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ORIGINAL ARTICLE

Antibacterial activity of *dali ni horbo* protein extract modified with citrus and pineapple juice

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ABSTRACT

Background: The global rise in antibiotic resistance highlights the urgent need to identify novel, natural antimicrobial agents. Fermented dairy products represent potential sources of bioactive peptides with inherent antibacterial properties. *Dali ni horbo* is a traditional fermented buffalo milk product from North Sumatra, Indonesia. This study aimed to evaluate the *in vitro* antimicrobial activity of a crude protein extract from *dali ni horbo*, modified with orange and pineapple juice, against selected pathogenic microorganisms.
Methods: This experimental laboratory study employed disk diffusion and well diffusion methods on agar media to assess the extract's activity against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Propionibacterium acnes*, and *Candida albicans*.
Results: The results showed no inhibition zones for any treatment against all tested microorganisms. The absence of detectable antimicrobial activity may be attributed to the low concentration or biological activity of antibacterial compounds in the crude extract, the possible degradation of bioactive peptides during extraction, and the limited diffusion capacity of high-molecular-weight proteins in solid agar media.
Conclusion: In conclusion, under the experimental conditions and methods applied, the crude protein extract of *dali ni horbo* did not exhibit antimicrobial activity against the tested bacterial and fungal species. Further studies involving protein purification, bioactive peptide isolation, and alternative liquid-based susceptibility assays are recommended.

Keywords: antimicrobial activity, *dali ni horbo*, disk diffusion, protein extract, well diffusion

Introduction

Infectious diseases caused by pathogenic microorganisms remain a major global health concern.[1] The increasing prevalence of antibiotic resistance has reduced the effectiveness of conventional therapies and elevated the risk of treatment failure for many infections.[2] This situation highlights the urgent need to develop alternative antibacterial agents that are safe, naturally derived, and sustainable.[3] One promising strategy involves the use of fermented foods as sources of bioactive compounds with antimicrobial properties.[4] Fermentation processes can produce a variety of secondary metabolites with biological activities, including antibacterial effects, through microbial metabolism.[5]

Dairy-based fermentations are of particular interest because milk proteins can undergo proteolysis by lactic acid bacteria (LAB), generating bioactive peptides.[6] These peptides are typically small, positively charged molecules that interact with bacterial cell membranes, disrupt cellular integrity, and inhibit the

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growth of pathogenic microorganisms.[7] However, the formation and biological activity of these peptides are strongly influenced by the type of fermenting microorganisms, the environmental conditions during fermentation, and the post-fermentation treatments applied.[6]

Indonesia possesses a remarkable diversity of traditional fermented foods across its regions, shaped by variations in raw materials, geography, and local processing techniques.[8,9] One such traditional product from North Sumatra is *dali ni horbo*, a food produced from buffalo milk through natural coagulation and fermentation. *Dali ni horbo* has a solid, chewy texture and is rich in nutrients, especially protein.[10] The relatively high protein content of buffalo milk suggests that *dali ni horbo* may serve as a potential source of bioactive peptides with various biological activities, including antibacterial properties.[11]

Previous study has shown that protein fractions or peptides isolated from *dali ni horbo* exhibit antibacterial activity against pathogens such as *Staphylococcus aureus*.[12] This activity is typically observed after the protein undergoes purification or further hydrolysis, which promotes the optimal formation and detection of small bioactive peptides. In contrast, crude protein extracts have limited activity due to the relatively large molecular size of the proteins and the low diffusion capacity of active compounds within solid testing media.[13] Antibacterial activity refers to the ability of a bioactive compound to inhibit the growth or induce the death of pathogenic bacteria through specific interactions that disrupt the structural integrity or physiological processes of microbial cells.[28] Proteins and peptides derived from traditional food sources are increasingly being investigated as alternative natural antimicrobial agents due to their diverse biological activities and their lower tendency to promote resistance compared to conventional synthetic antibiotics[28].

Modifying protein extracts with natural additives such as citrus and pineapple juice represents a potential strategy to enhance antibacterial potency. Citrus fruits contain phenolic compounds and flavonoids with inherent antibacterial properties, while pineapple contains bromelain, an enzyme capable of hydrolyzing proteins into smaller peptides.[14,15] The combination of these additives may promote the release or activation of bioactive compounds bound within the protein matrix.[16] However, the effectiveness of such modifications largely depends on the concentration of active compounds, the stability of bioactive components, and the antimicrobial testing methods used.[17]

Given the potential of *dali ni horbo* as a source of bioactive peptides and the need to optimize their biological activity, a systematic investigation is warranted. The antibacterial activity of *dali ni horbo* protein extract is generally evaluated in vitro using disc diffusion and well diffusion methods to determine the ability of the compound to inhibit the growth of test bacteria, as indicated by the formation of an inhibition zone on agar media.[28] Therefore, the objective of this study was to evaluate the *in vitro* antibacterial and antifungal activities of crude protein extracts from *dali ni horbo* modified with citrus and pineapple juice against a representative panel of microorganisms.

Method

This experimental laboratory study aimed to evaluate the in vitro antimicrobial activity of the *dali ni horbo* protein extract. The research was conducted at the Integrated Research Laboratory, Universitas Prima Indonesia, beginning in January 2025. The study population consisted of traditional fermented products derived from buffalo milk. Samples of *dali ni horbo* were collected from Durin Tunggal Village, Pancur Batu District, using a purposive sampling technique based on criteria of authenticity and traditional fermentation methods. The independent variable was the *dali ni horbo* protein extract modified with citrus and pineapple juice. The dependent variables were antibacterial and antifungal activities, identified by the presence or absence of inhibition zones. Controlled variables included the test microorganism species, growth media, incubation conditions, and the implementation of both positive and negative controls.

Protein extraction was conducted using a phosphate buffer solution, followed by centrifugation and protein precipitation without subsequent fractionation or hydrolysis. Antimicrobial activity was assessed using the disk diffusion and well diffusion methods. Mueller Hinton Agar was employed for bacterial assays, and appropriate media were used for Candida albicans. After incubation at 37°C for 24 to 48 hours, observations were made based on the presence and diameter of inhibition zones around the treatment sites. Data were collected through detailed visual examination and measurement of inhibition zones on the agar plates.

Data were analyzed descriptively and presented in both tabular and narrative formats. Inferential statistical tests were not conducted, as this preliminary investigation primarily aimed to provide an initial

assessment of antimicrobial potential. The results consistently demonstrated the absence of inhibition zones in all tests against the evaluated microorganisms.

Results

Antibacterial activity test using the disk diffusion method (6 µL)

Antibacterial testing using the disc diffusion method was carried out with a volume of 6 µL for each treatment. The treatments included a positive control consisting of lysozyme and protease enzymes, a negative control using NaCl solution, and the DNH protein extract combined with pineapple and orange extracts. Antibacterial activity was determined by the presence or absence of clear inhibitory zones surrounding the discs.

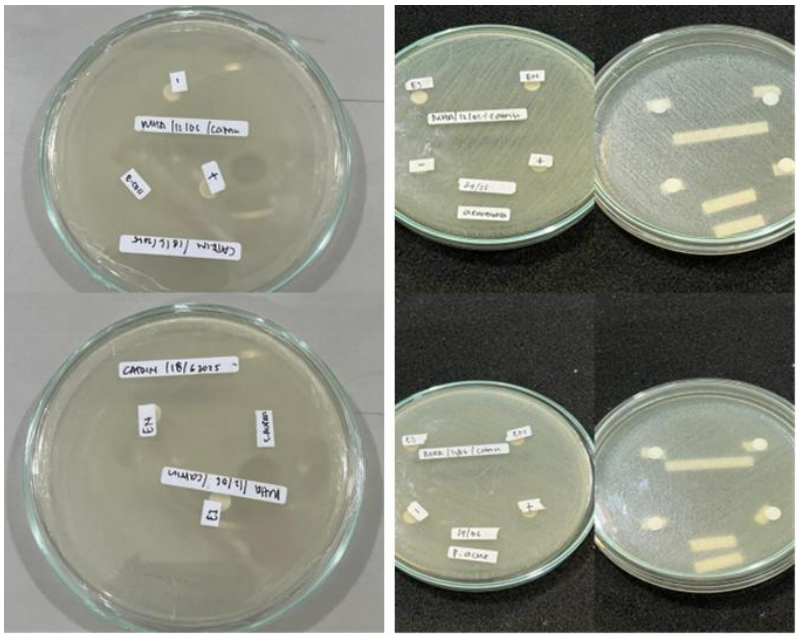

Figure 1. Results of antibacterial activity testing using the disc diffusion method.

According to the results presented in Table 1, no inhibition zones were observed in any of the treatments for the tested bacteria. Bacterial growth was evenly distributed on the agar surface with no evidence of clear zones surrounding the discs.

Table 1. Antibacterial activity by disc diffusion method (6 µL)

Test Microorganism	Positive Control (Lysozyme & Protease)	Negative Control (NaCl)	DNH + Pineapple & Orange Extracts
Escherichia coli	6 mm	6 mm	6 mm
Staphylococcus aureus	6 mm	6 mm	6 mm
Pseudomonas aeruginosa	6 mm	6 mm	6 mm
Propionibacterium acnes	6 mm	6 mm	6 mm

Antibacterial activity test using the well diffusion method (20 µL)

Antibacterial activity testing using the well diffusion method was performed with a sample volume of 20 µL for each treatment. The results of the inhibition zone observations are presented in Table 2.

Table 2. Antibacterial activity by well diffusion method (20 µL)

Test Microorganism	Positive Control (Lysozyme & Protease)	Negative Control (NaCl)	DNH + Pineapple & Orange Extracts
Escherichia coli	20 mm	20 mm	20 mm
Staphylococcus aureus	20 mm	20 mm	20 mm
Pseudomonas aeruginosa	20 mm	20 mm	20 mm
Propionibacterium acnes	20 mm	20 mm	20 mm

As shown in Table 2, none of the treatments exhibited inhibition zones against the tested bacteria. The bacterial colonies grew uniformly on the agar surface without any clear zones indicating antibacterial activity.

Antifungal activity test against *Candida albicans* (6 µL)

Antifungal activity testing using the well diffusion method was carried out with a sample volume of 6 µL per treatment. The results of the inhibition zone observations are displayed in Table 3.

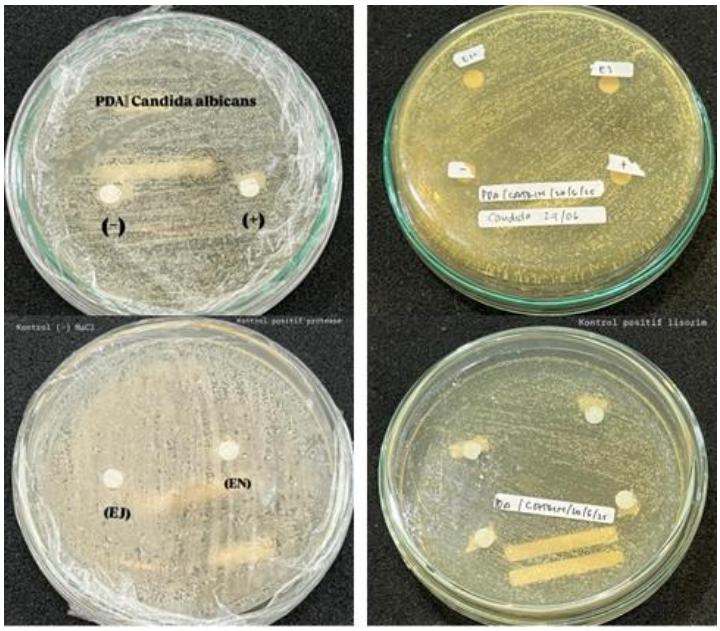

Figure 3. Results of antifungal activity testing

Based on the results, neither the *dali ni horbo* protein extract nor the controls demonstrated any inhibitory activity against *Candida albicans*.

Table 3. Antifungal activity against *Candida albicans* (6 µL)

Treatment	Inhibition Zone Diameter (mm)
Positive Control (Lysozyme)	6 mm
Positive Control (Protease)	6 mm
Negative Control (NaCl)	6 mm
DNH + Pineapple Extract	6 mm
DNH + Orange Extract	6 mm

Antifungal activity test against *Candida albicans* (20 µL)

Antifungal activity was further assessed using the well diffusion method with a sample volume of 20 µL for each treatment. The results are presented in Table 4.

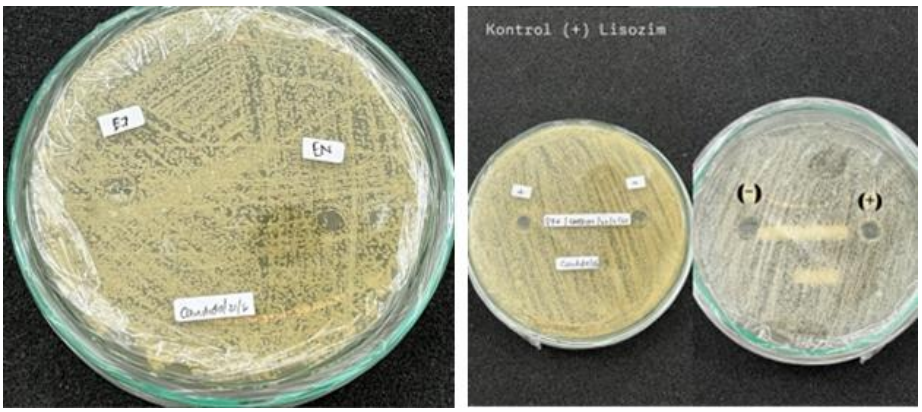

Figure 4. Results of antifungal activity testing

According to the findings, neither the *dali ni horbo* protein extract nor the control groups showed inhibitory effects against *Candida albicans*.

Table 4. Antifungal activity against *Candida albicans* (20 µL)

Treatment	Inhibition Zone Diameter (mm)
Positive Control (Lysozyme)	20 mm
Positive Control (Protease)	20 mm
Negative Control (NaCl)	20 mm
DNH + Pineapple Extract	20 mm
DNH + Orange Extract	20 mm

Discussion

The minimal to absent antimicrobial response of the *dali ni horbo* protein extract observed in this study warrants a detailed evaluation of the underlying chemical and methodological factors. Recent literature confirms that fermented milk products can produce antimicrobial peptides through the activity of lactic acid bacteria during fermentation. These peptides are typically characterized by low molecular weight and a strong capacity to penetrate microbial membranes.[6] Peptides of lower molecular weight are generally more effective in disrupting cell membrane integrity and exerting antibacterial activity compared to larger protein fragments.[18] In this study, the crude protein fraction was not subjected to controlled proteolysis or further purification, steps that several studies have identified as crucial for generating effective bioactive peptides.

The choice of antimicrobial testing method plays a critical role in the detection and interpretation of compound activity. Diffusion-based techniques on solid media, such as disk and well diffusion assays, are generally well suited for small, highly diffusible compounds but have limited capacity to detect bioactivity from large or complex molecules.[13] In contrast, liquid media-based methods, such as Minimum Inhibitory Concentration (MIC) assays, have been reported to offer greater sensitivity in detecting larger antimicrobial peptides.[17] Large-molecular-weight antimicrobial compounds show more apparent activity when assessed using MIC assays compared to diffusion-based methods.[19,20]

Environmental testing conditions also significantly influence the activity of natural products. Factors such as pH, incubation temperature, and compound stability strongly affect biological activity.[21,22] Suboptimal pH influences antimicrobial peptide activity from various sources, including milk hydrolysates. For hemoglobin-derived peptides from bovine and porcine sources hydrolyzed with pepsin, lower pH (2-3) enhanced peptide release and antibacterial activity against Gram-negative E. coli and others, with higher pH reducing hydrolysis efficiency.[23] This finding may help explain the low antimicrobial activity observed in the present study despite the traditional association of *dali ni horbo* with functional properties.

Some studies propose that additives rich in enzymes or phyto-compounds, such as fruit extracts, can enhance the release of bioactive peptides when appropriately processed.[14] Pineapple extract containing bromelain and phenolic or flavonoid compounds from citrus sources has the potential to augment antimicrobial activity when extracted under suitable conditions.[15] However, other research indicates that in complex protein matrices, such compounds may interact in ways that reduce their bioavailability and efficacy.[24] These findings underscore that the successful activation or synergy of bioactive compounds is highly dependent on extraction technique, concentration, and assay conditions.

In contrast to reports describing antifungal activity in hydrolyzed protein fractions from fermented dairy products[25,26], the present study found that the crude protein fraction of *dali ni horbo* exhibited no antifungal effect against *Candida albicans*. This observation aligns with evidence suggesting that non-protein metabolites, such as organic acids, volatile compounds, or other fermentation-derived products, often play a more dominant role in antifungal activity than crude protein fractions.[26,27]

The limitations of this study include the use of crude protein extract without additional proteolysis or purification, the limited sensitivity of diffusion methods for detecting large-molecular-weight compounds, and the absence of optimization for extract concentration and environmental test parameters. Future studies should focus on isolating and characterizing bioactive peptides, employing liquid-based methods such as MIC assays, and optimizing testing conditions to reveal the antimicrobial potential of *dali ni horbo* more effectively and comprehensively.

Conclusion

This study concludes that the crude protein extract of *dali ni horbo*, in the form tested, exhibited no antibacterial activity against *E. coli*, *S. aureus*, *P. aeruginosa*, or *P. acnes*, and no antifungal activity against *Candida albicans* in *in vitro* assays. Therefore, the study objective to evaluate the antimicrobial potential of this extract was not fully achieved under the conditions used. These results suggest that both the inherent characteristics of the crude extract and the limitations of the diffusion assay contributed to the low level of detectable biological activity. It is recommended that subsequent research include protein purification or isolation of specific bioactive peptides, increased extract concentrations, optimized environmental test conditions, and the use of alternative liquid-based methods such as MIC assays. These approaches would provide a more comprehensive understanding of the antimicrobial potential of *dali ni horbo* and contribute to the broader application of natural materials as sources of bioactive compounds in pharmaceutical science.

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