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Antimicrobial activity of plantaricin IIA-1A5 on whey growth medium againts Pseudomonas aeroginosa: a scanning electron microscopy (SEM) study

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ABSTRACT

The study aimed to understand antimicrobial properties of plantaricin derived from *L. plantarum* IIA-1A5 and its inhibitory mechanism against *Pseudomonas aeruginosa* ATCC 27853 under SEM (scanning electron microscopy) observation. The inhibition of microbial growth was evaluated according to diameter of inhibitory zone. Scanning electron microscopy was then applied to observe the microstructure. The diameter of antimicrobial activity against *Pseudomonas aeruginosa* ATCC 27853 reached 6.52 mm in whey medium and 8.45 mm in whey+ medium (added with sucrose, tryptone and yeast extract). The experimental result clearly demonstrated the inhibition *Pseudomonas aeruginosa* ATCC 27853. Observation by SEM suggested that the antimicrobial activity of *Pseudomonas aeruginosa* ATCC 27853 related destruction of cellular morphology. The cell membrane was destroyed as indicated by the release of nucleic acid and reduction of membrane potential. In short, our experiment provided meaningful evidence on the future use of plantaricin IIA-1A5 as food preservative through disruption of cellular membrane causing leading to cell dying.

Keywords: Plantaricin, Lactobacillus plantarun, Pseudomonas aeruginosa, antimicrobial activity

INTRODUCTION

Lactic acid bacteria (LAB) has been massively distributed in animal products (e.g. milk and fresh meat), vegetables, and other processed foods. LAB strains have been applied as probiotics since they exert many beneficial effects, such as immune system activation,anticancer activity, maintenance of mucosal integrity, and antagonistic role again pathogens (Grosu-Tudor et al., 2014). The application of LAB for culture starter in manufacturing fermented meat, processed milk and vegetables is regarded as one of the oldest food processing method, which aims to stabilize food storability as well as produce a specific flavor (Germani et al., 2014). Bacteriocin is a protein capable of serving as antimicrobial agent, and not toxic compound. The need for new strain of LAB with probiotic features increases greatly, including *Lactobacillus plantarum* IIA-1A5. The bacteria are obtained from local beef (I. Isnafia Arief et al., 2015). *Lactobacillus plantarum* IIA-1A5 is cultured using a special commercial medium, i.e. de mann ragosa sharp broth, enabling to induce growth of LAB. However, it is less preferable due to high cost and limited supply. Therefore, finding an alternative to such conventional medium with affordable cost and abundance material such as whey is required. *Lactic acid bacteria* (LAB) are characterized as gram-positive cocci or rods, aerotolerant, and able to ferment carbohydrates for energy with the production of lactic acid (Singh et al., 2021)

Pseudomonas aeruginosa is reported to cause various infections, primafily when attacks in immunocompromised hosts.In addition, the bacteria exhibit numerous virulance factors and s high resistance to antibacterial agents.Beside natural resistance to many drugs, they enable to produce biofilms, complex adherent structured microbial communities (Shokri et al., 2018).

Additionally, pseudomonas closely related to food and milk-spoilage microorganism. Hence, probiotics are considare as appreciable attempt to eradicate antibiotic-resistant and food spoilage microorganisms,being an safer option towards the use of antibiotics and certain chemical preservatives (Chatterjee et al., 2016).

Pseudomonas refers to pathogenic bacteria commonly found in foods, while *P. aeruginosa* often contaminates meat and water (Vitale & Schillaci, 2016). *P. aeruginosa* produces a exotoxin and enterotoxin easily transmitting into foodstuffs which cause diseases in human (Hugo & Hugo, 2015). To date, natural and chemical preservatives are often applied to extend food shelf. Nevertheless, some researches have revealed that the use of chemical preservative may cause deleterious side effects on human health at various extent. In addition, it possibly alters sensory and taste of the foods. Thus, researches discussing the use of natural preservatives have escalated remarkably. Current preservative often originated from animal, plant and bacteria. Among these sources, LAB are famous for their capability in producing antimicrobial compounds, exopolysaccharides; and bacteriocin from such bacteria is popular as active compound to inhibit pathogenic bacteria (Le et al., 2019).

Plantaricin obtained from *L. plantarum* IIA-1A5 showed a noticeable inhibition towards *Staphylococcus aureus*, while it also exerted bactericidal effects on *S. aureus*(Irma Isnafia Arief et al., 2015). The antibacterial effects of plantaricin from *L. plantarum* IA-1A5 have been reported by many researchers; however, they focused mostly on E. coli and Staphylococcus aureus. Therefore, this present work aimed to determine the capability of the plantaricin to inhibit P. Aeruginosa, evaluated using scanning electron microscope (SEM) observation. The further investigation on antimicrobial mechanism by the plantaricin against P. Aeruginosa is essential as basic evidence for the application of the plantaricin-producing bacteria in food industries.

MATERIAL AND METHOD

Production of Bacteriocin and Purification of Ammonium Sulfate

The culture for bacteriocin cultivation was made in cheese whey (1000 mL). For harvesting, the bacteriocin culture was centrifuged (Himac CR21G) at speed of 30623× g at 4 °C for 20 min. Filtration of cell-free supernatant used 0.20 µm filter. NaOH 1 N was added to adjust pH of 6.8. Heidolph VV micro evaporator was applied to evaporate supernatant for 48 h until reaching 50% of the initial volume. Afterwards, addition of ammonium sulfate was carried out to reach concentration of 40%. Sediment (bottom layer) was collected and centrifuged at 30623 × g for 20 min at 4°C, resulting in crude bacteriocin. Furthermore, the crude bacteriocin was analyzed by using dialysis membrane (diameter of 20 µm) and submerged in phosphate buffer, i.e. K2HPO4 and KH2PO4 at concentration of 20 mM at pH 6.8 for 24 h. The buffer was replaced 6 times each 4 h. All experimental stages were conducted at 4 °C. Further step was chromatography purification using sepharose HiTrap FF (GE Healthcare). Fractions containing the eluted protein were checked by spectrophotometer UV-VIS at wavelength of 280 nm. Last, antimicrobial activity of the plantaricin was tested in a following stage (Arief et al., 2015)

Antimicrobial Activity

E. coli and S. aureus were applied as the testing bacteria, diluted to standard 0.5 McFarland (10^8) cfu mL^{-1}). The bacteria were cultivated on MHA medium. Sterilized paper disc was submerged to the 50 µL of the plantaricin, then inserted into medium. The incubation was performed for 48 h at 37°C. Clear zone was formed and measured using a calliper (Hosni et al., 2013)

SEM Observation

Observation by using SEM was performed to investigate the destruction of cell morphology. The bacterial suspension test was carried out after exposure with the antimicrobial agent for 4 h. Protocol for bacterial extraction followed method of (Goldstein et al., 2017). To remove supernatant, the suspension was centrifuged at 3500 rpm for 20 min, while the pellet containing cells was collected. It was then washed with buffer twice and submerged in 2% glutaraldehyde for 24 h, added with chocodylate buffer and soaked for 20 min. After centrifuged, the supernatant was removed and the pellet was mixed with 1% osmium tetraoxide, followed with soaking for 1 h. Subsequently, it was dried in a series of alcohol at concentration of 70%, 80%, 95% ad absolute alcohol for 20 min, respectively. The pellet was then mixed with butanol and dropped over a cover slip coated with gold under vacuum condition (Goldstein et al., 2017).

Statistical Analysis

Factorial randomized block design was arranged. Data were analyzed using analysis of variance. Tukey test was applied to compare significant difference between means by Statistical Package for Social Science (SPSS, 2020).

RESULT AND DISCUSSION

Antimicrobial Activity

Bacteriocin plantaricin IIA-1A5 demonstrated a strong antimicrobial activity, indicating that lactose in whey was properly fermented. The inhibitory effect against *Pseudomonas* varied, i.e. 6.52 mm using whey medium and 8.45 mm using whey+ containing sucrose, tryptone and yeast extract (Table 1). Difference in clear zones is influenced by some factors. A previous study suggested that plantaricin IIA-1A5 showed powerful antibacterial activities in inhibition of pathogenic bacteria *S. aureus* ATCC 25923 and *E. coli* ATCC 25922, indicated by clear zone diameter of about 10 mm (Kia et al., 2016). In this experiment, inhibition against *Pseudomonas aeruginosa* ATCC 27853 as Gram-negative bacteria is presented in Table 1. The result is in accordance with previous report by (Kyriakou et al., 2016). Plantaricin SLG1 demonstrated a considerable inhibition towards Gram-negative bacteria and fungi, producing the strongest effect compared with other bacteriocins. Similarly, (Lv et al., 2018) found that *Pseudomonas spp*. and Shewanella spp could be most inhibited by bacteriocin DY4-2. Nevertheless, (Júnior et al., 2015) found no relationship between LAB antagonistic activity and Gram status of pathogens tested.

Table 1. Antagonistic activity of plantaricin IIA-1A5 against *Pseudomonas aeruginosa* ATCC 27853

Media	Clear zone (mm)	Sig
Whey	6.52±0.32	*a
Whey+	8.45±0.51	*b
MRSB	9.35±0.44	*c

Note: Different superscripts following the means in similar column indicated significance at $p<0.05$; whey+ (sucrose, tryptone and yeast extract)

Clear zone diameter varied greatly among growth media, depending on their composition. As presented in Table 1, MRSB medium showed the greatest diameter zone, significantly differed in comparison with the others. In former experiment, *L. plantarum* IIA1A5 cultured in a MRSB commercial medium for plantaricin production demonstrated a satisfied antimicrobial activity towards pathogenic bacteria including *E. coli* TCC 25922, *S. thypimurium* ATCC 14028, *S Aureus* ATCC 25923, with diameter of clear zone ranging from 6.86 to 12.38 mm (Irma Isnafia Arief et al., 2015). similar finding was also reported in chaeacterization of LAB strains only by enriching and isolating strains from raw milk samples, using MRS medium(Yazdi et al., 2017). Additionally, the inhibition against *P. aeruginosa* by bacteriocin GM3 was discussed (Avaiyarasi et al., 2016). The clear zone was reduced in whey+ and whey, suggesting that antimicrobial activity of plantaricin against bacteria diminished as nutritional composition of the growth medium changed. (Faridah et al., 2017) also reported similar finding, in which *L. fermentum* strain A323L, B323K, C113L isolated from dangke showed lower bacterial antagonistic activity in whey medium. Additionally, (Soenarno et al., 2019) reported that *Lactobacillus plantarum* IIA-1A5 was revealed able to synthesize a bacteriocin showing a noticeable antimicrobial activity against pathogenic bacteria such as *Staphylococcus aureus* and *Escherichia coli*, at 20 h of fermentation in whey and skim media, he production of bacteriocin from *L. Plantarum* IIA-1A5 in the whey medium have potential as naturalpreservatives in the food industry (Mutmainna et al., 2021).

L. plantarum strains showed antagonistic activities against *Staphylococcus aureus*, *Listeria monocytogenes, Bacillus cereus*, and Micrococcus luteus strains. PCR assay, using specific primers, showed the presence of bacteriocin (plantaricin) encoding genes in all *L. plantarum* strains tested. On the other hand, one strain showed weak nitrate reductase activity, and four strains produced acetoin from glucose. In addition, all strains were lactic acid producers. (Kamiloğlu et al., 2020). This finding supports a former report (Armas et al., 2017), reporting antimicrobial activities of LAB strain against an array of pathogens using the agar spot assay. Antimicrobial compounds including bacteriocins, organic acids (e.g., acetic, lactic, propionic, succinic acids), short-chain fatty acids, hydrogen peroxide, and other low-molecular-weight substances produced by LAB aaccounted for their antimicrobial activity (Grosu-Tudor et al., 2014)

SEM Observation

SEM observation enables to evaluate morphology of *L. plantarum* IIA1A5 and *Pseudomonas aeruginosa* ATCC 27853. Principally, SEM releases high-energy electrons and exposes them to surface of particular object.he SEM micrographs of *Lactobacillus Plantarum* IIA-1A5 and *Pseudomonas aeruginosa* sensitive ATCC 27853 strain displayed a particular on cellular wall, including, deep craters, burst cells, and lysis. Most of the walls showed broken structurws which led to distorted shapes. Outer membrane thickening indicated severe damage *in Pseudomonas aeruginosa* sensitive. strain of control *Lactobacillus Plantarum* IIA-1A5 because there were no signs of cell wall destruction as well as sensitive IIA-1A5 strains (Fig. 1).

The normal appearing cocci of control Lactobacillus Plantarum IIA-1A5 could be found in the control in comparison to the treated *Lactobacillus plantarum* IIA-1A5 and *Pseudomonas aeruginosa* ATCC 27853. As the results, the object sends back them, producing secondary electrons spread in various directions (Mikrajuddin & Khairurrijal, 2009). SEM experiment showed morphological change in cells of *Pseudomonas aeruginosa* ATCC 27853. The release of nucleic acid and reduction of membrane potential strongly indicated. Anti-biofilm activity of LBA isolated from kefir membrane damage. Lactobacilli strains have been extensively studied in this regard, as well, because of their remarkable ability to inhibit the growth of pathogenic bacteria by producing bactericidal compound (Raras et al., 2019).

As depicted in Figure 1, the cellular morphology of *L. plantarum* IIA1A5 and Pseudomonas aeruginosa ATCC 27853s was damaged. This is augmented by Volk and Wheeler (1993), finding that Gram-negative bacteria (such as Pseudomonas) underwent partial removal of their cell walls, in which the outer membrane consisting of protein-lipopolysaccharide was partially removed, while peptidoglycan layer was completely removed with spheroplast. Meanwhile, cell walls of Gram-positive bacteria were entirely removed, and their membrane was unaltered, which is called as protoplast. Similarly, Arief et al. (2015) found that plantaricin IIA-IA5 enabled to deactivate targeted cells through destabilizing cellular membrane, inducing ion release and other essential molecules such as protein and genetic materials. Possibly, plantaricin IIA-1A5 induced lethality to the targeted cells. Using results of scanning electron microscopy observation (Pei et al., 2018) also stated that inhibition mechanism by plantaricin SLG1 was induction of cell damages by destabilizing membrane integrity.

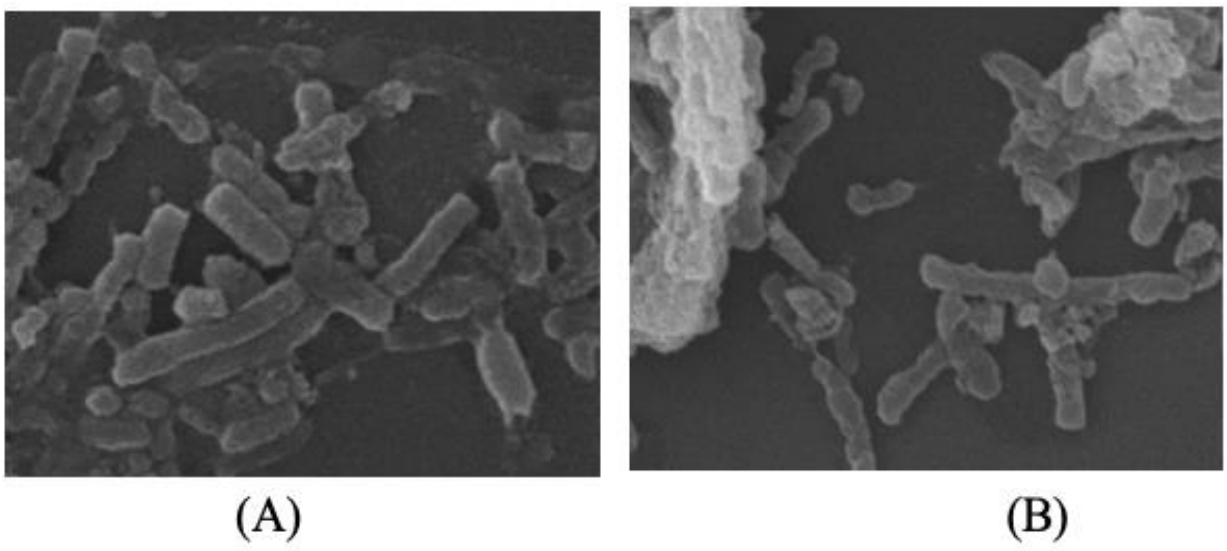

Figure 1. Cellular morphology of cells captured by SEM. (A) control *Lactobacillus Plantarum* IIA-1A5 and (B) *Lactobacillus Plantarum* IIA-1A5 and *Pseudomonas aeruginosa* ATCC 27853.

CONCLUSION

Plantaricin IIA-1A5 displayed antimicrobial activity by inducing detrimental effects on cellular morphology of Pseudomonas aeruginosa ATCC 27853. Regarding its bacteria antagonistic properties, this experiment provided basic scientific evidence for further studies on plantaricin IIA-1A5 as antibacterial agent applicable in food industries.

CONFLICT OF INTEREST

There is no financial, personal, and organizational conflict of interest related to the material discussed in this article.

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