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BIOACTIVE METABOLITES OF *Lactiplantibacillus plantarum* ISOLATED FROM *Sonchus arvensis* AS AN ANTIMICROBIAL AGENT

Metabolit Bioaktif *Lactiplantibacillus plantarum* yang Diisolasi dari *Sonchus arvensis* sebagai Agen Antimikroba

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ABSTRACT
Sonchus arvensis is a plant widely found in Southeast Asia and often used in traditional medicine. *Lactiplantibacillus plantarum* is a bacteria isolated from *S. arvensis* and categorized as qualified presumption of safety by the European Food Safety Authority and the US Food and Drug Administration. Therefore, this study aimed to determine the bioactive metabolites of *Lpb. plantarum* as antimicrobial agents. Lactic acid bacteria (LAB) were isolated using the serial dilution method, followed by isolation and amplification of the DNA through a 16S rRNA universal primer. Antimicrobial activity was screened using the well-diffusion method. Plantaricin gene identification was performed using PCR and the determination of lactic acid content was conducted using Spectrophotometric. In addition, the titration method was used to measure and determine the hydrogen peroxide. The results showed that *Lpb. plantarum* had higher inhibition toward pathogen bacteria than *Lc. lactis. Lpb. plantarum* had the largest inhibition zone against *B. subtillis,* followed by *S. aureus* and *E. coli,* respectively. It was discovered that *Lpb.* plantarum precipitated with ammonium sulfate had a greater protein content and antibacterial activity. Furthermore, *Lpb. plantarum,* which encodes *plnA* and *plnEF*, produced lactic acid and hydrogen peroxide at concentrations of 3.0158±0.2774 mg/mL and 0.195±0.04 mg/mL, respectively.

Keywords: *Bioactive metabolites, Hydrogen peroxide, Lactic acid, Lpb. Plantarum, Plantaricin*

ABSTRAK
Sonchus arvensis adalah tanaman yang banyak ditemukan di Asia Tenggara dan sering digunakan dalam pengobatan tradisional. Lactiplantibacillus plantarum merupakan bakteri yang diisolasi dari S. arvensis dan dikategorikan sebagai qualified presumption of safety oleh Otoritas Keamanan Pangan Eropa dan Badan Pengawas Obat dan Makanan Amerika Serikat. Oleh karena itu, penelitian ini bertujuan untuk mengetahui metabolit bioaktif Lpb. plantarum sebagai agen antimikroba. Bakteri asam laktat (BAL) diisolasi menggunakan metode pengenceran serial, dilanjutkan dengan isolasi dan amplifikasi DNA menggunakan primer universal 16S rRNA. Aktivitas antimikroba dilakukan dengan menggunakan metode well-diffusion. Identifikasi gen plantaricin dilakukan dengan menggunakan PCR dan penentuan kadar asam laktat dilakukan dengan menggunakan

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Spektrofotometri. Selain itu, metode titrasi digunakan untuk mengukur dan menentukan hidrogen peroksida. Hasil penelitian menunjukkan bahwa Lpb. plantarum memiliki daya hambat yang lebih tinggi terhadap bakteri patogen dibandingkan dengan Lc. lactis. Lpb. plantarum memiliki zona hambat terbesar terhadap B. subtillis, diikuti oleh S. aureus dan E. coli. Ditemukan bahwa Lpb. plantarum yang diendapkan dengan amonium sulfat memiliki kandungan protein dan aktivitas antibakteri yang lebih besar. Selanjutnya, Lpb. plantarum, yang mengkode plnA dan plnEF, menghasilkan asam laktat dan hidrogen peroksida pada konsentrasi masing-masing 3,0158 ± 0,2774 mg / mL dan 0,195 ± 0,04 mg / mL.

Kata kunci: *Asam laktat, Hidrogen peroksida, Metabolit bioaktif, Lpb. Plantarum, Plantarisin*

INTRODUCTION

A common plant in many parts of Southeast Asia, including Indonesia, is called *Sonchus arvensis*. Many cultures use this plant as traditional medicine, especially for digestive issues (Harahap 2020). The medicinal properties related to digestion of this plant known to be associated with the Lactic acid bacteria (LAB). LAB is mainly found in fermented foods, the digestive tracts of humans and animals, as well as natural environments, such as soil, freshwater, and plants (Mokoena 2017). *Lactiplantibacillus plantarum* (*Lpb. plantarum*), previously referred to as *Lactobacillus plantarum*, is the most common LAB found in plants (Zheng et al. 2020). The European Food Safety Authority has validated a qualified presumption of safety for *Lpb. plantarum* while The US Food and Drug Administration confirmed as safe (Syaputri and Iwahashi 2020).

Lpb. plantarum has been found to reduce cholesterol levels (Seddik et al. 2017), exhibit antimicrobial properties (Kim et al. 2020), act as diarrhea preventive (Kaźmierczak-Siedlecka et al. 2020), possess anti-inflammatory properties (Stankovic et al. 2022), and aid in the healing of intestinal inflammation (Jung et al. 2022). These properties are attributed to the production of organic acids by *Lpb. plantarum*, such as lactic acid, acetate, phenyl lactate, hydroxy phenyl lactate (Di Biase et al. 2022), hydrogen peroxide (Chen et al. 2023), and bacteriocins (Syaputri et al. 2023).

The previous studies documented an extensive exploration of lactic acid, bacteriocin, and hydrogen peroxide production for antibacterial effects against various pathogenic bacteria. Lactic acids synthesized by LAB can enter the lipid membrane of bacterial cells in undiluted phase, subsequently splitting into anions and protons (Szczerbiec et al. 2022). The protons induce cytoplasmic acidification and interfere with specific cell functions. Meanwhile, the buildup of anions disrupts metabolic processes, increases osmotic pressure, and causes cell demise (Szczerbiec et al. 2022; Syaputri et al. 2023). The bacteria *Lpb. plantarum* produces bacteriocins, specifically plantaricin. The plantaricin-coding gene is located in an operon cluster, found in the chromosome, plasmid, or transposons (Malik et al. 2016). Each plantaricin exhibits unique mechanisms and characteristics that vary according to the amino acid sequence encoded by its gene. Some of these genes include *plnW, plnV, plnU, plnS, plnR, plnP, plnO, plnN, plnM, plnL, plnK, plnJ, plnH, plnG, plnF, plnE, plnD, plnC, plnB* and *plnA* (Syaputri et al. 2021a). The mechanism of inhibiting pathogenic bacteria is also affected by the distinct characteristics of plantaricin. Genes exhibit differences in inhibiting pathogenic bacteria. However, this compound, plantaricin, disrupts the outer shell of the target cell (cell wall) and hinders its ability to build proteins or replicate its genetic material (nucleic acid synthesis) (Ahmad et al. 2017), leading to pore formation or cell lysis (Syaputri and Iwahashi 2020). Plantaricin belongs to class II bacteriocins, also known as two-peptide bacteriocins. This class has high efficiency in inhibiting pathogenic bacteria and is over 1000 times more active than other plantaricins (Bengtsson et al. 2020; Zhang et al. 2016).

Therefore, this study focused exclusively on class IIb plantaricin, specifically two-peptide plantaricin, due to the superior characteristics.

Catalase was synthesized by aerobic organisms and facultative anaerobes equipped with cytochrome systems. This synthesis protects cells against internally generated hydrogen peroxide by breaking the components into water and oxygen (Ezraty et al. 2017). A previous study showed that the acquisition of individual electrons by oxygen (O_2) caused the formation of partially reduced reactive oxygen species (ROS), such as superoxide anions ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH•). These components were found to potentially harm cells and induce cellular mortality (Erttmann and Gekara 2019; Ezraty et al. 2017). Therefore, this study investigated the bioactive metabolites produced by *Lpb. plantarum* isolated from *S. arvensis* as antimicrobial agents toward *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus*. The main metabolites of interest include plantaricin, lactic acid, and hydrogen peroxide, which inhibit pathogens bacteria.

MATERIAL AND METHODS

Isolation of Lactic Acid Bacteria from *S. arvensis*

Sonchus arvensis leaves were mixed with Man, Rogosa, and Sharpe (MRS) broth (Becton, Dickinson and Company, Franklin Lakes NJ, USA) at a ratio of 1:9 and subsequently diluted by serial dilution up to 10^{-5}. The mixture was inoculated on MRS agar (Becton, Dickinson and Company, Franklin Lakes NJ, USA) and incubated at 37°C for 24 hours in an anaerobic condition. The colonies were identified as LAB by their milky white, circular, and convex shape. Subsequently, the LAB were inoculated into MRS Broth and preserved at -80°C in MRS broth with 20% glycerol (Wako Pure Chemical Industries, Osaka, Japan).

DNA Isolation of Lactic Acid Bacteria

LAB was cultured until obtaining a biomass of 10^8 CFU/mL and then subjected to DNA isolation using the Extrap Soil DNA Kit Plus Ver.2. (Nippon Steel Eco-Tech Corporation, Tokyo, Japan). Total DNA quality testing was carried out using 1% agarose gel electrophoresis in 1x TAE buffer followed by dyeing of gel mixture with Ethidium Bromide and observed under ultraviolet light.

Identification and Sequencing Analysis of *Lpb. plantarum* using 16S rRNA

16S rRNA was employed to identify LAB using primer 27 F: (5'-GAGTTTGATCCTGGCTAG-3') and 1525 R: (5'-AGAAAGGAGGTGATCCAGCC-3') (Miranti et al. 2022). The Polymerase chain reaction (PCR) reaction components included 12.5 µL of 2× Green Master Mix PCR (Promega, Madison, USA), 9 µL of nuclease-free deionized water (Promega, Madison, USA), 1.25 µL of 0.05 pmol/µL for each primer, and 1 µL of template DNA. PCR was operated with pre-denaturation at 95°C for 5 minutes, followed by 25 cycles of amplification (denaturation for 1 minute at 94°C, annealing for 1 minute at 56°C, extension for 1.5 minute at 72°C), and a final extension at 72°C for 7 minutes. PCR products were analyzed using electrophoresis (Mupid-exU, Shiga, Japan) with 1% agarose gel in 1× TAE buffer at 100 V for 30 min. Furthermore, the estimation of DNA fragment size was observed using FastGene 100 bp DNA Ladder (Nippon Genetics, Düren, Germany) to identify the presence of a 1,500 bp band. Prior to sequencing, DNA purification was performed using the FastGeneTM Gel/PCR Extraction kit (Nippon Genetics, Düren, Germany). The purified PCR products were sequenced by DNA Sequencing Analysis Service of the Genomic Research Division, Gifu University, using the Multicapillary DNA Sequencer ABI Prism 3100/3130 Genetic Analyzer (Thermo Fisher Scientific, Waltham, USA). This process was followed by editing and assembling the sequences into contigs using BioEdit and connected using Bioedit-ClustalW Multiple Alignment. The sequences were read and compared with the NCBI database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Isolates were identified as the same species as the database with ≥97% sequence similarity (Johnson et al. 2019).

Antimicrobial Screening of Lactic Acid Bacteria

The well diffusion method was performed to assess antibacterial activity (Miranti et al. 2022) and characterized based on the presence of clear zones around the wells. LAB were incubated for 24 hours at 37°C in MRS Broth. The indicator bacteria used for antimicrobial screening were *B. subtilis*, *E. coli*, and *S. aureus*. The bacteria were grown overnight in a broth called Luria Bertani Broth (LB Broth) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 30°C. Then, holes were punched into Mueller-Hinton agar Becton, Dickinson and Company, Franklin Lakes, NJ, USA) plates, and 100 µL of a cell-free liquid (called cell-free supernatant) from the bacteria culture were added to each hole. A 100 µg/mL of sodium ampicillin (Avantor, USA) was used as the positive control. Positive results for antimicrobial activity were shown by the clear zone produced by isolates suggesting growth inhibitor. Antimicrobial screening was performed in triplicate and the highest clear zones were selected for further testing.

Crude Protein Precipitation and Antimicrobial Determination

The cell-free supernatant (CFS) of LAB was filtered using 0.20-µm pore size filters obtained from Advantec Toyo Roshi Kaisha, Tokyo, Japan. Crude protein extraction was carried out overnight with continuous stirring at 4°C using 80% (w/v) ammonium sulfate (Merck, Darmstadt, Germany). The obtained precipitate was collected via centrifugation at 14,000 g for 30 minutes at 4°C and then resuspended in 10 mM phosphate buffer. This solution was dialyzed to remove salts and the total protein content was measured using the Takara Bradford protein assay kit protocol (Takara Bio, Shiga, Japan). Crude protein was used for antimicrobial determination and assessed twice for each experiment.

Detection of Plantaricin Genes in *Lpb. plantarum*

Plantaricin gene identification was carried out using PCR and the reaction components included 12.5 µL of 2× Green Master Mix PCR (Promega, Madison, USA), 1.25 µL of 0.05 pmol/µL for each primer, 9 µL of nuclease-free deionized water (Promega, Madison, USA), and 1 µL of template DNA. Table 1 shows the plantaricin-specific primers and annealing temperatures used in this study. PCR products were evaluated by electrophoresis (Mupid-exU, Shiga, Japan) using 1% agarose gel in 1× TAE buffer at 100 V for 30 min. The estimation of DNA fragments size was conducted using FastGene 100 bp DNA Ladder (Nippon Genetics, Düren, Germany).

Table 1. Specific primers and annealing temperatures used in this study

Primer	Sequence (5′ → 3′)	Annealing Temperature (ºC)	References
plnA-F	TAGAAATAATTCCTCCGTACTTC	55	(Azizi et al. 2017)
plnA-R	ATTAGCGATGTAGTGTCATCCA		
plnEF-F	TATGAATTGAAAGGGTCCGT	54	
plnEF-R	GTTCCAAATAACATCATACAAGG		
plnW_F	CACGTCACAGCTAATCTGG	61,5	(Lages et al. 2015)
plnW_R	CTAATTGCTGAATGGTTGGT		
plnS_F	GCCTTACCAGCGTAATGCCC	56	(Stephens et al. 1998)
plnS_R	CTGGTGATGCAATCGTTAGTTT		
plnC8α-F	CGGGGTACCGATGATGATGATAAAG	56	(Jiang et al. 2018)
plnC8α-R	CATGCCATGGCTAAAATTGAACATA		
plnC8β-F	CGGGGTACCGATGATGATGATAAAT	56	
plnC8β-R	CATGCCATGGTTAATGATAAAAGCCTT		
plnNJK_F	CTAATAGCTGTTATTTTTAACC	55	(Cho et al. 2010)
plnNJK_R	TTATAATCCCTTGAACCACC		
pln423_F	GTCGCCCGGGAAATACTATGGTAATGGGG	55	(Van Reenen et al. 2003)
pln423_R	GCGTCCCGGGTTAATTAGCACTTTCCATG		
plnZJ5_F	AGATTCCAGGCAATG	55	(Song et al. 2014)

The Determination of Lactic Acid Content in *Lpb. plantarum*

Lactic acid content in *Lpb. plantarum* was determined using a protocol based on Spectrophotometry (Borshchevskaya et al. 2016) with CFS from *Lpb. plantarum* strain and iron (III) chloride hexahydrate ($FeCl_3.6H_2O$). This content was employed as a standard and determined using spectrophotometry with three replications for each experiment.

Hydrogen Peroxide Measurement

Hydrogen peroxide measurement was conducted by titration, including the dilution of 1 mL CFS with distilled water to a total volume of 25 mL, then 25 mL of sulfuric acid (H_2SO_4) was added. The solution was then titrated with 0.1N potassium permanganate ($KMnO_4$) until decolorization occurred. Each ml of 0.1N $KMnO_4$ was equivalent to 1.701 mg of hydrogen peroxide (H_2O_2).

Data Analysis

The experiments were conducted two to three times to ensure that the data was suitable for statistical analysis. The data was presented as the mean ± standard error, and significant differences were evaluated using one-way analysis of variance (ANOVA). This was followed by Tukey's post hoc test, conducted with GraphPad Prism 10.2.3 (Boston, Massachusetts, USA) on Windows 10. A p-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Isolation and Identification of *Lpb. plantarum* using 16S rRNA

LAB was isolated from *S. arvensis* with a total colony count of $31x10^6$ CFU/mL. During this phase, four LAB were selected with specifications of milky white color, round, and convex shape. Subsequently, DNA isolation was performed, followed by PCR using universal primer 16S rRNA for LAB, which confirmed a band length of approximately ~1500bp (Figure 1). PCR results were clear and without smears as higher DNA resolution implies better quality (Green and Sambrook 2019). PCR products were then sequenced to determine the nucleotide base sequence. In this study, a universal primer targeting 16S rRNA gene was used to identify the bacteria. 16S rRNA is a component of the bacterial ribosome, specifically the small subunit (Bose and Moore 2023).

In molecular biology, 16S rRNA gene is widely used as a marker for bacterial identification and phylogenetic analysis due to the highly conserved nature across bacterial species (Regueira-Iglesias et al. 2023; Varliero et al. 2023). 16S rRNA gene can be compared not only in bacteria but also with archaea (Bose and Moore 2023). Four isolates of LAB were sequenced, and the sequencing results identified two species, namely *Leuconostoc lactis* and *Lpb. plantarum*, which were compared with GenBank data at NCBI. In this research, we observed that the query sequence similarity percentage exceeded ≥97%. The objective was to optimize the search for highly similar sequences. This method was considered the most effective for finding the exact match with the query sequence (Choudhuri 2014).

As shown in Figure 2, eleven closely related species were designated to construct a phylogenetic tree based on genetic similarity using MegaX Construct/Test Neighbor-Joining Tree. Bootstrap certainty intervals are typically evaluated, particularly for complex data structures, given the fundamentally higher degree of precision compared to a simple standard error. A computer program can be used to assess the precision of almost any device that provides factual data. The phylogenetic tree was built using a cut-off esteem of <95%, which was indicative of well-supported resampling (Miranti et al. 2022). The bootstrap results in this study showed a confidence level of ≥99%.

Lpb. plantarum and *L. lactis* are species of LAB commonly found in a variety of environments, including fermented food (Syaputri et al. 2021a), soil (Syaputri et al. 2021b), plant material (Li et al. 2021), human gastrointestinal tract (Nordström et al. 2021), and animal intestines (Dell'Anno et al. 2021; Zhang et al. 2013). In general, LAB plays several significant roles in food production, human health, and environmental sustainability. The versatile capabilities make LAB invaluable in various industrial, agricultural, and biomedical applications.

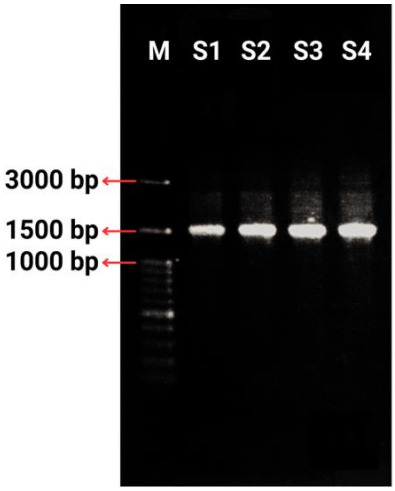

Figure 1. PCR results using 16S rRNA primers specific to LAB. M: Marker; S1: Sample 1; S2: Sample 2; S3: Sample 3; S4: Sample 4

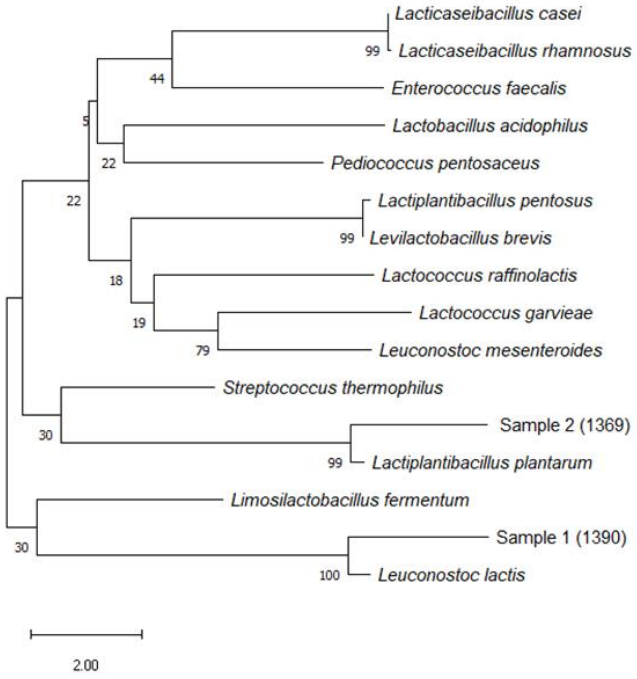

Figure 2. LAB phylogenetic tree was constructed and tested based on 16S rRNA sequence analyses using the MegaX Neighbor-Joining Tree method

Antimicrobial Screening of Lactic Acid Bacteria

Antimicrobial screening was performed to assess the capability of LAB to inhibit pathogenic bacteria, namely *B. subtilis*, *E. coli*, and *S. aureus*. The positive control used in this assessment was sodium ampicillin 100 µg/mL. The results showed that the isolates exhibited significant inhibitory zones against the indicator bacteria, with a p-value of <0.05. *Lpb. plantarum* had the largest inhibition zone against *B. subtillis*, averaging 12.7 cm, followed by *S. aureus* and *E. coli*, respectively. *Lc. lactis* has an inhibition zone against *E. coli*, averaging 3,3 cm, followed by *B. subtillis*, and did not give any antimicrobial activity on *S. aureus* (shown in Figure 3). However, *Lpb. plantarum* has higher inhibition than *Lc. lactis*. Subsequent studies should focus solely on *Lpb. plantarum*.

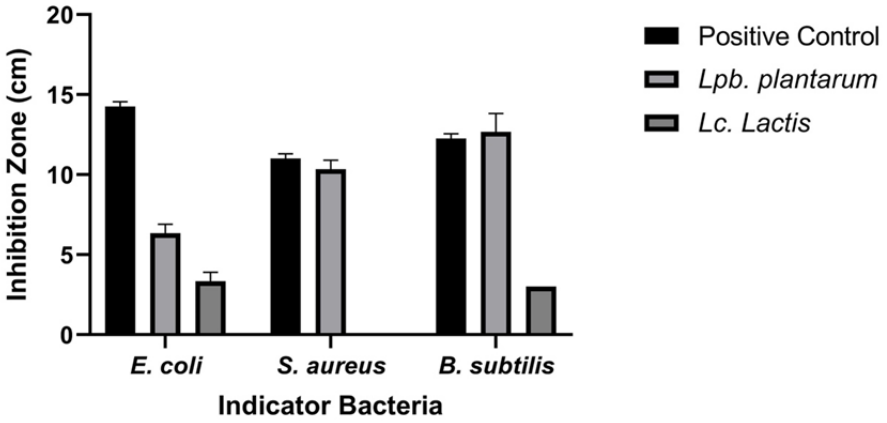

Figure 3. Inhibition Zone of LAB against indicator bacteria. The data were presented as mean±standard error with $p<0.05$ (n=3)

Pathogenic bacteria invade and multiply, as well as cause disease in the host, often producing toxins or triggering immune responses that lead to illness (Li et al. 2020). *E. coli* is a gram-negative bacterium and naturally forms part of the normal flora in the gut of humans and other animals. The majority of *E. coli* was considered harmless to humans. However, certain pathogenic *E. coli* strains can infect the gut area and cause severe illness. According to a previous study, pathogenic *E. coli* infection often causes severe diarrhea (Yang et al. 2017). *B. subtilis* and *S. aureus* are gram-positive bacteria responsible for numerous uncomplicated skins, respiratory tract, surgical site, prosthetic joint, and cardiovascular infections (Cheung et al. 2021; Stülke et al. 2023). Inhibiting pathogenic bacteria can be achieved through several methods, such as using probiotics. In this study, *Lpb. plantarum* and *Lc. lactis* were reported as probiotics.

Lpb. plantarum isolated from Chinese traditional dairy products had antimicrobial activity toward *Salmonella typhimurium*, *E. coli*, and *S. aureus*. This activity may be attributed to the production of organic acid, namely tartaric, lactic, acetic, citric, and malic acid (Hu et al. 2019), as well as plantaricin (Syaputri and Iwahashi 2020) and hydrogen peroxide (Sulistiani 2017). *Lc. lactis* also inhibits *Bacillus cereus*, *Enterococcus faecalis*, *Flavobacterium* sp., *Listeria monocytogenes*, *S. aureus*, *Salmonella enterica*, *Alcaligenes xylosoxidans*, *E. coli* O157:H7 and *Vibrio parahaemolyticus* by producing organic acid and bacteriocin (Moon et al. 2023). These LAB with antimicrobial properties can assist in natural food preservation, prevention of intestinal infections, and are useful as active ingredients in antimicrobial and probiotic product formulations.

Crude Protein Concentration and Antimicrobial Determination

CFS containing bacteriocin synthesized by *Lpb. plantarum* and *Lc. lactis* was collected and precipitated using 80% ammonium sulfate. As shown in Figure 4, the concentrations of the crude precipitated protein were 4663,7 and 3479,2 µg/mL, respectively. These concentrations were used for analyzing antimicrobial activity. Precipitation methods have been used for the past century, with ammonium sulfate continuing to be the most common. Ammonium sulfate is highly stabilizing to protein structure, very soluble, relatively inexpensive, and readily available in pure form (Burgess 2009). At the end of the experiment, the protein was found in a high concentration of salt. According to a previous study, dialysis is typically the best method for removing ammonium sulfate from a sample (Duong-Ly and Gabelli 2014). Consequently, pure crude protein can be obtained without impurities.

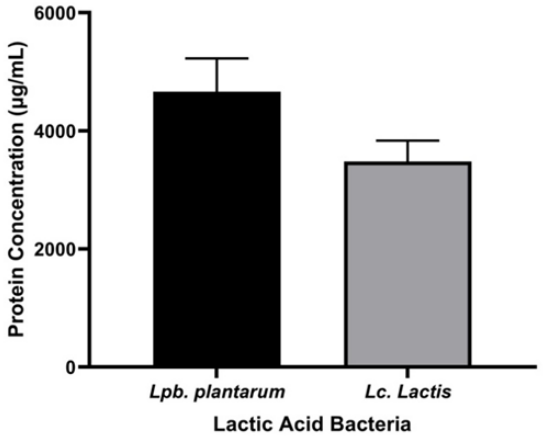

Figure 4. The crude protein concentrations were precipitated using 80% ammonium sulfate. The data includes mean values with standard deviation (n = 2)

The product of the crude protein precipitation was followed by antimicrobial screening using the pure crude protein. The result showed that *Lpb. plantarum* had better inhibition to *B. subtillis*, averaging 4.6 cm, followed by *S. aureus* and *E. coli*, respectively. Crude protein of *Lc. lactis* only have an inhibition zone on *E. coli*, as shown in Figure 5. *Lpb. plantarum* also had better inhibition than *Lc. lactis*. Therefore, subsequent studies should focus solely *on Lpb. plantarum*.

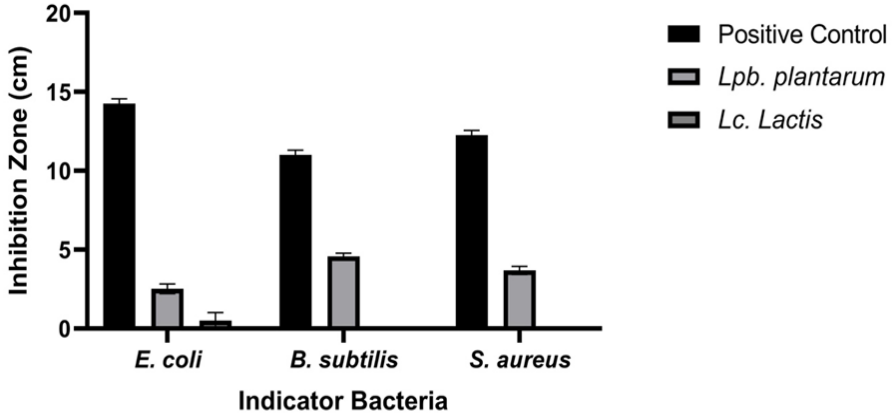

Figure 5. Inhibition Zone of crude protein isolated from *Lpb. plantarum* and *Lc. lactis* against indicator bacteria. The data were presented as mean±standard error with $p<0.05$ (n=2)

Lei et al. (2020) reported that the antibacterial activity of the crude extract *of Lpb. plantarum* strain zrx03 increased with higher ammonium sulfate concentrations. This activity reached maximum diameter when reconstituted with 80% ammonium sulfate and a pH 7 phosphate buffer solution. The inhibition zone diameter of the crude bacteriocin extract precipitated with ammonium sulfate was less than 18 mm (Lei et al. 2020). In this study, both LAB produced a small inhibition zone on the crude protein. This is because bacteriocins are not solely antimicrobial agents and require other components to work synergistically as antimicrobial agents. Therefore, future studies will focus on detecting plantaricin, lactic acid, and hydrogen peroxide in *Lpb. plantarum*.

Detection of Plantaricin Gene in *Lpb. plantarum*

The bacteriocin produced by *Lpb. plantarum*, known as plantaricin, was detected to ascertain the presence of specific genes isolated from *S. arvensis*. The template used for gene detection was the total DNA of *Lpb. plantarum*. The detected plantaricin genes include two-peptide

plantaricins such as *plnEF*, *plnW*, *plnS*, *plnC8α*, *plnC8β*, *plnNJK*, *pln423*, and *plnZJ5*. These genes show high efficacy, lack drug resistance, display 10^3 times greater activity when combined with the corresponding peptides than when used independently. The genes are readily modifiable through bioengineering to augment either the activity or specificity against the target microorganism (Bengtsson et al. 2020; Zhang et al. 2016). In addition, *plnA* served as the precursor protein of plantaricin. Table 2 shows the results of plantaricin gene detection present in *Lpb. plantarum*.

Table 2. Detection of plantaricin gene in *Lpb. Plantarum*

Genes Detection	*plnA*	*plnEF*	*plnW*	*plnS*	*plnC8α*	*plnC8β*	*plnNJK*	*pln423*	*plnZJ5*
Results	+	+	-	-	-	-	-	-	-

The results showed that *plnA* and *plnEF* were encoded in *Lpb. plantarum* isolated from *S. arvensis*. However, *plnW*, *plnS*, *plnC8α*, *plnC8β*, *plnNJK*, *pln423*, and *plnZJ5* were not encoded in *Lpb. plantarum*. Plantaricin, classified as a class II bacteriocin, exhibits antimicrobial effects even under conditions of increased temperature, extreme acidity or alkalinity, and high salinity concentrations (Syaputri et al. 2021a). *PlnA* is a cationic bacteriocin, mainly a peptide pheromone that regulates the production of antimicrobial peptides in *Lpb. plantarum*. These bacteriocins also directly permeabilize specific bacterial strains (Meng et al. 2022). *PlnA* is unstructured in solution but adopts an α-helical structure upon interacting with membrane lipids. Following this non-chiral interaction with the target cell membrane, *PlnA* binds to the receptor, thereby mediating the pheromone effect (Sand et al. 2010). Additionally, *PlnEF* is a class-IIb two-peptide bacteriocin that consists of the 33-residue *PlnE* and the 34-residue *PlnF* peptides. These two components are required in about equimolar amounts in order to obtain maximal antimicrobial activity (Ekblad et al. 2016). The high potency of two-peptide bacteriocins suggests that the components function by binding to a specific membrane protein, known as a bacteriocin receptor. Heeney et al. (2019) found that membrane leakage and cell death are caused by the interaction between the bacteriocin and the receptor protein.

Two-peptide plantaricin formed relatively specific pores by eliminating transmembrane potential, lowering pH, and inhibiting enzymatic processes (Ekblad et al. 2016; Oppegård et al. 2016). A study by (Goel and Halami 2023) showed that plantaricin had a bactericidal effect on other closely related microorganisms. This component is a bactericidal agent with several mechanisms of action, such as inhibiting protein and nucleic acid synthesis or disrupting the integrity of the cell wall (Ahmad et al. 2017), binding to lipid components through surface molecule binding sites, and facilitating pore formation through specific or non-specific receptor binding (Jiang et al. 2018). Therefore, antimicrobial properties make plantaricin a promising tool for enhancing food safety, combating antibiotic-resistant bacteria, and supporting sustainable agricultural practices.

The Synergistic of Lactic Acid and Hydrogen Peroxide

Lpb. plantarum is a facultative heterofermentative LAB, which produces lactic acid and hydrogen peroxide as the metabolic end product from the fermentation of carbohydrates (Zhang et al. 2022). Both compounds play an important role in antimicrobial activity, specifically in food fermentation. *Lpb. plantarum* exhibited strong and broad-spectrum antibacterial activity, capable of inhibiting the growth of indicator bacteria in agriculture and food production. Furthermore, *Lpb. plantarum* isolated from *S. arvensis* produced lactic acid and hydrogen peroxide at a level of 3.0158±0.2774 mg/mL and 0.195±0.04 mg/mL, respectively. *Lpb. plantarum* isolated from fish produced the highest organic acid, namely lactic, acetic, propionic, and succinic acid than *Enterococcus gallinarum*, *Lactobacillus brevis*, *Pediococcus acidilactici* and *Streptococcus* spp. (Kuley et al. 2020). In addition, *Lpb. plantarum* strain Su-ls520, Su-ls530, and Su-ls537 isolated from fermented salted

vegetables also produced 67-72 mg/mL and 0.17-0.25 mg/mL lactic acid and hydrogen peroxide, respectively (Sulistiani 2017)

Lactic acid and hydrogen peroxide had mechanisms in inhibiting pathogenic bacteria by disrupting cell membrane permeability and produce hydroxyl radicals through intracellular iron ions that caused DNA alteration and apoptosis (Zhang et al. 2019). Recent findings indicated that combined lactic acid/hydrogen peroxide demonstrated increased synergistic efficacy in eliminating pathogens. The synergistic effect was studied across various microbial species, showing that low concentrations of lactic acid and hydrogen peroxide could effectively kill both Gram-negative and Gram-positive bacteria.

CONCLUSIONS

In conclusion, *Lpb. plantarum* isolated from *S. arvensis* had the potential to be an effective antimicrobial agent against *B. subtilis*, *E. coli,* and *S. aureus*, *Lpb. plantarum*, recognized as safe by global food authorities. The components produced various bioactive metabolites, such as lactic acid, hydrogen peroxide, and bacteriocins (plantaricin) that effectively inhibit pathogenic bacteria. Lactic acid and hydrogen peroxide altered the permeability of the cell membrane and generated free radicals that damaged DNA, while cell wall integrity was disrupted by plantaricin, and protein or nucleic acid synthesis was also inhibited.

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