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Assessment on the Antimicrobial Activity of *Lactiplantibacillus plantarum* SU-KC1a against *Listeria monocytogenes* ATCC-7644

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Abstract

***Lactobacillus* species are particularly noteworthy due to their ability to synthesize a variety of antimicrobial substances, including organic acids and bacteriocins. *Lactiplantibacillus plantarum* SU-KC1a, a member of the genus of *Lactobacillus*, had been previously isolated from human breast milk by our group. A preliminary *in silico* investigation had identified that *Lpb. plantarum* SU-KC1a possessed genes responsible for plantaricin, which is a type of bacteriocin produced by *Lpb. plantarum* strains. This study therefore aimed to assess the antibacterial activity of *Lpb. plantarum* SU-KC1a against pathogenic *Listeria monocytogenes* ATCC-7644 and to induce the biosynthesis of plantaricin by *Lpb. plantarum* SU-KC1a through co-culture with *L. monocytogenes* ATCC-7644 as the inducer strain. Our results showed that the cell-free supernatants from both co-culture and monoculture of *Lpb. plantarum* SU-KC1a exhibited a modest antibacterial activity against *L. monocytogenes* ATCC-7644. However, it was further determined that the inhibitory effect was not due to the production of plantaricin. Instead, it was primary attributed to the production of organic acids, which decreased the pH and inhibited the growth of *L. monocytogenes* ATCC-7644. In conclusion, our study demonstrated that *Lpb. plantarum* SU-KC1a could exert a modest antibacterial activity towards *L. monocytogenes ATCC-7644*, but not through the induction of plantaricin.**

Keywords: *Lactiplantibacillus plantarum*, lactic acid bacteria, *Listeria monocytogenes*, antimicrobial activity, co-culture

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Introduction

The discovery of penicillin and the further development of antibiotics are arguably one of the biggest scientific accomplishments of the 20th century in terms of impact on human wellbeing (Palmer & Foster, 2022). However, the misuse and overuse of antibiotics coupled with the lack of novel antibiotic discoveries have caused a widespread antimicrobial resistance, which in return creating a major public health crisis. As a result, antibiotic-resistant infections require prolonged and/or costly treatments, extended hospitalization, necessitate additional healthcare visits and consequently higher mortality and morbidity rates (Michael *et al.,* 2014). Thus, a novel invention is urgently required to adequately treat bacterial infections and to control antibiotic resistance.

A promising approach involves extracting antibacterial compounds produced by specific bacterial species. *Lactobacillus* species are of interest as they are known to produce several antimicrobial molecules, including (i) lactic acid and other organic acids; (ii) phenolic compounds; (iii) metabolic by-products, such as hydrogen peroxide; and (iv) synthesized peptides, i.e., bacteriocins (Rocchetti *et al*., 2021). Bacteriocins are a class of peptides with antimicrobial properties naturally synthesized by bacteria. As a part of their inherent defense system, bacteriocins are

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released extracellularly with bactericidal or bacteriostatic effects to closely related (narrow inhibitory spectra) or non-related (broad inhibitory spectra) microbiota (Selegård *et al*., 2019; Palmer & Foster, 2022). A particular member of the *Lactobacillus* species, *Lactiplantibacillus plantarum*, a lactic acid bacterium natively found in the human gut, has been reported to improve the gastrointestinal health especially for people with irritable bowel syndrome. Interestingly, various strains of *Lpb. plantarum* strains were capable of producing bacteriocins, known as plantaricin (Echegaray *et al*., 2023). Therefore, the antimicrobial activities by *Lpb. plantarum*, mediated by various bioactive molecules, will be of great importance to adequately treat bacterial infections.

Listeria monocytogenes, a facultative intracellular pathogen that contaminates foods like dairy, poultry, meat, fruits, and vegetables, is responsible for causing listeriosis, an intermittent but severe disease, with symptoms ranging from meningitis and septicemia to stillbirths and abortion (Jibo *et al.,* 2022). The most recent listeriosis outbreak happened in South Africa in 2018, infecting 1,060 individuals and resulting in 216 fatalities. This is particularly concerning as a recent survey reported the presence of *L. monocytogenes* in 15% of foods sampled (Jibo *et al.,* 2022). Moreover, the pathogen has developed resistance to several antibiotics, including fluoroquinolones, cephalosporins and fosfomycin (Olaimat *et al*., 2018).

Lactiplantibacillus plantarum SU-KC1a, had been isolated from human breast milk sample at the Department of Biology of Universitas Pelita Harapan (Rachmah, 2020; Sugata *et al*., 2024). The whole-genome analysis of the isolate had also shown a presence of the genes encoding plantaricin (Timotius, 2021). However, the antimicrobial activity of *Lpb. plantarum* SU-KC1a has not been fully elucidated (Widjaja *et al*., 2022), including whether it was capable to generate plantaricin. In this study, the antimicrobial activity of *Lpb. plantarum* SU-KC1a towards pathogenic *L. monocytogenes* ATCC-7644 and the induction of plantaricin expression through co-culture method were evaluated.

Methods and Materials

Bacterial strains

Lactiplantibacillus plantarum strain SU-KC1a had been isolated from human breast milk from a consenting donor at Rumah Sakit Anak dan Bunda Harapan Kita, Jakarta (PP.04.03/XXI.2/922/2020) and stored in an 80% glycerol solution at -20°C (Rachmah, 2020; Sugata *et al*., 2024). The strain was re-cultured in deMan Rogosa Sharpe/MRS (Himedia, India) agar and incubated for 24 hours at 37°C under microaerophilic condition. *Listeria monocytogenes* strain ATCC-7644 was purchased from PT. Intralab Ekatama (Bogor). The bacterium was sub-cultured in tryptic soy (TS) agar (Himedia, India) for 24 hours at 37°C under aerobic condition.

Preparation of cell-free supernatant

Co-cultures were prepared based on Liu *et al*. (2022) with modifications. *Lpb. plantarum* SU-KC1a and *L. monocytogenes* ATCC-7644 were co-inoculated into MRS broth for 48 hours at 37°C in microaerophilic condition with an inoculum ratio of 1:1 (at final concentrations of 1×10^7:1×10^7 CFU/mL). Aliquots of the culture were collected every 12 hours. Cell-free supernatant/CFS was obtained through centrifugation at 5,000 × g at 4°C for 15 minutes. In addition, monoculture of *Lpb. plantarum* SU-KC1a and *L. monocytogenes* ATCC-7644 were grown separately in MRS broth for 48 hours at 37°C in microaerophilic condition with final concentration of 1×10^7 CFU/mL. The CFS from each monoculture was obtained as described above. The pH of each culture was also measured using a pH indicator strip (Merck, Germany).

Antibacterial activity of cell-free supernatant

The antibacterial activity of CFS samples against the growth of *L. monocytogenes* ATCC-7644 were assessed using the well-diffusion method (Afrin *et al*., 2021). One mL of *L. monocytogenes* ATCC-7644 were sub-cultured into 10 mL of TS agar by using pour-plate method. Subsequently, wells with diameter of 5 mm were created on the agar plate and filled with 100 µL aliquots of (i) co-culture between *Lpb. plantarum* SU-KC1a and *L. monocytogenes* ATCC-7644 (1×10^7:1×10^7 CFU/mL); (ii) monoculture *Lpb.*

plantarum SU-KC1a (1×10^7 CFU/mL); (iii) monoculture *L. monocytogenes* ATCC-7644 (1×10^7 CFU/mL); (iv) sterile MRS as negative control; and (v) amoxicillin (Capri, Indonesia) in sterile MRS with concentration of 2.5 mg/mL as positive control. Upon incubation of varying periods (0, 12, 24, 36 and 48 hours) at 37 °C under aerobic condition, the inhibition zones were measured. The results were subsequently subtracted from 5 mm (i.e., the diameter of well). Tests were performed in three replicates.

Attenuation of the antibacterial activity through an adjustment of pH or temperature

The antibacterial activity of the collected CFS from all tested conditions were subsequently assessed upon adjustment of pH or temperature. The pH of each sample was adjusted to 7 and 9 by using 1 Molar of Tris solution. Any difference in volume upon pH adjustment was normalized by adding sterile water into the solution, hence the volumes across samples would be consistent. With regard to the adjustment of temperature, sample from each tested condition was aliquoted into three parts, in which the samples would be heated at 50°C, 75°C or 100°C for 30 minutes. One-hundred µL of treated samples were transferred into wells in TS agar plate that had been inoculated with *L. monocytogenes* ATCC-7644. A negative control of sterile MRS and a positive control of 2.5 mg/mL amoxicillin in MRS were included into the agar plate as well. Inhibition zones were observed upon incubation at 37°C for 24 hours under aerobic condition. Inhibition zones were measured and the results were subtracted from 5 mm (i.e., the diameter of well). Tests were performed in three replicates.

Evaluation of a co-culture method to induce plantaricin

The co-culture method between *Lpb. plantarum* SU-KC1a and *L. monocytogenes* ATCC-7644 (for 48 hours at 37°C in microaerophilic condition with an inoculum ratio of 1×10^7:1×10^7 CFU/mL) was evaluated to detect any production of plantaricin. Monoculture of *Lpb. plantarum* SU-KC1a (1×10^7 CFU/mL) was used as a control. The cell-free supernatant was harvested from both conditions. Secreted proteins by *Lpb. plantarum* SU-KC1a were precipitated through acetone based on Liu *et al*. (2022) with modifications. Three mL of each sample were precipitated with 12 mL of cold acetone (Mallinckrodt Chemicals, U.S.A) and incubated overnight at -30°C. The resulting precipitates were collected via centrifugation at 10,000 × g for 15 minutes and subsequently solubilized in 200 µL of phosphate-buffered saline/PBS. Next, quantification of proteins was based on Katharopoulos *et al*. (2016) with modification. A standard curve showing the correlation between OD_{595} and protein concentration was prepared. This was achieved by diluting a protein standard (i.e., bovine serum albumin) with PBS to concentrations of 1.0, 0.8, 0.6 and 0.2 mg/mL. Five µL of extracted protein from each condition were transferred into a 96-well plate and were inoculated with 250 µL of Bradford reagent (Himedia, India). Upon incubation for 10 minutes at room temperature, the OD_{595} of each sample was measured. The protein concentration of each condition was calculated based of the standard curve. Finally, the antibacterial activity of extracted protein was assessed using the well diffusion method based on Afrin *et al*. (2021) with modifications. One-hundred µL of each condition were transferred into the wells of tryptic soy agar plate that had been inoculated with *L. monocytogenes* ATCC-7644. An amoxicillin in MRS with concentration of 2.5 mg/mL was used as the positive control, while sterile MRS broth was used as the negative control. Upon incubation of 24 hours at 37 °C under aerobic condition, the inhibition zones were measured. The results were subsequently subtracted from 5 mm (i.e., the diameter of well). Tests were performed in three replicates.

Results and Discussion

Antibacterial activity of *Lactiplantibacillus plantarum* strain SU-KC1a

The antibacterial activity of *Lpb. plantarum* SU-KC1a was analyzed in this study by using the well-diffusion assay. Specifically, cell-free supernatant/CFS harvested from the co-culture between *Lpb. plantarum* SU-KC1a and *L. monocytogenes* ATCC-7644 was compared with CFS collected from monoculture of *Lpb. plantarum* SU-KC1a or *L. monocytogenes* ATCC-7644 in inhibiting the growth of *L. monocytogenes* ATCC-7644. The inhibition zones were measured upon incubation at 0, 12, 24, 36 and 48 hours (Table 1).

The CFS extracted from various conditions containing *Lpb. plantarum* SU-KC1a upon incubation of 24 hours or beyond would exhibit a modest antibacterial activity. There was no substantial difference between the antibacterial activity of co-culture and monoculture of *Lpb. plantarum* SU-KC1a, suggesting that the observed antibacterial activity is not dependent on the addition of *L. monocytogenes* ATCC-7644 in the co-culture.

As shown in Table 2, the culture conditions of *Lpb. plantarum* SU-KC1a experienced a pH reduction from 5.5 at the starting period to 4.0 after 24 hours of incubation. The pH remained at 4.0 until 48 hours of incubation. Interestingly, this coincided with the observed antimicrobial activity from 24 hours onward (Table 1). This suggested that the antibacterial activity could be attributed to the reduction of pH, as *L. monocytogenes* ATCC-7644 was reported to be sensitive to pH levels of 4 or lower as well as above 9.6 (Maggio *et al*., 2022; Suphandi *et al*., 2023).

Table 1. The antibacterial activity against pathogenic *L. monocytogenes* ATCC-7644 using well-diffusion assay

Condition	Concentration	Inhibition Zone (cm)				
		0 hour	12 hours	24 hours	36 hours	48 hours
Co-culture of *Lpb. plantarum* SU-KC1a and *L. monocytogenes* ATCC-7644	10^7:10^7 CFU/mL	0	0	0.40±0.10	0.43±0.06	0.43±0.06
Monoculture of *Lpb. plantarum* SU-KC1a	10^7 CFU/mL	0	0	0.40±0.10	0.40±0.17	0.47±0.12
Monoculture of *L. monocytogenes* ATCC-7644 [b]	10^7 CFU/mL	0	0	0	0	0
Amoxicillin [c]	2.5 mg/mL	1.70±0.27	1.70±0.17	1.73±0.12	1.87±0.12	1.93±0.15
MRS [b]	-	0	0	0	0	0

Data are shown as mean of triplicates ± standard deviation. Amoxicillin resuspended in deMan Rogosa Sharpe/MRS was used as the positive control, while MRS was used as the negative control.

Table 2. The pH of cell-free supernatant from various cultures

Condition	Concentration	pH				
		0 hour	12 hours	24 hours	36 hours	48 hours
Co-culture of *Lpb. plantarum* SU-KC1a and *L. monocytogenes* ATCC-7644	10^7:10^7 CFU/mL	5.5	5.0	4.0	4.0	4.0
Monoculture of *Lpb. plantarum* SU-KC1a	10^7 CFU/mL	5.5	5.0	4.0	4.0	4.0
Monoculture of *L. monocytogenes* ATCC-7644	10^7 CFU/mL	5.5	5.5	5.5	5.5	5.5
Amoxicillin	2.5 mg/mL	5.5	5.5	5.5	5.5	5.5
MRS	-	5.5	5.5	5.5	5.5	5.5

Data are shown as mean of triplicates. Amoxicillin resuspended in deMan Rogosa Sharpe/MRS was used as the positive control, while MRS was used as the negative control.

Table 3. The antibacterial activity upon pH adjustment against pathogenic *L. monocytogenes* ATCC-7644

Condition	Concentration	Inhibition Zone (cm)	
		pH of 7	pH of 9
Co-culture of *Lpb. plantarum* SU-KC1a and *L. monocytogenes* ATCC-7644	10^7:10^7 CFU/mL	0	0
Monoculture of *Lpb. plantarum* SU-KC1a	10^7 CFU/mL	0	0
Monoculture of *L. monocytogenes* ATCC-7644	10^7CFU/mL	0	0
Amoxicillin	2.5 mg/mL	1.83±0.15	1.80±0.00
MRS Broth	-	0	0

Data are shown as mean of triplicates ± standard deviation. Amoxicillin resuspended in deMan Rogosa Sharpe/MRS was used as the positive control, while MRS was used as the negative control.

Table 4. The antibacterial activity upon temperature adjustment against pathogenic *L. monocytogenes* ATCC-7644

Isolate	Concentration	Inhibition Zone (cm)		
		50°C	75°C	100°C
Co-culture of *Lpb. plantarum* SU-KC1a and *L. monocytogenes* ATCC-7644	10^7:10^7 CFU/mL	0.37±0.06	0.4±0.00	0.47±0.6
Monoculture of *Lpb. plantarum* SU-KC1a	10^7 CFU/mL	0.4±0.00	0.47±0.06	0.47±0.6
Monoculture of *L. monocytogenes* ATCC-7644	10^7CFU/mL	0	0	0
Amoxicillin	2.5 mg/mL	1.77±0.15	1.9±10.17	1.83±0.06
MRS	-	0	0	0

Data are shown as mean of triplicates ± standard deviation. Amoxicillin resuspended in deMan Rogosa Sharpe/MRS was used as the positive control, while MRS was used as the negative control.

This observation was further tested by adjusting the pH of collected CFS to 7 and 9 (Table 3). As expected, no antibacterial activity was observed at pH 7 and 9. The study by Arrioja-Bretón *et al*. (2020) reported that the antimicrobial activity of *Lpb. plantarum* weakened once the pH value is near neutral or base. This was also in line with a study by Peh *et al*. (2020), which stated that the inhibitory activity of lactic acid, the most common form of organic acid produced by *Lpb. plantarum*, was most effective at pH of less than 4.2.

Of note, the collected CFS samples from various conditions were heated to analyze whether the antibacterial activity against *L. monocytogenes* ATCC-7644 would be attenuated. The inhibitory activity of antibacterial compounds, such as lactic acid, should not be affected by temperature, but the inhibitory activity of antimicrobial peptides, such as plantaricin, might be susceptible to high temperatures (Oesterreicher *et al*., 2019). Table 4 shows that despite the heating, the antibacterial activity of CFS did not change. Taken together, this suggested that the observed modest antimicrobial activity of *Lpb. plantarum* SU-KC1a in this study was contributed by molecule(s) that is pH-sensitive but temperature-resistant (Widjaja *et al*., 2022).

Evaluation of plantaricin production by Lactiplantibacillus plantarum strain SU-KC1a

Antibacterial activity of *Lpb. plantarum* has been associated with

biosynthesis of plantaricin or other proteinaceous compounds (Rocchetti *et al*., 2021; Wiman *et al*., 2023). Therefore, a co-culture method with an inducer/pathogenic bacterium was designed to induce production of plantaricin by *Lpb. plantarum* SU-KC1a. The inducer strain in this co-culture method was *L. monocytogenes* ATCC-7644. First, the proteins were precipitated from the CFS harvested from the co-culture between by *Lpb. plantarum* SU-KC1a and *L. monocytogenes* ATCC-7644 at a ratio of 1:1. As a control, the CFS was harvested from monoculture of *Lpb. plantarum* SU-KC1a. The quantification of precipitated proteins is shown in Table 5. There was a gradual increase in protein concentration every 12 hours for both co-culture and monoculture, in which the highest concentration was 0.89 mg/mL after 48 hours of incubation. Intriguingly, the presence of *L. monocytogenes* ATCC-7644 as an inducing strain did not increase the protein production by *Lpb. plantarum* SU-KC1a.

The antibacterial activity of the precipitated proteins was subsequently tested via well-diffusion assay in an agar plate previously inoculated with *L. monocytogenes* ATCC-7644. As shown in Table 6, there was no inhibition zone from both co-culture and monoculture of *Lpb. plantarum* SU-KC1a. This finding suggested that despite the presence of genes encoding plantaricin within the genome of *Lpb. plantarum* SU-KC1a (Timotius, 2021), our experimental method was not able yet to activate the production of plantaricin.

Table 5. The protein concentration from various cultures.

Condition	Concentration	Protein Concentration (mg/mL)				
		0 hour	12 hours	24 hours	36 hours	48 hours
Co-culture of *Lpb. plantarum* SU-KC1a and *L. monocytogenes* ATCC-7644	10^7:10^7 CFU/mL	0.08±0.04	0.55±0.17	0.53±0.01	0.67±0.06	0.89±0.10
Monoculture of *Lpb. plantarum* SU-KC1a	10^7 CFU/mL	0.01±0.02	0.54±0.08	0.79±0.13	0.80±0.00	0.89±0.02
PBS	-	0	0	0	0	0

Data are shown as mean ± standard deviation (n=3). Phosphate-buffered saline/PBS was used as the negative control.

Table 6. The antibacterial activity of precipitated protein samples against *L. monocytogenes* ATCC-7644.

Condition	Concentration	Inhibition Zone (cm)				
		0 hour	12 hours	24 hours	36 hours	48 hours
Co-culture of *Lpb. plantarum* SU-KC1a and *L. monocytogenes* ATCC-7644	10^7:10^7 CFU/mL	0	0	0	0	0
Monoculture of *Lpb. plantarum* SU-KC1a	10^7 CFU/mL	0	0	0	0	0
Amoxicillin	2.5 mg/mL	1.70±0.10	1.73±0.17	1.73±0.12	1.83±0.06	1.83±0.06
MRS	-	0	0	0	0	0

Data shown as mean of triplicates ± standard deviation. Amoxicillin resuspended in deMan Rogosa Sharpe/MRS was used as the positive control, while MRS was used as the negative control.

Various studies had shown that microbial co-culture was indeed a favorable method for bacteriocin production and had been extensively used in *Lpb. plantarum* strains (Lei

et al., 2020). Co-culturing *Lpb. plantarum* with a specific bacterial strain(s) as its environmental stimuli indeed offers a potential mechanism to exploit the protein-synthesis pathways of *Lpb. plantarum* strains (Liu *et al.,* 2021).

Plantaricin biosynthesis is regulated by multiple quorum-sensing (QS) regulatory system. QS is an intercellular communication system within and between species that regulates gene expression in a concerted manner, in response to changes in the surrounding environment. The QS system of *Lpb. plantarum* is mediated by two classes of signaling molecules, i.e., autoinducer-2/AI-2 and autoinducing peptide/AIP of PlnA. In general, the plantaricin operons usually consist of three main components: (i) the induction factors (AI-2 and AIP) (Gutiérrez-Cortés *et al*., 2018); (ii) genes encoding histidine protein kinase (HPK); and (iii) two response regulator (RR) genes. When concentration of AI-2 or AIP reaches a detectable level by extracellular HPK receptors, it will bind and activate the signaling cascade that triggers autophosphorylation of a histidine residue within the cytoplasmic domain of HPK. Upon the phosphorylation, the HPK will act as a kinase and phosphorylate RR, which in return activates their roles as transcriptional factors that bind to gene encoding for bacteriocin (Meng *et al*., 2021). In addition, the AIP of plnA is a density-dependent signaling molecule produced when the cells are growing. When plnA reaches a detectable level by extracellular HPK (presumably during the stationary phase of bacterial growth), the plantaricin's production can be initiated (Rizzello *et al.*, 2014).

In a study conducted by Man & Xiang (2021), only five strains out of 300 tested bacterial strains that were able to induce the production of plantaricin when co-cultured with *Lpb. plantarum* NMD-17. All five strains were lactic acid bacteria and belong to the genera of *Lactobacillus*, *Lactococcus*, or *Enterococcus*. This study showed that inducing strains were able to act as environmental stimulus (presumably through AI-2) for *Lpb. plantarum* NMD-17 to produce plantaricin. The molecule AI-2 is synthesized through the activity of *luxS* AI enzyme, which is encoded by the *luxS* gene. The widespread presence of *luxS* gene among bacterial species might suggest that AI-2 is a "universal language" of interspecies communication (Raut *et* al., 2013). However, the exact role of AI-2 in plantaricin biosynthesis in *Lpb*. *plantarum* and the reason of only specific strains of bacteria that can induce plantaricin production are elusive yet. Thus, there was a possibility that the inability to induce plantaricin in our co-culture method could be due to a fact that *L*. *monocytogenes* ATCC-7644 was not a suitable inducer.

Conclusion

Our study demonstrated that *Lpb. plantarum* SU-KC1a exhibited a modest ability to inhibit the growth of *L. monocytogenes* ATCC-7644. This inhibitory activity was presumably contributed to the production of organic acids, which reduced the pH of cell culture's medium. The low pH indeed inhibited the growth of of *L. monocytogenes* ATCC-7644. Detectable expression of plantaricin via a co-culture of *Lpb. plantarum* SU-KC1a and *L. monocytogenes* ATCC-7644, however, was not successfully achieved. This could be attributed to the incorrect usage of *L. monocytogenes* ATCC-7644 as an inducing strain. Subsequent studies will attempt to use bacterial strains that are closely related or both belonging to the lactic acid bacterial group. Co-culture of *Lpb. plantarum* SU-KC1a with related species, hopefully, will increase the likelihood of achieving cross-talk QS interspecies, which results in successful production of plantaricin.

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