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ANTIBACTERIAL ACTIVITY AND PEPTIDES CHARACTERIZATION OF PARTIAL-PURIFIED NEUTRALIZED CELL-FREE SUPERNATANT OF BACTERIAL ISOLATES FROM PADO FERMENTED FISH

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Abstract

Pado is a traditional fermented food from West Sumatra, made by combining pelagic fish with grated coconut and dried seeds of *Pangium edule*. Lactic acid bacteria are the predominant microorganisms present and generally produce metabolites that contribute to prolonging the shelf life of the product. The neutralized cell-free supernatant (NCFS) of these isolates may contain bioactive peptides, such as antibacterial peptides. This study aimed to obtain partially purified NCFS from pado isolates with the best inhibitory activity against *Salmonella* Typhimurium and *Staphylococcus aureus* using agar diffusion methods. Crude NCFS was obtained from seven isolates from pado by centrifuging their growth media, and the supernatant was neutralized. The proteinaceous nature of crude NCFS was confirmed after artificial digestion with proteolytic pepsin. Ultrafiltration was used to partially purify crude NCFS. Antibacterial peptides were further analyzed using liquid chromatography-high resolution mass spectrometry (LC-HRMS). All NCFS showed antibacterial activity against *S.* Typhimurium, but only three isolates showed apparent inhibitory activity against *S. aureus*. The *Lactiplantibacillus plantarum* B3Iw4 isolate was selected for further study. The low-molecular-weight NCFS fraction (in the range of 317.20–1,494.70688 Da) after ultrafiltration by a 3 kDa membrane exhibited a threefold increase in specific activity against *S. aureus* compared to the crude NCFS. LC-HRMS analysis identified 20 peptides in the partially purified NCFS. The peptide, AAERAGAAALAMHGR, was predicted to have antibacterial activity after matching with the BIOPEP-UWM database, with a matched sequence AA. This peptide has relatively high hydrophobicity and is interesting for further study.

Keywords: antibacterial peptide, fermented fish, *Lactiplantibacillus plantarum*, specific activity, ultrafiltration

Aktivitas Antibakteri dan Karakterisasi Peptida Supernatan Murni Parsial dari Isolat Bakteri Asal Ikan Fermentasi Pado

Abstrak

Pado adalah pangan fermentasi tradisional dari Provinsi Sumatra Barat, terbuat dari ikan pelagis yang dicampur dengan ampas kelapa dan cacahan daging biji picung (*Pangium edule*) kering. Bakteri asam laktat merupakan bakteri yang dominan ditemukan pada produk fermentasi dan umumnya dapat menyekresikan metabolit yang berperan dalam membantu memperpanjang masa simpan produk pangan. Supernatan bebas sel yang dinetralkan (NCFS) dari isolat tersebut diduga mengandung peptida bioaktif, yaitu peptida antibakteri. Penelitian ini bertujuan untuk menentukan NCFS murni parsial yang dihasilkan oleh isolat asal pado dengan aktivitas penghambatan terbaik terhadap *Salmonella* Typhimurium dan *Staphylococcus aureus* menggunakan metode agar difusi. NCFS kasar diperoleh dari tujuh isolat pado dengan sentrifugasi media

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pertumbuhannya dan menetralkan supernatannya. Sifat protein dari NCFS kasar dikonfirmasi dengan memaparkan enzim pepsin proteolitik. Ultrafiltrasi digunakan untuk memurnikan NCFS kasar secara parsial. Peptida antibakterinya dikarakterisasi menggunakan l*iquid chromatography-high resolution mass spectrometry* (LC-HRMS). Semua NCFS menunjukkan aktivitas antibakteri terhadap *S.* Typhimurium, tetapi hanya tiga isolat yang menunjukkan aktivitas penghambatan terhadap *S. aureus*. Isolat *Lactiplantibacillus plantarum* B3Iw4 dipilih untuk penelitian lanjut. Fraksi NCFS dengan berat molekul rendah (dalam rentang 317,20 Da hingga 1.494,70688 Da) setelah ultrafiltrasi dengan membran 3 kDa menunjukkan peningkatan aktivitas spesifik tiga kali lipat terhadap *S. aureus* dibandingkan dengan NCFS kasar. Analisis LC-HRMS mengidentifikasi 20 peptida dalam NCFS murni parsial. Satu peptida, AAERAGAAALAMHGR, diprediksi memiliki aktivitas antibakteri setelah dicocokkan dengan basis data BIOPEP-UWM, dengan sekuen yang cocok AA. Peptida ini memiliki sifat hidrofobisitas yang relatif tinggi dan menarik untuk diteliti lebih lanjut.

Kata kunci: aktivitas spesifik, ikan fermentasi, *Lactiplantibacillus plantarum*, peptida antibakteri, ultrafiltrasi

INTRODUCTION

Pado is a local fermented food product from West Sumatra, made from a mixture of tamban (*Sardinella* sp.) or sarai (*Rastrelliger* sp.) fish, grated coconut, and dried *Pangium edule* seeds. Pado is rare compared to other fermented fish products such as bekasam, rusip, and fish paste. Currently, pado sellers can be found solely in Tandikat, Malalak, Gunung Padang Alai, V Koto Kampung Dalam, and Bukittinggi. Previous research has reported that neither homemade pado nor pado purchased in certain districts of West Sumatra contained *Staphylococcus aureus*, a pathogenic bacterium (Hasbullah *et al*., 2016a). These findings are comparable to those of buduk, a fermented fish food from West Kalimantan, which is known to contain no pathogenic bacteria, such as *Bacillus cereus*, *Clostridium perfringens*, Enterobacteriaceae, *Staphylococcus aureus*, and *Listeria monocytogenes* (Nofiani *et al*., 2019). The absence of pathogenic bacteria could be attributed to the role of lactic acid bacteria (LAB) in these foods.

Lactic acid bacteria are the predominant bacteria in pado, a fermented fish (Syafitri *et al*. 2022). According to Cleveland *et al*. (2001), LAB has been comprehensively investigated as a source of antimicrobial peptides for food applications. Lactic acid bacteria are a group of lactic acid-forming bacteria that are generally recognized as safe (GRAS), non-respiring, non-sporulating, and facultatively anaerobic (Liu *et al*., 2021). Antimicrobial peptides have attracted the attention of many researchers. They are found in many fermented foods. Moreover, they are known as potential antimicrobial agents with high thermostability and selectivity; therefore, they can be used in the food industry as food preservatives to prevent spoilage caused by microorganisms and foodborne pathogens (Kamal *et al*., 2023).

Antimicrobial peptides from microorganisms applied in the food industry as biopreservatives are known as bacteriocins. Bacteriocins are metabolites mainly produced by lactic acid bacteria that can act against pathogenic and food spoilage bacteria (Ageni *et al*., 2017). In addition to being used to preserve food safety and prolong shelf life, these antimicrobial peptides also help maintain the nutritional composition, antioxidant parameters, and sensory attributes of food, such as the application of RSQ01 (a bacteriocin from *Lactococcus lactis*) in milk (Zhang *et al*., 2023). Furthermore, bacteriocins are easily digested by proteases in the human gastrointestinal tract. Thus, bacteriocin-based food preservatives have been permitted for food application by the Food and Drug Administration (FDA), for example, nisin, a lanthionine-containing peptide produced by certain strains of *Lactococcus lactis*. The European Food Safety Authority (EFSA) stated that no adverse effects were found in animal toxicity studies; therefore, it was considered safe for human consumption.

Some bacteriocins produced from fermented food isolation are known as pentocin 31-1 produced by *L. pentosus* 31-1 from traditional Chinese fermented Xuan-Wei ham (Zhang *et al*., 2009), bacteriocin 163-1 produced by *L. plantarum* 163-1 from Chinese fermented salted radish (Hu *et al*., 2016), and plantaricin LPL-1 produced by *L. plantarum* LPL-1 from fermented fish (Wang *et al*., 2018). The antimicrobial peptides have sequences of

VIADYGNGVRXATLL, YVCASPW, and VIADKYYGNGVSCGKHTCTVDWGEA FSCSVSHLANFGHGKC, respectively, which can reveal the peptides' characteristics and profiles. However, it has been no report on peptide profiles with antimicrobial activity from LAB of pado origin.

The process of identifying bacteriocins is tedious and difficult; therefore, the initial step in finding the metabolites is to produce a neutralized cell-free supernatant (NCFS) and then purify it through a series of purification stages to analyze substances that are similar to bacteriocins. Cell-free supernatant production attempts to obtain extracellular metabolites present in the culture medium. Silva *et al.* (2018) stated that compared to the direct application of bacteriocinogenic cultures, purified and concentrated bacteriocins as food additives have become the preferred preparation due to their efficacy. The ultrafiltration step has been widely used to obtain a partially purified sample by separating components based on their molecular weights. The peptide sequences contained in the ultrafiltration fraction were identified using liquid chromatography-high resolution mass spectrometry. This is a convenient method for revealing peptide profiles in the fraction.

The fact that pado can be stored at room temperature for 14 weeks (Hasbullah *et al.*, 2016b) has led to the investigation of its antibacterial peptides. During the fermentation process of pado, the species of LAB that are consistently found have the closest relationship with *Lactiplantibacillus plantarum,* based on molecular analysis (Syafitri *et al.*, 2022). This LAB is known to produce bacteriocins as antibacterial agents. This study aimed to obtain partial-purified NCFS from pado isolates with the best inhibitory activity against *Salmonella* Typhimurium and *Staphylococcus aureus* using agar diffusion methods.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Lactic acid bacteria were isolated from pado using the method described by Syafitri *et al.* (2022). Previous researchers isolated these bacteria from the making of pado in the laboratory of IPB University, and pado obtained from the Tandikek market in Padang Pariaman Regency and Malalak market in Agam Regency, West Sumatera Province. Seven LAB isolates (APd3_2U1, APd5_2U1, APd7_2U2, A2Aw4, B1Aw4, B3Iw4, Id3_1U2) were successfully identified molecularly, with a high number of hits closely related to *Lactiplantibacillus plantarum*. The isolates were cultivated on DeMan Rogosa Sharpe (MRS) media (Oxoid, UK) at 37 °C for 24 h. *Salmonella* Typhimurium ATCC 14028 and *Staphylococcus aureus* ATCC 25923 were selected as the test bacteria. They are both pathogenic bacteria commonly associated with food poisoning in fish. *S.* Typhimurium and *S. aureus* represent Gram-negative bacteria and Gram-positive bacteria, respectively. They were cultivated in Tryptone Soya Broth (TSB) media (Oxoid, UK) at an incubation temperature of 35 °C for 24 h.

Production of Crude NCFS of LAB Isolates from Pado

Seven cultivated LAB isolates were inoculated at 1% v/v into 50 mL of liquid MRS medium (Oxoid, UK) and incubated for 48 h at 37 °C. The bacterial cultures were centrifuged at 6,000 rpm for 30 min. The supernatant was separated from the pellet to obtain a cell-free supernatant (CFS). The pH of CFS was adjusted to 6.5 using 1 M NaOH (Merck, Germany) to exclude the antimicrobial effect due to organic acids, and then filtered using a 0.2 μm membrane filter (Sartorius, Germany). Crude NCFS was obtained according to the method described by Liu *et al.* (2019), with modifications. The neutralized cell-free supernatants were freeze-dried using a freeze-dryer (Labconco, US) under vacuum conditions for 48 h. Each dried sample (0.25 g) was dissolved in 1 mL of 0.9% physiological NaCl solution and tested for antibacterial activity.

Proteinaceous Nature Confirmation of Crude NCFS of *L. plantarum* B3Iw4

The proteinaceous nature of crude NCFS was tested by exposing 250 μL of

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crude NCFS to 750 µL of pepsin (1 mg/mL) dissolved in sodium acetate buffer (pH 4.6), followed by homogenization and incubation for 2 h at 37 °C (Kumar *et al*., 2016; Sari *et al*., 2018). The sample was heated at 100 °C for 5 min and then cooled to room temperature. The samples were then tested for antibacterial activity.

Partial Purification of Crude NCFS of *L. plantarum* B3Iw4

Crude NCFS was partially purified using the ultrafiltration membrane technique based on the procedure described by Kurnianto *et al*. (2021), with modifications. Before partial purification, the crude NCFS was diluted to a particular concentration to ensure that the sample was not too concentrated. The components in the sample were separated based on their molecular weight using a regenerated cellulose membrane with a molecular weight cut-off (MWCO) of 3 kDa (Merck, Germany) by centrifugation for 60 min at 1,320 × *g* at 4°C. This process separates the sample into two fractions with molecular weights (MW) of <3 kDa and >3 kDa.

Each fraction was concentrated using a freeze-dryer, and the dried sample was dissolved in aquabidest water. The fractions were analyzed for protein content and tested for inhibitory activity against the test bacteria. Total protein concentration was analyzed by injecting 2 µL of crude NCFS, the fraction with MW <3 kDa, and >3 kDa, into the Microvolume UV-Vis Spectrophotometer NanoDrop One (Thermo Scientific, US).

Antibacterial Activity Assay of Crude NCFS and Partial-purified NCFS

The antibacterial activity of crude NCFS was tested using the disc and well agar diffusion methods, whereas partially purified NCFS (NCFS UF) was analyzed using the disc diffusion method. The disc diffusion method was performed based on the guidelines of the Clinical and Laboratory Standards Institute (2012) by first preparing a fresh test bacterial inoculum, and then a sterile cotton swab was dipped into the inoculum suspension. Furthermore, a cotton swab was streaked over the entire surface of the Mueller Hinton Agar (MHA) media (Oxoid, UK). A 6 mm disc (HiMedia, India) that had been dripped with 30 µL of sample was placed on the surface of the agar media and incubated overnight at 35 °C. Chloramphenicol (30 µg/mL) was used as a positive control. The diameter of the inhibition zone was measured using a digital caliper (Krisbow, Indonesia), including the diameter of the disc, and expressed in millimeters.

The well diffusion method was carried out by inoculating 1% v/v of the test bacteria into 25 mL of MHA media, which was then poured into a sterile Petri dish until the agar media solidified (Kurnianto *et al*., 2021). A well with a 6 mm diameter was created using a sterile cork borer. This well diffusion assay used crude NCFS volume treatments, namely 60 and 100 µL, to determine the volume of the sample that best inhibited the growth of the test bacteria. The sample at each volume was then dripped into the well and placed in the refrigerator at 4°C for 1-2 hours. The Petri dish was incubated for 24 h at 35°C, and the clear zone formed was observed. The total antibacterial peptide activity, antibacterial peptide-specific activity, and purification level were calculated according to Kurnianto *et al*. (2021).

LC-HRMS Analysis of Partial-purified NCFS

The NCFS fraction with MW <3 kDa was analyzed using liquid chromatography-high resolution mass spectrometry (LC-HRMS). The test was carried out by filtering the NCFS fraction using a 0.22 µm membrane filter and then injecting it into the NanoLC Ultimate 3,000 Series System Tandem Q Exactive Plus Orbitrap HRMS instrument (Thermo Scientific) equipped with a PepMap RSLC C18 capillary column (75 µm × 15 cm, 3µm, 100 Å) (Thermo Scientific) and a Thermo Scientific™ 164649 trap column (30 µm × 5 mm). The sample was eluted with 0.1% (v/v) formic acid in water (mobile phase A) and 0.1% (v/v) formic acid in acetonitrile (mobile phase B) at a flow rate of 300 nL/min. The elution program was as follows: 2% for 30 minutes, 2-35% B for 27 minutes, 35-99% B

for 10 min, 99% B for 15 min, and 2% B for 10 min. A mass range of 200-2,000 m/z was used for the spectra collection.

Data generated from LC-HRMS in Xcalibur software (Thermo Fisher Scientific) were analyzed using Proteome Discoverer 2.2 (Thermo Fisher Scientific) with Sequest HT as the search engine for peptide identification. The UniProt (https://www.uniprot.org/peptide-search) and Findpept (https://web.expasy.org/findpept/) databases were used to predict peptide sequences containing 3-5 amino acids. PepDraw (https://www.pepdraw.com/) was used to determine the molecular weights and hydrophobicities of the detected peptides. The identified peptides were matched with the sequences of peptides in Biopep-UWM (https://biochemia.uwm.edu.pl/biopep-uwm/) with antibacterial activity to predict the antibacterial peptides.

Data Analysis

Antibacterial activity experiments were performed in duplicate, and the results were expressed as mean ± standard deviation using Microsoft Excel. These data were also used to calculate the total antibacterial peptide activity, antibacterial peptide-specific activity, and purification level using Microsoft Excel. Significant differences between the means of the antibacterial activity data were determined using the Shapiro-Wilk test for normality and the Kruskal-Wallis test (95% confidence interval) using IBM SPSS Statistics 30.0.0 software.

RESULTS AND DISCUSSION

Antibacterial Activity of Crude NCFS

The inhibition zone of crude NCFS indicated antibacterial activity against *Salmonella* Typhimurium and *S. aureus*. All crude NCFS inhibited the growth of *Salmonella* Typhimurium (Table 1). The results of the disc diffusion assay in this study were comparable to those of Heredia-Castro *et al*. (2015), who reported that 20 µL of crude extract of *Lactobacillus pentosus* J37 showed an inhibition zone of approximately 6 mm against *S.* Typhimurium. Usually, the sample volume added to the test disc for antimicrobial testing using agar disc diffusion is 20-30 µL, to prevent flooding over the disc that can lead to uneven diffusion and inaccurate results. A small sample volume allows for even diffusion of the substance from the disc into the agar, ensuring consistent results. On the other hand, the well agar diffusion method, which is commonly used for screening the antimicrobial activity of different extracts from plants or microorganisms, usually uses a larger volume of sample, namely upto100 µL (Balouiri *et al*., 2016). As shown in Table 1, there were significant differences in the diameter of the inhibition zone between the sample volumes in the well diffusion agar test. The 100 µL crude NCFS showed a larger inhibition zone diameter, ranging from 7.63±0.19 mm to 7.95±0.16 mm. Nguyen *et al*. (2014) found that the neutralized cell-free supernatant of *Lactobacillus plantarum* T8 and *Lactobacillus plantarum* T13, isolated from traditional Vietnamese fermented cabbage, could inhibit *S.* Typhimurium Sal1 by 5-7.5 mm. There was no significant difference between the crude NCFS isolates using disc or agar well diffusion assays.

The antibacterial activity test against *S. aureus* using the well diffusion method revealed no differences in the diameter of the zone of inhibition between the crude NCFS with volumes of 60 and 100 µL (Table 2). In the disc diffusion treatment, the overall zones of inhibition were not excessively large. The crude NCFS of LAB isolates from pado exhibited relatively lower antibacterial activity in inhibiting the growth of *S. aureus* than that reported in other studies. The crude extract of *L. plantarum* J25, isolated from artisanal Mexican Cocido cheese, demonstrated an antibacterial activity of 8 mm against *S. aureus* ATCC 29213 using the disc diffusion method (Heredia-Castro *et al*., 2015). Using agar diffusion treatment, the inhibition zone towards *S. aureus* ATCC 6538 was 12.55 mm from purified bacteriocin of *L. plantarum* C8 isolated from traditional Chinese pickle (Zhou *et al*., 2014).

Crude NCFS from all *L. plantarum* isolates exhibited better antibacterial activity against gram-negative bacteria than gram-positive bacteria in the well diffusion assay

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Table 1 Antibacterial activity of crude NCFS against *Salmonella* Typhimurium

Crude NCFS	The diameter of the inhibition zone (mm)		
	Volume for disc diffusion assay (µL)	Volume used for well diffusion assay (µL)	
	30	60	100
L. plantarum APd3_2U1	6.11±0.16^{aA}	6.37±0.52^{aA}	7.74±0.24^{aB}
L. plantarum APd5_2U1	6.04±0.05^{aA}	7.65±0.13^{bB}	7.69±0.21^{aB}
L. plantarum APd7_2U2	6.35±0.22^{abA}	6.92±0.37^{abA}	7.76±0.26^{aB}
L. plantarum A2Aw4	6.31±0.14^{aA}	6.53±0.74^{aA}	7.63±0.19^{aB}
L. plantarum B1Aw4	6.41±0.36^{abA}	7.06±0.88^{abA}	7.95±0.16^{aB}
L. plantarum B3Iw4	6.42±0.40^{abA}	6.86±0.95^{abA}	7.90±0.06^{aB}
L. plantarum Id3_1U2	6.04±0.05^{aA}	6.89±0.79^{abB}	7.71±0.04^{aB}
Positive control (Chloramphenicol 30 µg/mL)	6.75±0.49^{bA}	11.37±0.20^{cC}	16.70±0.19^{bD}
Negative control (0.9% physiological NaCl)	-	-	-

Different small letter marks on the same column and different capital letter marks on the same row indicated significant differences (p<0.05).

Table 2 Antibacterial activity of crude NCFS against *S. aureus*

Crude NCFS	The diameter of the inhibition zone (mm)		
	Volume for disc diffusion assay (µL)	Volume used for well diffusion assay (µL)	
	30	60	100
L. plantarum APd3_2U1	6.00±0.00^{aA}	6.00±0.00^{aA}	6.00±0.00^{aA}
L. plantarum APd5_2U1	6.09±0.13^{aA}	6.00±0.00^{aA}	6.00±0.00^{aA}
L. plantarum APd7_2U2	6.01±0.02^{aA}	6.00±0.00^{aA}	6.00±0.00^{aA}
L. plantarum A2Aw4	6.05±0.07^{aA}	6.00±0.00^{aA}	6.46±0.64^{aA}
L. plantarum B1Aw4	6.00±0.00^{aA}	6.00±0.00^{aA}	6.53±0.75^{aA}
L. plantarum B3Iw4	6.11±0.15^{aA}	6.00±0.00^{aA}	6.41±0.57^{aA}
L. plantarum Id3_1U2	6.75±1.06^{bA}	6.00±0.00^{aA}	6.00±0.00^{aA}
Positive control (Chloramphenicol 30 µg/mL)	6.96±0.67^{bA}	14.93±0.23^{bB}	16.07±0.09^{bC}
Negative control (0.9% physiological NaCl)	-	-	-

Different small letter marks on the same column and different capital letter marks on the same row indicated significant differences (p<0.05).

(sample volumes of 60 and 100 µL). The results were similar to those obtained from antibacterial testing using the disc diffusion assay on crude NCFS from *L. plantarum* APd3_2U1, APd7_2U2, A2Aw4, B1Aw4, and B3Iw4 strains. These isolates were isolated from a mixture of grated coconut with dried *Pangium edule* seeds on days 3, 5, and 7 of laboratory-scale pado fermentation production, fish on day 3 of laboratory-scale pado fermentation production, a mixture of grated coconut with dried *Pangium edule* seeds from products taken from a market in Tandikek (week 4 storage), a mixture of grated coconut with dried *Pangium edule* seeds, and fish from products taken from a market in Malalak (week 4 storage) (Syafitri *et al*., 2022). The findings of this study differ from those reported in the literature. Nisin and pediocin A have bactericidal activity against a wide range of gram-positive bacteria, whereas other bacteriocins (diplococcin, lactacin B&F, lactocin 27, helveticin J, and lactostrepcins) have limited activity against species that are closely related to the producer strain (Klaenhammer, 1988). The relatively lower inhibitory ability of *S. aureus* in this experiment might be due to the crude NCFS obtained, which was not yet pure.

The inhibition zone diameters of all crude NCFS against both *S*. Typhimurium and *S. aureus* in this study were classified as slight, based on the classification applied by Afrin *et al*. (2021). According to Afrin *et al*. (2021), the diameter of the inhibition zone from the results of antibacterial testing with the well diffusion method could be classified as slight (x<4 mm), medium (x=4-8 mm), high (x=8-12 mm), and very high (x>12 mm). x is the difference between the inhibition zone diameter and the well diameter (6 mm). Morales et al. (2003) categorized antibacterial activity in the disc diffusion assay as follows: - (no activity), + (6-10 mm inhibition zone diameter), ++ (11-20 mm inhibition zone diameter), and +++ (21-30 mm inhibition zone diameter). Thus, the antibacterial activities of all crude NCFS against *S*. Typhimurium and *S. aureus* were classified as +, except for the crude NCFS from *L. plantarum* APd3_2U1 and B1Aw4, which exhibited no antibacterial activity against *S. aureus*. Since the crude NCFS of *L. plantarum* B3Iw4 inhibited the growth of the two test bacteria in both disc and well diffusion assays, this isolate was selected for crude NCFS production and partial purification. The crude NCFS of *L. plantarum* B3Iw4 showed antibacterial activity against *S*. Typhimurium, which was 6.42±0.40 mm by disc diffusion and 7.90±0.06 mm by well diffusion (a sample volume of 100 µL) and against *S. aureus*, the activity was 6.11±0.15 mm and 6.41±0.57 mm, respectively.

Confirmation of the proteinaceous nature of crude NCFS of *L. plantarum* B3Iw4 showed the loss of an inhibition zone against *S*. Typhimurium and *S. aureus* after treatment with pepsin (data not shown). Pepsin is a protease that hydrolyzes proteins and peptides into amino acids and short-chain peptides, respectively. As a result of this process, the ability of the metabolite to inhibit the growth of the test bacteria may be reduced or lost entirely. The proteinaceous nature of the bacteriocin was confirmed when the antimicrobial activity of the bacteriocin extract was completely lost after treatment with proteolytic enzymes such as proteinase K, chymotrypsin, trypsin, papain, pronase, and pepsin (Hassan *et al*., 2020). It was assumed that proteinaceous compounds were the active metabolites in crude NCFS of *L. plantarum* B3Iw4 and played a role in inhibiting the growth of the test bacteria.

Antibacterial Activity of Partial-purified NCFS

The zone of inhibition diameter, total antibacterial peptide activity, and antibacterial peptide-specific activity of NCFS from *L. plantarum* B3Iw4 are presented in Table 3. After partial purification using ultrafiltration, NCFS UF with MW <3 kDa (filtrate) and >3 kDa (retentate) inhibited *S. aureus* growth better than crude NCFS (6.14±0.17 mm and 6.12±0.12 mm). The NCFS fraction with low molecular weights exhibited the highest total antibacterial peptide activity against *S. aureus*. Compared to crude NCFS, the antibacterial peptide-specific activities of NCFS UF with MW <3 kDa and >3 kDa increased to 1.85 AU/mg and 1.92 AU/mg, respectively.

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Table 3 Total proteins/peptides, the diameter of the inhibition zone, total antibacterial peptides activity, antibacterial peptides-specific activity, and purification level of crude NCFS produced from *L. plantarum* B3Iw4 and its fraction

Parameter	Test bacteria	Sample		
		Crude NCFS	NCFS UF <3 kDaa	NCFS UF >3 kDaa
Total proteins/peptides (mg)	-	452.49	192.54	158.50
The diameter of the zone of inhibition (mm)	*S. aureus*	6.11±0.15	6.14±0.17	6.12±0.12
	S. Typhimurium	6.42±0.40	6.00±0.00	6.00±0.00
Total antibacterial peptides activity (AU)b	*S. aureus*	278.85	355.78	304.45
	S. Typhimurium	1091.97	-	-
Antibacterial peptides-specific activity (AU/mg)[c]	*S. aureus*	0.62	1.85	1.92
	S. Typhimurium	2.41	-	-
Purification level[d]	*S. aureus*	1.00	3.00	3.12
	S. Typhimurium	1.00	-	-

[a]NCFS UF is an ultrafiltration fraction of crude NCFS; [b]Total antibacterial peptides activity was determined by disc diffusion; [c]Antibacterial peptides-specific activity was determined by the ratio between total antibacterial peptides activity and total proteins/peptides; [d]Purification level was determined by the ratio between antibacterial peptides-specific activity of NCFS UF and antibacterial peptides-specific activity of crude NCFS.

Based on the antibacterial peptide-specific activity, the components in the two fractions separated by MWCO 3 kDa became more active than those in the unseparated NCFS in inhibiting the growth of *S. aureus*. The antibacterial peptide-specific activity explains the value of the activity of 1 mg of protein contained in the fraction. These findings are consistent with those of other studies. In another study, the specific activities of BLIS UF *Streptomyces labedae* SCA-8 <3 kDa and 3-10 kDa were 167.9 AU/µg and 266.2 AU/µg, respectively, which were higher than that of crude BLIS (136.2 AU/µg) (Kurnianto *et al*., 2021). In another study, bacteriocin Bac-IB45 of *Lactobacillus plantarum* KIBGE-IB-45 isolated from cheddar cheese was purified using desalting, Sepharose CL-6B, and Centricon 10 kDa, and it showed higher specific activity than crude bacteriocin, 17.73 AU/mg, 25.68 AU/mg, and 123.07 AU/mg, respectively (Ibrahim *et al*., 2019). Several factors could influence the differences in results between studies and the literature, such as the strain of bacteria used, the strain's ability to produce metabolites, and the stages of purification performed.

According to Kurnianto *et al*. (2022), BLIS-specific activity can demonstrate the effectiveness of the purification process. The antibacterial peptide-specific activity of crude NCFS was increased up to three times, indicating that partial purification using ultrafiltration was effective. High purification level results can be obtained by performing various purification stages. Wang *et al*. (2018) reported that precipitation with ammonium sulfate, SP-Sepharose Fast Flow cation exchange, Sephadex 105 gel filtration chromatography, and reverse-phase high-performance liquid chromatography (RP-HPLC) had higher purification rates, 2.56 times, 37.16 times, 61.66 times, and 86.63 times, respectively, compared to *L. plantarum* LPL-1 culture supernatant.

In contrast to the antibacterial activity against *S. aureus*, the NCFS fraction showed no zone of inhibition against *S.* Typhimurium. Therefore, the NCFS UF with MW <3 kDa and >3 kDa exhibited no total antibacterial peptide activity or antibacterial peptide-specific activity. Al-Hammam *et al*. (2023) also discovered that a bacteriocin-like substance produced by *Lactobacillus* sp. GMP1 isolated

from fermented fish products exhibited greater bacteriocin activity against *Staphylococcus aureus* ATCC 6538 than *Salmonella* sp. 230C. In this study, a combination of NCFS fractions with low and high molecular weights was likely required to inhibit the growth of *S.* Typhimurium. *S.* Typhimurium is a Gram-negative bacterium that is more resistant to antimicrobial agents than gram-positive bacteria because of the presence of an outer membrane that acts as a protective layer.

LC-HRMS profile of Partial-purified NCFS

The data obtained from LC-HRMS analysis were chromatograms showing peptide peaks (Figure 1). Peptides from peaks with retention times of less than 17 min were identified using Proteome Discoverer 2.2, and the remaining peptides were identified by predicting their mass spectra using the Uniprot and Findpept databases. A total of 20 peptides were found in NCFS UF with MW <3 kDa, 14 of which were identified using Proteome Discoverer 2.2.

The peptides found in the NCFS fraction (with low molecular weights) had a molecular mass range of 317.20 – 1,494.70688 Da and peptide length range of 3-15 amino acid residues (Table 4). In addition, the peptides had a percentage of hydrophobic amino acids of up to 60%, with hydrophobicity ranging from +9.14 to +22.90 kcal/mol. These results were similar to those of Kurnianto *et al*. (2021), who reported that the BLIS UF *Streptomyces labedae* SCA-8 <3 kDa peptides had hydrophobic amino acid percentages of up to 75% and hydrophobicity values of up to 26.50 kcal/mol. Antimicrobial peptides typically contain hydrophobic residues that enable them to cause cell death (Cao *et al*., 2021).

The peptides were examined for their potential as antibacterial peptides using the Biopep-UWM database. The Ala-Ala (or AA) antibacterial peptide sequence found in the Biopep-UWM database was also found in a peptide, AAERAGAAALAMHGR, at a 16.338-minute retention time. The peptide had a length of 15 amino acid residues and a hydrophobic amino acid content of 60%.

The complete sequences of all peptides did not match the sequences listed in the Biopep-UWM database. The mismatch of all peptides from LC-HRMS results with the Biopep-UWM database could be due to the possibility that the sequences have never been registered by other researchers in the database. Some sequences that have been reported as antibacterial peptides in the form of bacteriocins from *L. plantarum*, such as YVCASPW (Hu *et al*., 2016), PGWAVAAA GALGGTAAIVWIARQFGVHLTTKLTQKA LDLLSSGASLGT-VAAVILGVTL (Golneshin *et al*., 2020), and KYGNGLSRIFSALK (Pei *et al*., 2021), were not registered in the Biopep-UWM database; therefore, these sequences were not included in the search.

CONCLUSION

Among the seven LAB isolates from pado, *L. plantarum* B3Iw4 produced crude NCFS with potential antibacterial activity. The crude NCFS was fractionated by ultrafiltration, which effectively increased the antibacterial peptide-specific activity against *S. aureus*. The low-molecular-weight NCFS fraction (<3 kDa) contained 20 peptides, one of which was predicted to be an antibacterial peptide. This fraction is an object of interest for further research, particularly in optimizing the synthesis and predicting peptide characteristics using bioinformatics tools.

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Table 4 Peptide profiles in partial-purified NCFS (MW <3 kDa) from analysis using LC-HRMS

No	Retention time	Accession No.	Sequence	Molecular mass (Da)	Description	Reported organism	Hydrophobicity (kcal/mol)
1	14.9339	A0A349MY07	TSPAIDHPSSQR	1,294.63871	RNA-binding protein	*Lactobacillus* sp	+17.74
2	15.1346	A0AAD0TRW0	WQLADTATFER	1,336.65876	Uncharacterized protein	*Lactiplantibacillus paraplantarum*	+14.20
3	15.4235	A0A0R1YNA6	VGSYSLGmRQR	1,268.62464	Lantibiotic transport ATP-binding protein srtF	*Amylolactobacillus amylophilus DSM 20533 = JCM 1125*	+12.42
4	15.7508	A0A2R3JX27	ELTASATAVDFQR	1,407.69434	Uncharacterized protein	*Lactobacillus* sp. CBA3606	+16.79
5	15.7553	E1QWE8	ADVGASNFTITDER	1,494.70688	Polar amino acid ABC transporter, inner membrane subunit	*Olsenella uli* ATCC 49627 / DSM 7084 / CIP 109912 / JCM 12494 / NCIMB 702895 / VPI D76D-27C	+21.29
6	15.9063	A0A1S6QK79	IQQGGSLMVYISR	1,450.76542	Serine/threonine protein kinase	*Lentilactobacillus curieae*	+9.14
7	15.9591	A0A1Z5IAJ0	QDLVDQPTVYGNK	1,475.7313	Carboxylate--amine ligase	*Secundilactobacillus mixtipabuli*	+19.03
8	16.0985	A0AA46QEU5	TLGSGNPGSTNTFR	1,407.69489	Glycerol-3-phosphate acyltransferase	*Lactobacillus* sp. c10Ua232AE	+13.71
9	16.1865	W9EJS5	LNGAVSQHTQR	1,209.62229	Prepilin-type N-terminal cleavage/ methylation domain-containing protein	*Fructilactobacillus florum* 8D	+15.08

Table 4 Peptide profiles in partial-purified NCFS (MW <3 kDa) from analysis using LC-HRMS (cont)

No	Retention time	Accession No.	Sequence	Molecular mass (Da)	Description	Reported organism	Hydrophobicity (kcal/mol)
10	16.338	A0A426D6W0	AAERAGAAALAMHGR	1,467.74634	tRNA-dihydrouridine synthase	*Lactiplantibacillus garii*	+21.36
11	16.3403	G2KV71	KANQMTSLNNR	1,275.6319	Glycosyltransferase	*Fructilactobacillus sanfranciscensis* TMW 1.1304	+15.12
12	16.4826	A0A4V1P037	WGNIPSFEFEPK	1,449.69031	Serine--tRNA ligase	*Lacticaseibacillus chiayiensis*	+14.07
13	16.6176	A0A2P4R5G6	KNETAPTVSHGPGR	1,449.74433	ABC transporter ATP-binding protein	*Companilactobacillus formosensis*	+22.90
14	16.8552	A0A5P0ZJ72	GKGNSKWSQMNPK	1,460.71326	Zinc-ribbon domain-containing protein	*Companilactobacillus mishanensis*	+19.37
15	17.85	F9UPX2	VHTGR	568.29	Transposase	*Lactiplantibacillus plantarum ATCC BAA-793 / NCIMB 8826 / WCFS1*	+12.98
16	20.59	F9UN82	NSYK	510.23	Cell surface SD repeat protein, membrane-anchored	*Lactiplantibacillus plantarum ATCC BAA-793 / NCIMB 8826 / WCFS1*	+11.30

Table 4 Peptide profiles in partial-purified NCFS (MW <3 kDa) from analysis using LC-HRMS (cont)

No	Retention time	Accession No.	Sequence	Molecular mass (Da)	Description	Reported organism	Hydrophobicity (kcal/mol)
		F9URL4			ABC transporter, permease protein	*Lactiplantibacillus plantarum ATCC BAA-793 / NCIMB 8826 / WCFS1*	
17	22.55	F9US71	HTR	412.20	Nitrobenzoate reductase	*Lactiplantibacillus plantarum ATCC BAA-793 / NCIMB 8826 / WCFS1*	+12.29
		F9UTG8			Amino acid transport protein	*Lactiplantibacillus plantarum ATCC BAA-793 / NCIMB 8826 / WCFS1*	
18	27.38	F9UMW2	KHSK	498.27	ATP-dependent RNA helicase	*Lactiplantibacillus plantarum ATCC BAA-793 / NCIMB 8826 / WCFS1*	+16.29
		F9UU07			Bacteriocin peptide PlnF	*Lactiplantibacillus plantarum ATCC BAA-793 / NCIMB 8826 / WCFS1*	
19	28.97	F9UTQ8	YSAR	495.28	Extracellular transglycosylase	*Lactiplantibacillus plantarum ATCC BAA-793 / NCIMB 8826 / WCFS1*	+9.96
		Q6LWF7			*Uncharacterized protein (Fragment)*	*Lactiplantibacillus plantarum ATCC BAA-793 / NCIMB 8826 / WCFS1*	
		F9UN46	HTR		Extracellular protein	*Lactiplantibacillus plantarum ATCC BAA-793 / NCIMB 8826 / WCFS1*	
20	30.35		SGR	317.20			+11.32
		F9UT23			ABC transporter, ATP-binding protein	*Lactiplantibacillus plantarum ATCC BAA-793 / NCIMB 8826 / WCFS1*	

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