.coli* colonies that grew on EMBA media.

Total Bacteria *Staphylococcus aureus [8]*

A total of 1 mL of dangke was reacted with 9 mL of sterile 0.1% buffer peptone water (BPW) solution in a sterile container aseptically for dilution 10-1. Then repeated up to 10-3. Furthermore, each dilution was taken 1 mL by pour plate method on Baird Parker Agar (BPA) media plus potassium tellurite and egg yolk. Then, incubated at 35°C for 24 hours. Then the number of *S.aureus* colonies that grew was counted.

Antioxidant Capacity Analysis by DPPH Method

Sample Extraction

The dange extraction method was carried out using methanol as the extraction agent. A total of 10 g of dangke sample in each treatment was taken and then homogenized with 5 ml of methanol for 1x24 hours and measured pH. The mixed solution will then be centrifuged at 10,000 rpm for 10 minutes at 4^{o}C until separated. Then, the precipitate will be separated and the supernatant will be stored at cold temperature in a refrigerator.

Antioxidant Activity Testing with DPPH Method [17]

DPPH testing by adding 0.15 mL of sample extract with 0.9 mL of DPPH and incubated using a waterbath in the dark room for 30 minutes at 37 ^{o}C. Then readings were taken with a spectophotometer at a wavelength measurement (λ) of 517 nm until the reading was constant. A standard curve was prepared with the same procedure but the extract was replaced using ascorbic acid at concentrations of 0.0; 0.5; 1.0; 1.5; 2.0; 2.5 mg 100 mL.

Antihypertensive Testing Through ACE Inhibition [18]

Antihypertensive activity test was performed using *Hippuryl-L-Histidyl-L-Leucine* (HHL, Sigma, USA) and also ACE solution. The first thing to do is to make ACE solution (25mU) in sodium borate buffer. Next, 50µL of ACE solution was added and then pre-incubated at 37°C in a waterbath for 10 minutes. After that, HHL substrate solution was added (Substrate was made by mixing 8.3 mM HHL in 50 mM sodium borate buffer containing 0.5 M NaCl at pH 8.3) as much as 150µL and again incubated at 37°C in a waterbath for 30 minutes. The reaction was then stopped by adding 250 µl of 1 M HCL. Afterwards, 0.5 mL of ethyl acetate was added and homogenized using a vortex. This process was done to extract the hippuric acid (HA) produced in the reaction mixture due to the hydrolysis of the substrate catalyzed by ACE. Next, the dangke sample was centrifuged for 15 minutes at 800 gx at 4°C. Then, two layers of solution were produced and the ethyl acetate solution at the top was taken as much as 0.8 µl which will be transferred into a clean tube and will be evaporated at 85°C for 60 minutes. Then, the HA in the tube will be dissolved using 1 mL of distilled water and cooled at room temperature. Then, the absorbance was calculated using spectophotometry with a wavelength (λ) of 228 nm.

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Peptide profile analysis by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Peptide profiles were examined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [19]. Peptide profile analysis was only conducted on dangke samples before storage to ensure that proteolysis occurred during the dangke-making process by the microbes . Its hydrolyzate were visualized through this technique with a modified approach. The gel concentration for the stacking gel was 4% (v/v), and for the separating gel, it was 15% (v/v). Electrophoresis was carried out for 3 hours and 15 minutes at a voltage of 70 V and 20 mA. Following the electrophoresis, the gel was taken out, washed twice with deionized water, and then subjected to staining. The marker PM 2700 from Smobio Technology, Inc. served as the standard. Band distribution was visualized using Coomassie Brilliant Blue staining (CBBR-250).

Research Design

The design used in this study was a group randomized block design (RBD) factorial pattern (2x2). The first factor was the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 which consisted of 2 levels (0% and 10%). The second factor was the length of storage (0 days, 5 days and 10 days) at temperature 10^0C. The data obtained were analyzed using Minitab19 software, the level of significance set at α=0.05. The analysis was continued with Duncan's test if P<0.05 was obtained to see different variables. Each experiment was carried out as many as 4 replicates, duplo.

3.RESULTS AND DISCUSSION

The addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 bacteria as much as 10% produces dangke products that contain probiotics. Meanwhile, dangke without the addition of bacteria *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 also has probiotic content from Indegeous LAB. To determine the effect of the addition of probiotic bacteria *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 as much as 10% and without the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 in dangke, several types of tests were carried out, namely, physical characteristics test, microbiological quality (LAB, *E. coli*, and *S. auereus*) antioxidant activity with DDPH method, and antihypertensive activity with ACE inhibition method at 0 days, 5 days, and 10 days of storage.

Physical Characteristics

The test of dangke characteristics includes testing the pH value, Total Titratable Acid, Water activity (aw), and Texture using Texture Analyzer. The characteristics of buffalo milk dangke with the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 at 10% with storage duration of 0 days, 5 days, and 10 days are presented in Table 1.

Table 1. Physical characteristics of buffalo milk dangke

Parameters Testing	Period Storage (Days)	Dangke	Dangke + 10% LP
pH	0	6.99 ± 0.00^{c}	5.73± 0.05^{a}
	5	7.02 ± 0.02^{c}	6.09±0.40^{a}
	10	7.05 ± 0.03^{c}	6.87± 0.01^{b}
TTA (%)	0	0.42±0.03^{d}	0.63±0.03^{e}

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Parameters Testing	Period Storage (Days)	Dangke	Dangke + 10% LP
	5	0.26±0.01[c]	0.48±0.12[d]
	10	0.22±0.00[a]	0.23±0.00[b]
A_w (%)	0	0.86±0.02[a]	0.87±0.03[a]
	5	0.91±0.04[ab]	0.89±0.03[ab]
	10	0.93±0.00[b]	0.90±0.00[b]
Texture (g/force)	0	2.30±0.00[a]	2.38±0.01[b]
	5	2.32±0.01[a]	2.35±0.00[ab]
	10	2.33±0.01[a]	2.34±0,00[a]

Different superscript letters per parameter indicate significant difference (P<0.05)
LP = *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5

pH Value

The pH value is a value that indicates the degree of acidity or basicity of a solution by measuring the number of concentrations of Hydrogen ions (H+) and (OH-) contained therein. The pH of buffalo milk used in making dangke is at a value of 6.18 while the pH of the papain enzyme used is 7.2. The pH value of dangke with the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 as much as 10% during cold temperature storage (±4 °C) ranged from 5.73-6.87. Meanwhile, the pH of dangke without the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 bacteria ranged from 6.99-7.05. The pH value obtained in this study is in line with the pH value of dangke conducted by Musra *et al.*, [20] which is 6.13-7.24. ANOVA test results showed the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 10% bacteria and the length of storage had a significant effect (P<0.05). So that an interaction occurs between the addition of bacteria and the duration of storage on the pH value of dangke. It underscores the importance of considering both factors in optimizing the production and quality of buffalo milk dangke.

In Table 1, it can be seen that cold storage of dangke can increase the pH value. This is because the activity of LAB produced by *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 or indigenously undergoes an inhibitory process. According to Pangestu low temperature can inhibit or reduce LAB metabolism in producing lactic acid [16]. During the storage process, nutrient consumption by LAB is reduced while their metabolic activities continue. This can lead to a decrease in lactic acid production and an increase in pH in dangke. In addition, changes in environmental conditions such as temperature and humidity in the refrigerator can also affect LAB activity. LAB will grow optimally at 30°C-40°C. Setyawardani in their research stated that storage time affects the pH value of cheese. [21]

Total Titrated Acid (TTA)

The total titrated acid value is the total amount of lactic acid contained in the solution. Arkan *et al.*, [22] stated that total titratable acid is the determination of the total concentration of acid produced by lactic acid bacteria (LAB). The results of statistical analysis showed that the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 at the 10% level and the length of storage had a significant effect (P>0.05) on the TTA value of dangke. This lack of interaction can be explained by the possibility that *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 addition and storage duration independently impact the total titratable acidity of dangke. Probiotic addition may contribute to the

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production of certain titratable acids, while storage duration may affect the degradation of existing titratable acids. However, there was no significant interaction between the two factors. Table 1 shows that the TAT value of dangke with the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 10% and without the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 is inversely proportional to the pH value. The decrease in TTA value in dangke is related to the increase in pH value. Under lower temperature conditions, the metabolic activity of bacteria slows down, so that the production of organic acids such as lactic acid is also reduced. This causes a decrease in the TTA value in dangke along with the length of storage in the refrigerator. Meanwhile, the increase in pH value occurs due to a decrease in the concentration of organic acids in dangke. Reducted lactic acid production by LAB, the balance between acids and bases in dangke is disturbed, which then leads to an increase in pH value. Low acid production can be supported by low storage temperatures, so LAB will experience a decrease in cellular metabolic rate [10, 23].

Water activity (a_w)

The water activity (a_w) of dangke refers to the concentration of water available in the dangke product. Water activity is an important parameter that describes the ability of water to interact with other components in the food system. High aw values in dangke can accelerate the growth of microorganisms, including pathogenic bacteria, molds and yeasts that can cause product deterioration or spoilage. Cheese generally has an aw value of 0.89-0.99%. Fresh cheese such as cream or cottage cheese has a high percentage and moisture of water, resulting in a relatively shorter shelf life of fresh cheese [24]. Table 1 shows the increase of aw dangke during low temperature storage (±4°C) in closed containers.

The aw value of dangke without the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 ranged from 0.86-0.93. Meanwhile, in dangke with the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 is in the lower range of 0.87-0.90. ANOVA test results showed the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 as well as the length of storage of dangke at low temperatures had no significant effect ($P>0.05$). Based on these findings, there is no significant interaction between the factorial pattern of adding *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 and storage duration on the total water activity value of buffalo milk dangke. This is because closed conditions during storage do not affect the evaporation process and reduce the risk of contamination by external microorganisms that can affect product shelf life. Thus, probiotic bacteria in the form of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 that are added do not have a major influence on the shelf life of dangke because the environment is already protected from contamination, evaporation and humidity. According to Dardanela processed cheese products wrapped in packaging that has a good closing system can minimize the decline in sensory quality at cold and room temperature storage. [8,11,12, 25]

Texture

Texture parameters can include the chewiness, brittleness, softness, crunchiness or density of a food. Food texture plays an important role in the sensory experience and can influence taste and perception of the food. The chewiness parameter in the dangke texture test using the Texture Analyzer TVT 6700, was carried out to see whether dangke with or without the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 could return to its

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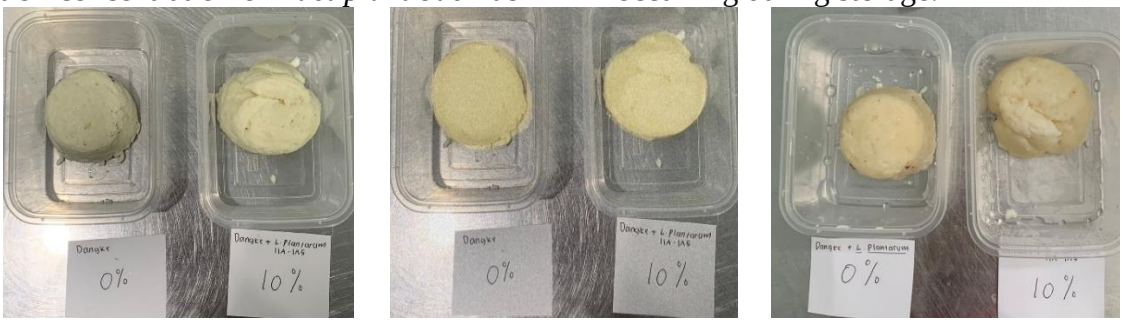
original shape after pressure was applied. In general, the texture produced by cheese is influenced by several factors, namely water content, fat and protein content, salt and pH [26]. Temperature is a key indicator of the quality of cheese during storage. Table 1 shows the changes in dangke's chewiness value during storage for 0 days, 5 days, and 10 days in low temperature (±4°C).

The chewiness value of dangke with the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 ranged from 2.34-2.38 g/f. Meanwhile, dangke without the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 has a relatively constant chewiness value. Statistical test results showed that the addition concentration of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 as much as 10% had a significant effect ($P<0.05$) on the chewiness value of dangke during storage for 0 days, 5 days and 10 days. We found a significant interaction between the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 and storage duration on the texture firmness of dangke. Our results indicate that the effect of increased firmness due to the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 is 10% more pronounced in shorter storage durations compared to longer storage durations (10 days). This interaction suggests that the probiotic effect on the texture firmness of dangke can be influenced by processes occurring during storage.



A **B** **C**

Figure 1: Appearance of dangke and dangke + *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 10% at storage time 0 days (A), 5 days (B), 10 days (C).

Microbiological Quality of Dangke

The microbiological quality of dangke refers to the evaluation of microorganisms present in dangke or the environment. It includes parameters such as the number, type and activity of microorganisms that can affect the safety, quality and durability of dangke. The microbiological quality test of dangke in this study includes total LAB test, total *E. coli* contamination and total *S. aureus* contamination at 0 days, 5 days and 10 days of storage at low temperature (± 4°C). The results of the microbiological quality test of dangke are shown in Table 2.

Table 2. Microbiological quality of buffalo milk dangke

Parameters Testing	Period Storage (Days)	Dangke	Dangke + 10% LP
Lactic Acid Bacteria (log CFU/g)	0	7.20 ± 0.40^{b}	7.77 ± 0.06^{a}
	5	7.11 ± 0.02^{c}	7.50 ± 0.08^{b}
	10	6.41 ± 0.00^{d}	7.18 ± 0.00^{b}
E. coli (log CFU/g)	0	0.59 ± 0.96^{a}	0.07 ± 0.01^{a}
	5	1.14 ± 0.07^{a}	0.40 ± 0.04^{a}

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	10	1.47±0.07[a]	0.70±0.01[b]
S. aureus (log CFU/g)	0	1.26±0.00[b]	0.07±0.01[e]
	5	1.45±0.03[a]	0.21±0.02[d]
	10	1.46±0.03[a]	0.64±0.03[c]

Different superscript letters per parameter indicate significant difference (P<0.05)
LP = *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5

Total Lactic Acid Bacteria (LAB)

Lactic acid bacteria belong to the gram-positive category that produce lactic acid as the main product of metabolism commonly found in fermented products such as yogurt. As presented in Table 2, all LAB populations were significantly different (P<0.05). Our results indicate a significant interaction between the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 and storage duration on the amount of LAB produced. We found that the addition of Lactiplantibacillus plantarum has a greater influence on the LAB count when the storage duration is shorter (0 days) compared to when the storage duration is longer (10 days). This interaction suggests that the effect of adding Lactiplantibacillus plantarum on the LAB count in dangke may be influenced by factors related to storage duration. The highest population of LAB was found in dangke with the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 as much as 10% at 0 days storage at 7.77 ± 0.06 log CFU/g. Followed by dangke with the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 10% on day 5 and day 10. Although the population decreased during storage, the total LAB present in dangke with the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 was still in the range of 10^7 CFU/g compared to dangke without the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5, which decreased to 6.41 ± 0.00 log CFU/g on day 10. This is in line with the addition *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 as much as 10% as the minimum threshold of LAB addition to the product, where *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 contributes to the total LAB population of dangke. The decrease in LAB during storage at ± 4 °C decreased the metabolic ability of LAB in converting lactose sugar in dangke into lactic acid. Long storage with low storage temperature does affect LAB metabolism, because the lower the storage temperature the activity of LAB in metabolism is inhibited so that their productivity slows down [8, 10, 12, 27].

Total *Escherichia coli*

Eschericia coli is a gram-negative bacterium that is pathogenic. Based on SNI No. 7388:2009 concerning the Maximum Limit of Microbial Contamination in Food, it is required that the bacterial/microbial contamination of *E. coli* is 1 x 10^3 CFU/g. Testing for bacterial contamination in fresh milk is done as an indicator of sanitation in the milk production or handling process and as an indicator of the health and safety of milk. Table 2 shows the changes in the number of *E. coli* contamination in dangke during storage of 0 days, 5 days, and 10 days in low temperature (±4 °C). ANOVA test results showed that the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 10% had a significant effect (P<0.05) on *E. coli* contamination at long storage and low temperatures. Our results indicate a significant interaction between the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 and storage duration on the amount of *E. coli* produced in dangke. Specifically, we found that the

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reduction effect of *E. coli* due to the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 is stronger in shorter storage durations (0 days) compared to longer storage durations (10 days). The addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 as much as 10% in dangke during day 0 storage is effective in reducing *E. coli* contamination with a total *E. coli* of 0.07 ± 0.05 log CFU/g. Meanwhile, the most *E. coli* contamination was shown in dangke without the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 at 1.47 ± 0.07 CFU/ml. This is because the buffalo milk used in making dangke has decreased in quality due to prolonged storage in the refrigerator. In addition, Indigenous LAB in dangke is not able to reduce the value of *E. coli* contamination such as the ability of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5. According to Arini *Lactobacillus plantarum* can also inhibit the growth of *E. coli* because it produces lactic acid where lactic acid produced by *Lactobacillus plantarum* is able to break down the bacterial membrane and acidify the environment in the cell which results in the release of H + ions from the bacterial cell. [10, 28]

Total *Staphylococcus aureus*

Different from *E. coli*, *Staphylococcus aureus* is a gram-positive pathogenic bacterium. The presence of *S. aureus* in food in high numbers can lead to poisoning. Thus, detection and measurement of the amount of *S. aureus* in dangke is important to ensure product safety and prevent the risk of poisoning. ANOVA test results showed that the addition of 10 *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 had no significant effect (P>0.05) on total *S. aureus* contamination in dangke with different lengths of simmering and low temperatures. In our analysis, that is not find a significant interaction between the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5, and storage duration, on the quantity of *S. aureus* bacteria produced. Our results indicate that the effects of each factor on the quantity of *S. aureus* bacteria tend to be independent of each other, without any significant interaction between these factors. However, the ability of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 bacteria significantly reduced the *S. aureus* contamination value in dangke compared to dangke without the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5. The addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 as much as 10% in dangke during day 0 storage is effective in reducing *S. aureus* contamination with a total *S. aureus* of 0.07 ± 0.03 CFU/ml. In addition to the ability of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 in reducing the value of *S. aureus*, the addition of papain enzyme in making dangke also plays a role in fighting and reducing the population of *S. aureus* bacteria. Papain enzyme derived from papaya fruit sap can inhibit the growth of *S. aureus* up to 90% [29].

Antioxidant and antihypertensive activity

Previous studies have shown that some strains of *Lactiplantibacillus plantarum* have potential as antioxidant, antibacterial anti-inflammatory and antihypertensive agents due to their ability to produce bioactive compounds. Therefore, testing of antioxidant activity through the DPPH method and ACE inhibition in dangke was done with the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 as a bioactive compound producing agent. Dangke microbiological quality test results are shown in Table 3.

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Table 3. Antioxidant and antihypertensive activity of buffalo milk dangke

Parameters Testing	Period Storage (Days)	Dangke	Dangke + 10% LP
Antioxidant activity of DPPH method (%)	0	30.74 ±0.05^{c}	83.45±0.03^{a}
	5	21.83±0.02^{e}	42.74±0.00^{b}
	10	17.52±0.02^{f}	27.31±0.02^{d}
Antihypertensive activity of ACE inhibition method (%)	0	34.86±0.65^{c}	45.60±0.92^{a}
	5	32.23±2.96^{c}	39.52±1.43^{b}
	10	25.50±0.17^{d}	32.46±1.36^{c}

Different superscript letters per paramater indicates significant difference (P<0.05)
LP = *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5

Antioxidant Activity

The Addition Of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 in dangke products can be considered as a strategy to increase antioxidant activity in dangke. Mardia *et al.*, [30] states that antioxidants are substances capable of slowing down or preventing the oxidation process. Antioxidants are also able to protect cells from the harmful effects of free radicals. Free radicals have high reactivity, so they can grab electrons from other molecules in an effort to get their electron pairs. The dangers of free radicals, among others, can trigger coronary heart disease, stroke, and cancer. One commonly used assay mechanism for evaluating antioxidant activity is the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. In this test, DPPH, which is a purple-colored compound, reacts with antioxidant compounds present in the sample, resulting in a change in color to pale yellow or colorless, depending on the compound's ability to capture free radicals. The higher the antioxidant activity of the sample, the faster the color change occurs.

From the DPPH test conducted on dangke with the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5, obtained results that showed significant antioxidant activity and higher (P<0.05) compared with dangke without the addition of probiotic bacteria. The analysis shows a significant interaction between the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 and storage duration. Our results indicate that the addition of probiotics contributes to an increase in antioxidant activity, but its effect may vary depending on the storage duration and the period of dangke production. For example, on day 0 of dangke production, the addition of probiotics can provide a more significant increase in antioxidant activity compared to day 5 or day 10.

The highest antioxidant activity was found in dangke with the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 10% at 0 days of storage time of 83.45%. This suggests that the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 effectively increases the content of antioxidant compounds in dangke products. These changes can be explained by the ability of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 to produce antioxidant compounds during fermentation, as well as possible interactions between probiotic bacteria and components in buffalo milk that can increase the production of antioxidant compounds. Thus, the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 in dangke products can be considered as an effective strategy to increase the antioxidant activity of such

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products, which can provide additional benefits to consumer health.

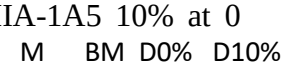
Antihypertensive Activity

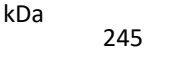
The ACE enzyme plays a role in increasing blood pressure by converting angiotensin I to angiotensin II, which leads to narrowing of blood vessels and an increase in blood pressure. Therefore, inhibition of ACE enzyme activity can be an effective strategy in lowering blood pressure and preventing hypertension. In the ACE inhibition assay, dangke samples with the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 was evaluated for its ability to inhibit ACE enzyme activity. The results obtained showed a significant and higher inhibitory activity of ACE ($P<0.05$) compared to dangke without the addition of probiotic bacteria. The analysis results revealed a significant interaction between the addition *of Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 and storage duration on anti-hypertensive activity. This interaction suggests that the increased anti-hypertensive activity due to probiotic addition can be influenced by storage duration. On day 0, probiotic addition showed a greater increase in anti-hypertensive activity compared to day 5 or day 10.

This suggests that the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 in dangke products effectively increases the antihypertensive activity of the product. The highest antioxidant activity was found in dangke with the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 10% at 0 days of storage time is 45.60 %. The mechanism behind the increased antihypertensive activity may be related to the production of bioactive compounds by *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 during the dangke fermentation process. These compounds may have inhibitory properties against the enzyme ACE, which may help lower blood pressure. Interactions between probiotic bacteria and components in buffalo milk may also play a role in increasing antihypertensive activity. This is confirmed by the statement of Nurul Afiyah *et al.,* [31] in her research, which states that *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 to produce secondary metabolite compounds with ACE inhibitory properties. According Raras dan Makrokopis *Lactobacillus plantarum* in the long term can play a major role in the mechanism of lowering blood pressure. [32]

Peptide Profile Analysis

Protein profile analysis is a process to study the composition and characteristics of proteins in dangke. This method can involve various techniques, including protein separation based on their molecular weight using electrophoresis. The purpose of protein profile analysis is to better understand the structure and function of proteins in a biological system or to detect differences in protein profiles between different samples, for example, between normal samples and samples infected with disease. Dangke profile protein test results are shown in Figure 2.





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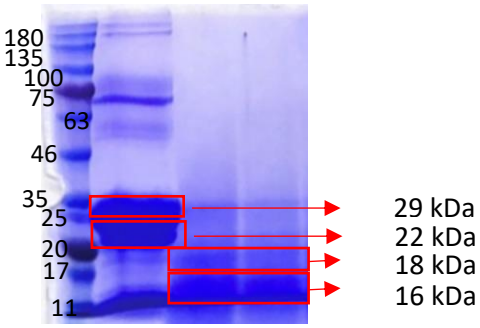

Figure 2. Dangke protein profiles analyzed using SDS-PAGE

Description:
M = Marker protein
BM = Buffalo milk
D0% = Dangke with addition 0% of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5
D10% = Dangke with addition 10% of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), or commonly referred to as SDS, is a technique used to separate polypeptide chains in proteins based on their ability to migrate in an electric current, which is a function of their polypeptide chain length or molecular weight. This is achieved by adding the detergent SDS and heating to disrupt the three-dimensional structure of proteins by breaking disulfide bonds, which are subsequently reduced to sulfhydryl groups. SDS will form complexes with proteins, and these complexes are negatively charged due to the organic groups of SDS [33]. According to Lestari, in her research on milk casein substrates, it is clearly observed and has a molecular weight of approximately 33, 29, and 22 kDa [34]. This is further supported by the statements of Wang and Costa that these three bands correspond to α-casein, β-casein, and κ-casein, respectively. Based on the electrophoresis results, the band patterns in buffalo milk are in the range of 22 and 29 kDa, which corresponds to κ-casein and β-casein protein. [35, 36]

The enzyme papain plays a crucial role in the process of making dangke, particularly in the context of hydrolyzing milk peptides. Dangke is a traditional Indonesian cheese typically made from buffalo or goat milk. The proteolysis mechanism of papain in this process can be explained as follows. Papain is a protease, an enzyme that breaks peptide bonds in proteins. In the production of dangke, papain is used to hydrolyze the proteins in milk, particularly casein, into smaller peptide fragments. Casein is the primary protein in milk responsible for curd formation in cheese. Papain works by disrupting the peptide bonds among amino acids in the polypeptide chain of casein. This results in smaller peptide fragments, enhancing the solubility of proteins. The proteolysis in the dangke processing process causes the casein protein with a size of 22-29 kDa to break down into smaller sizes, specifically into 16-18 kDa. This is demonstrated in the SDS PAGE results (Figure 2), where the thick protein band at 22-29 kDa, indicating buffalo milk casein protein, becomes thin in dangke, and a thick band appears at the protein size of 16-18 kDa. Those low-sized proteins are suspected to possess antihypertensive properties of dangke. In addition to hydrolysis by papain, proteolysis is also carried out by probiotic

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Lactiplantibacillus plantarum subsp *plantarum* IIA-1A5, as evidenced by the higher antihypertensive and antioxidant activities in dangke supplemented with this probiotic (Table 3). The hydrolysis by papain not only produces smaller peptide fragments but also modifies the taste and texture characteristics of the cheese. The generated peptide fragments can contribute to a distinctive taste and a smoother texture in the final product, such as dangke. Additionally, the small peptide fragments produced by papain can influence the aroma profile and organoleptic balance of dangke. The hydrolysis process can generate new aroma compounds, imparting unique characteristics to the dangke.

4.CONCLUSIONS

The addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 to buffalo milk dangke has no effect on water activity (aw) during storage, but has a significant effect on pH, TAT and texture values. Dangke with the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 10% is able to inhibit and reduce the growth of pathogenic bacteria such as *E. coli* and *S aureus*. In the test of its functional properties, dangke with the addition *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 10% or non-probiotic added are best consumed immediately. However, under appropriate conditions, the percentage of antioxidant properties in dangke with the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA 1A5 10% can still be preserved at 27.31% after 10 days, compared to the basline level of 83.45%. Furthermore, the antihypertensive properties, which were at 45.60% on day 0, can still be preserved at 32.46% on day 10. Thus the addition of *Lactiplantibacillus plantarum subsp. plantarum* strain IIA-1A5 as much as 10% at a storage time of 0 days with a temperature of ± 4°C proved to be the most effective in improving the functional, physical characteristics, and reducing pathogenic contamination in buffalo milk dangke. Based on the electrophoresis results, β-casein was found at a molecular weight of 29 kDa, and β-lactoglobulin was found in Dangke at a molecular weight of 18 kDa due to the presence of hydrolyzed casein protein.

5.ACKNOWLEDGMENTS

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