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Research Article

THE POTENTIAL OF BIOACTIVE PEPTIDES FROM GOAT MILK KEFIR WITH ANTIBACTERIAL ACTIVITY AS AN ANTIBIOTIC SUBSTITUTE

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Abstract

This study aimed to determine the optimal incubation time for goat milk kefir to produce the highest antibacterial peptides, and to determine the antibacterial activity of purified goat milk kefir. Protein levels were determined using a microassay method. Kefir, known to have the optimal incubation time for producing the highest antibacterial activity when ultrafiltered with a 10 kDa cut-off membrane, was analyzed for molecular weight by SDS-PAGE. The antibacterial activity was tested against pathogenic *E. coli* and *S. aureus*. The highest soluble protein content was observed in kefir incubated for 36 h, with 2.75% soluble protein and a pH of 4.37. The highest antibacterial activity was observed in kefir incubated for 36 h, with inhibition zones of 15.57 mm against *E. coli* and 12.10 mm against *S. aureus*. Goat's milk kefir purified to a molecular weight >10 kDa produced inhibition zones of 69.1 mm against *E. coli* and 41.3 mm against *S. aureus*, while goat's milk kefir with a molecular weight <10 kDa did not produce inhibition zones against pathogenic bacteria *E. coli* and *S. aureus*. Goat's milk kefir incubated for 36 h contained six bands. At K > 10, purified and filtered goat's milk kefir with a molecular weight > 10 kDa showed three protein bands.

Keywords: Antibacterial, Goat Milk Kefir, Antimicrobial Peptides

INTRODUCTION

Goat milk and its products are becoming increasingly popular in Indonesia. Goat milk contains proteins, fats, carbohydrates, minerals, and vitamins needed by the body. Goat milk has more benefits than cow milk. Additionally, goat milk is an alternative for individuals with cow milk intolerance due to its similar protein content (12.3%) (Lestari & Giordan, 2020). The protein composition of goat milk includes 80% casein (α-casein, β-casein, and γ-casein) and 20% whey protein (Mohanty et al. 2016; Setyawardani 2017). The proteins in goat milk are more readily digested and confer improved functional characteristics upon fermentation into kefir.

Kefir is a fermented milk beverage product obtained through lactose fermentation by symbiotic culture bacteria and yeast present in kefir grains. The microbial consortium generally includes *Lactococci* and *Leuconostoc spp*, *Lactobacilli,* and yeast (*Saccharomyces cerevisiae, Saccharomyces unisporus, Candida kefyr*, and *Kluyveromyces marxianus ssp. Marxianus)* (Rattray & O'Connell, 2022; Prado *et. al.*, 2015). In addition to using kefir grains, commercially available freeze-dried starters containing active bacterial cultures (*Lactococcus lactis, Lactococcus cremoris, Lactococcus diacetylactis, Lactobacillus acidophilus, Saccharomyces cerevisiae,* and *Kluyveromyces lactis*) are available. Historically, kefir has been used for the treatment of several diseases,

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including tuberculosis, hypertension, diabetes, inflammation, hypercholesterolemia, cancer, and gastrointestinal disorders (Azizi et al., 2021). Numerous studies also demonstrate the potential of kefir as an adjunct or substitute for antibiotics against *S. enteritidis, S. aureus, E. coli*, and *H. pylori*. (AbdEl-Mogheith et al., 2017; Bekar et al., 2011). A randomized clinical trial demonstrated that individuals receiving a 14-day combination therapy of lansoprazole (30 mg), amoxicillin (1000 mg), and clarithromycin (500 mg) for *Helicobacter pylori* infection achieved an eradication rate of 78.2%, whereas the control group exhibited a 50% eradication rate. Further studies indicated that the treatment group exhibited no significant adverse effects compare to the control group (Bekar et al., 2011). These findings indicate that kefir has the potential to be used as an alternative or in conjunction with antibiotic therapy.

Goat milk can be fermented into kefir, producing various bioactive peptides that are beneficial for health due to the large number of bacteria and the high protein concentration (Lestari & Giordan, 2020). The proteolytic microbes in the kefir starter hydrolyze goat's milk protein into amino acids (Rahayu et al., 2020). Peptide bonds are formed when amino acids bond with each other. Bioactive peptides are specific protein components that contain between two and twenty amino acid residues, some of which have multiple functions (Herlina et al., 2019). The resulting bioactive properties differ due to interactions among different amino acid types and sequences (Lestari & Giordan, 2020). The fermentation time of goat milk kefir also affects the peptide profile and bioactive potential (Dalabasmaz et al., 2023). Kefir bioactive peptides have antibacterial properties as one of their biological activities.

By disrupting bacterial membranes, altering metabolism, or interacting with cytoplasmic components, antibacterial peptides prevent pathogenic bacteria from growing or being destroyed. Positively charged substances and negatively charged phospholipids of the pathogenic bacterial membrane interact electrostatically to bind the antibacterial substance to precursors of the pathogenic bacterial cell wall biosynthesis. This results in the formation of a complex within the pathogenic bacterial cell membrane, resulting in pores of approximately 2 nm in width. This prevents the development of the peptidoglycan network and increases membrane permeability, allowing important cellular components to leak out, which in turn leads to the death of the pathogenic bacterial cell (Bahrami et al., 2019; Gharsallaoui et al., 2016; Punyauppa-path & Phumkhachorn, 2015). Pure kefir enhanced antibacterial activity compared to ampicillin at 10 mg/mL. Correspondingly, an in vitro study revealed higher antibacterial effect on *S. enteridis, S. Aureus,* and *E. coli* compared to ampicillin (AbdEl-Mogheith et al., 2017).

In contrast to earlier findings, antibacterial potential was identified in bioactive peptides generated from bromelain-hydrolyzed goat milk casein (Rosyani, 2018). The present study focused on the fractionation of bioactive peptides to isolate those with antibacterial activity. Membrane separation using molecular weight cut-off (MWCO) has been applied to facilitate the purification of bioactive peptides and the enrichment of target peptides based on molecular size (Kusumaningtyas et al., 2015). Bioactive peptides obtained from goat milk casein, particularly those within peptide fractions of less than 30 kDa, have been shown to exhibit antibacterial activity against *S. aureus* (Lestari & Giordan, 2020). Conversely, fractions possessing molecular weight greater than 10 kDa demonstrate higher inhibitory capacity against *E. coli, S. typhimurium* and *L. monocytogenes* (Kusumaningtyas et al., 2015). The current study focuses on bioactive peptide fractionation to isolate peptides with antibacterial activity. This research aims to address variation in antibacterial efficacy across molecular weight fractions, specifically investigating the activity of peptide fractions separated by MWCO filtration.

MATERIALS AND METHODS

Materials

In the present study Sapera goat milk was sourced from Alam Farm, Bandung Regency. The starter used was Yogourmet freeze-dried. *Escherichia coli* and *Staphylococcus aureus* were used as microorganisms. The test materials used include NaOH (Merck, German), MRSA (deMan Rogosa Sharp Agar)(Himedia, India), MEA (Malt Extract Agar)(Himedia, India),

MHA (Mueller Hinton Agar)(Himedia, India), 10% TCA (Merck, German), BSA (Merck, German), Coomasie brilliant blue G-250 (Himedia, India), 95% Ethanol (Merck, German), Orthophosphoric acid (Merck, German), Distilled water (Amidis, Indonesia), and NaCl (Merck, German).

Starter Preparation and Goat Milk Kefir Making

Goat milk was pasteurized using an autoclave with the High-Temperature Long-Time (HTLT) method at 100°C for 10 min. Pasteurized goat milk was sampled for testing with a lacto-scan (Milkotronic Ltd, Bulgaria) to determine its composition. The starter kefir used comes from freeze-dried kefir brand Yogourmet. Starter kefir contains active bacterial cultures (*Lactococcus lactis, Lactococcus cremoris, Lactococcus diacetylactis, Lactobacillus acidophilus, Saccharomyces cerevisiae,* and *Kluyveromyces lactis*) and is diluted to obtain an intermediate culture. Goat milk processed at a sterilization temperature of 100 ℃ for 10 min was then fermented with a starter from an intermediate culture (5%) for different time periods (0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 48 h), with non-fermented milk (0 h) used as a control. Fermentation was carried out in sterile jars at 27 ℃ in an incubator, under aerobic conditions without stirring.

Optimization of Goat Milk Kefir Incubation Time

Kefir was centrifuged at 15,770 × g for 20 min at 4℃ at each time point. The supernatant obtained, which is whey, was heated to 65℃ for 10 min to inactivate microbes and enzymes. Whey was measured for the degree of hydrolysis, protein content, pH and antibacterial activity at each time point. Protein content was measured by referring to the microassay method described by (Suhartono & Artika, 2017). The standard curve was determined using bovine serum albumin (BSA) with a concentration range of 0.01 – 0.1 mL/mL protein. The degree of hydrolysis was tested using the SN-TCA method and calculated using the following equation:

$$DH = \frac{\text{Soluble Protein Concentration TCA 10\%}}{\text{Total Protein Concentration of the Sample}} \times 100\%$$

Measurement of the kefir pH value was conducted using the AOAC (2006) method, where the pH value was measured using a cleaned and calibrated pH meter. The device was calibrated using standard buffer solutions of pH 4 and 7. After calibration, the pH of a 5 mL kefir sample was measured by immersing the pH meter probe into the sample. The pH value was measured in triplicate, and the average value for each sample was then calculated.

The antibacterial activity was assessed against *E.coli* and *S.aureus* as indicator pathogens. The turbidity of each bacterial suspension was standardized to the 0.5 McFarland standard (1.5 x 10^8 CFU/mL). A 40 µL sample of goat milk kefir was used as the test sample. The incubation was performed at 37 ℃ for 24 h. The formation of clear zones around the wells, indicated the antibacterial activity of the kefir starter on the test pathogen bacteria (Nurhayati et al., 2020). The diameters of both the inhibition zones and the wells were measured at three different points using a vernier caliper and average values were obtained (Afriani, 2017). The total and well areas were determined using the circular area formula. Subsequently, the bacterial inhibition zone area was determined using the following formula :

$$\text{Area of Bacterial Inhibition Zone} = \text{Total Zone Area} - \text{Area of the well zone}$$

Ultrafiltration of Antimicrobial Peptides

Kefir demonstrated optimal antibacterial activity after incubation and ultrafiltration through a 10 kDa cut-off membrane. The resulting peptide fractions (<10 and >10 kDa) were assessed for antibacterial activity (Kusumaningtyas et al., 2015). Determination of peptide molecular weight was carried out using the SDS-PAGE methode as outlined by Laemmli (1970). Electrophoresis was performed at 100 V. Staining was performed using Commassie blue.

Statistical Analysis

Data obtained from this exploratory study were collected in triplicate to ensure consistency and reliability. The results are expressed as the mean values ± standard deviation (SD). All descriptive statistical analyses, including the calculation of means and standard deviations, were performed using SPSS software (version 26). As this is an

exploratory study, the data were analyzed descriptively to compare inhibition zones and protein profiles across different fractions, with graphical representations generated in Microsoft Excel.

RESULTS AND DISCUSSION

The quality of kefir can be influenced by several factors, such as the type of milk, the fermentation process, and the type of microorganisms used. Adding different starters and percentages, as well as using different raw materials, can result in varying qualities of fermented milk and alter its nutritional value and physical properties, including texture (Triana et al., 2022). Milk of good quality will also produce kefir of good quality. The lacto-scan results of the goat milk used in making goat milk kefir showed that it contained 7.91% non-fat dry matter, 5.55% fat, 4.53% lactose, 4.2% protein, and had a pH value of 6.53. The composition of the goat milk meets the SNI standard for the quality requirements of pasteurized milk, where the minimum non-fat dry matter is 7.8%, fat is 3%, and protein content is 2.7% (SNI 3951, 2018).

Optimization of Goat Milk Kefir Incubation Time

The appropriate incubation time during the fermentation of goat's milk kefir will produce peptides with the highest antibacterial activity; therefore, it is necessary to optimize the incubation time. The fermentation time of goat milk kefir plays an important role in determining the resulting peptide profile and bioactive potential (Dalabasmaz et al., 2023). The results of optimizing the incubation time for goat milk kefir are presented in Table 1.

Based on the data in Table 1, kefir incubated for 36 h yielded the highest soluble protein content at 2.75%$\pm$0.01. The protein content can be influenced by the raw materials used. Milk with a relatively high protein content produces kefir with a relatively high protein content (Hardiansyah, 2020). The goat milk used was from Sapera goats that had been lacto-scanned and had a whole protein content of 4.2%. The soluble protein content of kefir is also influenced by the number of microbes it contains. The higher the number of microbes in goat milk kefir, the higher the protein content, as most microbial components are proteins (Hanum, 2016).

As presented in Table 1, the pH value of kefir incubated for 0-48 h ranged from 4.29 to 6.53. The pH is an important parameter that indicates the acidity level of goat's milk kefir. Acidity levels are influenced by the metabolites formed during the metabolic processes of kefir microbes. This aligns with the opinion of Prastujati, Hilmi, & Khirzin (2018), who stated that in kefir, lactose fermentation occurs into organic acids that can lower the pH value. During the incubation period of 0-28 h, the pH did not meet the standard, where the maximum pH standard for fermented milk according to the Australian Food Standard Code 2.5.3 is 4.5. This is because the kefir microbes have not yet reached optimal growth, so their metabolism is not fully efficient. The yeast used as a starter for making kefir has a lag phase duration of 20-32 h (Norberto et al., 2018). Only after an incubation period of 32-48 h did the pH value meet the standard and continue to decrease. This is consistent with the statement by Heni et al. (2021) that the longer the fermentation time, the lower the pH of the kefir whey. Kefir incubated for 12 – 48 h has a pH of 4.71 – 4.52 (Rizqiati et al. 2021).

As shown in Table 1, the highest degree of protein hydrolysis was found in kefir incubated for 36 h, with a protein hydrolysis degree value of 50.48%. The protein hydrolysis degree value is influenced by how much intact protein is broken down by microbes in kefir into amino acids. This is supported by Chen et al. (2017), who stated that proteolysis carried out by LAB intracellularly breaks down milk proteins into amino acids. The more protein that is broken down, the higher the degree of protein hydrolysis value produced. The amount of protein broken down can also be influenced by the number of microbes. At an incubation time of 0-32 h, the degree of protein hydrolysis in kefir did not reach its highest value. This is because the kefir microbes haven't reached optimal growth yet, which affects the degree of protein hydrolysis. BAL has a lag phase duration ranging from 1 to 20 h (Bancalari et al., 2016). The lag phase is the stage at which microorganisms adapt to their environmental conditions. A long lag phase can slow the metabolism of kefir microorganisms. At incubation times of 24 and 28 h, the degree of

protein hydrolysis continued to increase but had not yet reached the optimal point. This could be because the microbes have no fully completed the exponential phase. Rezvani, Ardestani, & Najafpour (2017) stated that LAB can experience an exponential phase for 25-45 h. Only at an incubation time of 32-40 h do the microbes enter a stationary phase, and they are optimal at an incubation time of 36 h. At an incubation time of 36 h, the number of microbes was at its highest, enabling optimal protein breakdown and producing the highest degree of protein hydrolysis. After reaching optimal conditions, the protein hydrolysis degree of goat milk kefir decreased further. This is because as the number of microbes increases, competition for nutrients increases, resulting in less protein degradation.

As depicted in Table 1, the highest antibacterial activity was found in kefir incubated for 36 h, with an inhibition zone against *E. coli* and S. aureus measuring 15.57 and 12.10 mm, respectively. The size of the clear zone determined the inhibition zone. The results show that goat milk kefir has a strong inhibition zone against *E. coli* and *S. aureus* bacteria. The antibacterial inhibition zones were classified into four categories: weak inhibition (<5 mm), moderate inhibition (5–10 mm), strong inhibition (10–20 mm), and very strong inhibition (>20 mm) (Fajeriyati & Andika, 2017). The inhibition zone produced during kefir fermentation is influenced by microbial metabolites. Organic acids (lactic acid), bacteriocins, and antibacterial peptides are synthesized during the complex and symbiotic fermentation process (Azizi et al., 2021; Kurniawan et al., 2025). The inhibition zone produced during kefir fermentation is influenced by microbial metabolites. The production of organic acids (lactid acid), bacteriocins and antibacterial peptides synthesized during the complex and symbiotic fermentation process (Azizi et al., 2021; Kurniawan et al., 2025) Since the supernatant was preheated to eliminate enzymatic activity, the metabolite responsible for antibacterial peptides. These bioactive peptides, consisting of small protein fragments, exhibit bactericidal or bacteriostatic effects against pathogenic bacteria (Marcos & Manzanares, 2013). Research has shown that certain microorganisms in kefir, such as bacteria and yeast, can produce antibacterial peptides (Miao et al., 2016). Moreover, prior investigations reveale that bioactive peptides derived from bromelain-hydrolyzed of goat milk casein identified to possess antibacterial acticity (Rosyani, 2018).

Ultrafiltration of Antimicrobial Peptides

The desired peptide concentration can be achieved using this method based on the molecular weight (Kusumaningtyas et al., 2015). Table 2 presents the results of the antibacterial test of goat milk kefir before and after the fractionation. This method allows the peptide concentration to be selectively obtained based on molecular weight (Kusumaningtyas et al., 2015). The results of the antibacterial test of goat milk kefir prior to the following fraction are summarized in Table 2.

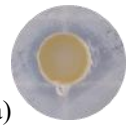
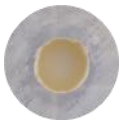
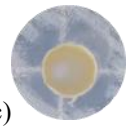
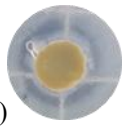
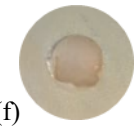
The results indicate that unpurified goat milk kefir following 36 h of incubation exhibited 15.57 ± 0.14 mm inhibition zones against *E. coli* and 12.10 ± 0.20 mm against *S. aureus*. The >10 kDa fraction showed significantly higher inhibition (69.1 ± 0.1 mm and 41.3 ± 0.2 mm, respectively), whereas the <10 kDa fraction showed no observable inhibition against either pathogenic bacterium. Table 2 illustrates antibacterial activity of goat milk kefir with an incubation time of 36 h under conditions (a) unpurified and unfiltered samples against pathogenic *E. coli*, (b) unpurified and unfiltered samples against pathogenic *S. aureus*, (c) purified >10 kDa fraction samples against pathogenic *E. coli*, (d) purified >10 kDa fraction samples against pathogenic *S. aureus*, (e) purified <10 kDa fraction samples against pathogenic *E. coli*, and (f) purified <10 kDa fraction samples against pathogenic *S. aureus*. According to the data, kefir fractions with molecular weights above 10 kDa showed an increase in inhibition zones against pathogenic bacteria. This suggests that the antibacterial activity in this study is not attributed to small peptides (<10 kDa), but rather to larger protein fragments or bacteriocin-like compounds retained in the retentate. The absence of activity in the <10 kDa fraction indicates that smaller peptides were either present in insufficient quantities or lacked the specific structural properties required for bactericidal action in this specific fermentation context. This observation supports the findings of Kusumaningtyas et al.

(2015), who reported that molecular weight-based fractionation enhances the concentration of peptides based on molecular size. The purification process demonstrated that goat milk exhibited a strong inhibitory effect against *E. coli* and *S. aureus*. The strength of antibacterial inhibitory power is considered very strong when it is > 20 mm (Fajeriyati & Andika, 2017).

Table 1. Goat milk kefir incubation time

Incubation Time (hour)	Soluble Protein Content (%)	pH	Degree of Protein Hydrolysis (%)	Antibacterial	
				E. colli	*S. aureus*
0	0.85 ± 0.01	6.53 ± 0.01	20.23 ± 0.12	-	-
4	1.07 ± 0.01	6.15 ± 0.01	25.48 ± 0.16	6.61 ± 0.03	-
8	1.20 ± 0.01	5.59 ± 0.01	28.57 ± 0.15	8.40 ± 0.05	-
12	1.21 ± 0.01	5.15 ± 0.01	28.81 ± 0.20	9.01 ± 0.05	-
16	1.21 ± 0.01	4.90 ± 0.01	28.81 ± 0.20	9.90 ± 0.05	-
20	1.26 ± 0.01	4.82 ± 0.01	30.00 ± 0.20	10.00 ± 0.05	-
24	1.46 ± 0.01	4.62 ± 0.01	34.76 ± 0.25	10.22 ± 0.05	-
28	1.63 ± 0.01	4.54 ± 0.01	38.81 ± 0.30	11.14 ± 0.05	-
32	2.28 ± 0.01	4.45 ± 0.01	54.28 ± 0.20	11.03 ± 0.05	8.55 ± 0.05
36	2.75 ± 0.01	4.37 ± 0.01	65.48 ± 0.25	15.57 ± 0.1	12.10 ± 0.1
40	2.60 ± 0.01	4.37 ± 0.01	61.90 ± 0.30	13.67 ± 0.1	10.48 ± 0.1
44	1.50 ± 0.01	4.37 ± 0.01	35.71 ± 0.30	12.79 ± 0.1	9.69 ± 0.1
48	1.21 ± 0.01	4.29 ± 0.01	28.81 ± 0.10	-	-

Table 2. Antibacterial test of goat milk kefir before and after fractionation

Molecular Weight (kDa)	Antibacterial		*E. colli*	*S. aureus*
	E. colli	*S. aureus*		
Kefir 36 h	15.57 ± 0.14	12.10 ± 0.20	 (a)	 (b)
> 10	69.1 ± 0.1	41.3 ± 0.2	 (c)	 (d)
< 10	-	-	 (e)	 (f)

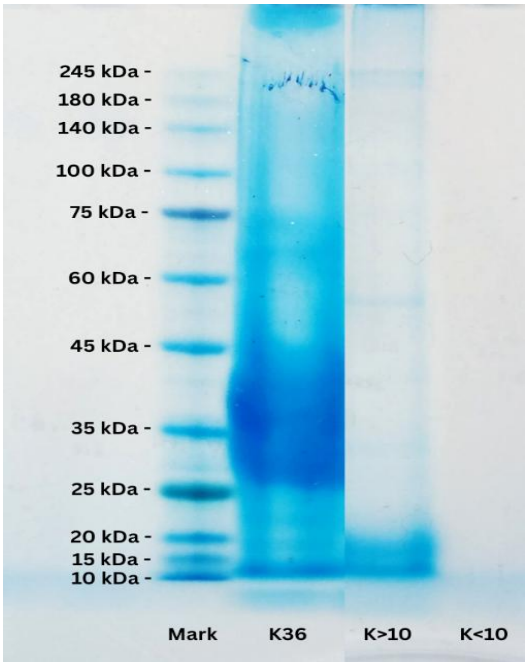


Figure 1. SDS-PAGE profile of goat milk kefir
Mark: LMW marker; K36: kefir incubated for 36 h without purification;
K>10: 36-hour kefir purified with molecular weight >10 kDa;
K<10: 36-hour kefir purified with molecular weight <10 kDa

Based on the SDS-PAGE gel results (Figure 1), K36, goat's milk kefir incubated for 36 h, contains six bands with approximate sizes of 78, 30, 24, 18, 16, and 14 kDa. K>10 and K<10 show the purified protein bands of goat milk kefir. At K>10, three protein bands were found with sizes of approximately 55, 15, and 13 kDa, while no protein bands were found at K<10. This absence of detectable bands in the <10 kDa fraction directly correlates with its lack of inhibitory zones, confirming that the active antimicrobial agents are concentrated within the 13–55 kDa range. The bands at 13 kDa and 15 kDa likely correspond to low-molecular-weight proteins or large bacteriocins, while the 55 kDa band may represent aggregated peptide complexes or larger functional proteins derived from the goat milk proteolysis.

These findings present a contrast to previous literature. While Lestari & Giordan (2020) reported that bioactive peptides derived from goat milk casein with molecular weights <30 kDa were effective against *S. aureus*, and Kusumaningtyas et al. (2015) found that fractions >10 kDa demonstrated higher inhibitory capacity against *E. coli* and *L. monocytogenes*, this study observed activity exclusively in the >10 kDa fraction. These discrepancies in molecular weight activity likely stem from variations in experimental conditions:

a. Proteolysis Extent and Fermentation Time: This study utilized a 36 h fermentation, reaching a degree of hydrolysis of 65.48%. Shorter or longer fermentation times in other studies would alter the cleavage patterns, potentially producing smaller active peptides that were not yet dominant in this study.
b. Starter Culture Composition: The specific microbial consortium used (*Lactococcus lactis, Lactobacillus acidophilus, Saccharomyces cerevisiae*, etc.) dictates the specific proteases and bacteriocins produced. Different strains used in other studies would generate different peptide profiles.
c. Milk Source: The use of Sapera goat milk may result in different initial protein structures compared to other goat breeds, influencing the final size of the bioactive fragments after hydrolysis.

According to Nitsche (2011), a protein with a molecular weight of 37 kDa was α-casein, while the protein with a molecular weight of 18 kDa was β-lactoglobulin, and the protein with a molecular weight of 14 kDa was

α-lactalbumin. However, according to Tay & Gam (2011), the protein bands with molecular weights of 30-60 kDa in goat milk were dominated by κ-casein.

CONCLUSIONS

The optimal incubation time for the fermentation of goat milk protein using a kefir starter was 36 h, with a soluble protein content of 2.75%, a pH value of 4.37, degree of hydrolysis of 50.48%, and antibacterial activity against *E. coli* and S. aureus of 15.57 and 12.10 mm, respectively. The antibacterial activity of bioactive peptides after purification with a molecular weight >10 kDa increased compared to that before purification, resulting in inhibition zones of 69.1 mm against *E. coli* and 41.3 mm against *S. aureus*. Three protein bands were found in peptides with a molecular weight >10 kDa, measuring approximately 55, 15, and 13 kDa. These results suggest that goat milk kefir produces antibacterial peptides that can potentially be used as adjuncts or substitutes for antibiotics.

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