

## Short Communication: Acute toxicity study of plantaricin from *Lactobacillus plantarum* S34 and its antibacterial activity

ARIDO YUGOVELMAN AHADDIN<sup>1,♥</sup>, SRI BUDIARTI<sup>2</sup>, A. ZAENAL MUSTOPA<sup>3,♥♥</sup>, HUDA S. DARUSMAN<sup>4</sup>,  
LITA TRIRATNA<sup>3</sup>

<sup>1</sup>School of Biotechnology, Institut Pertanian Bogor. Jl. Raya Dramaga, Bogor 16680, West Java Java, Indonesia. Tel./fax.: +62-251-8622642,

♥email: a.yugovelman@gmail.com

<sup>2</sup>Research Center for Bioresource and Biotechnology, Institut Pertanian Bogor. Jl. Raya Dramaga, Bogor 16680, West Java Java, Indonesia

<sup>3</sup>Research Center for Biotechnology, Indonesian Institute of Sciences. Jl. Raya Jakarta-Bogor Km. 46, Cibinong, Bogor 16911, West Java, Indonesia,  
Tel./fax.: +62-21-8754587, ♥♥email: azmustopa@yahoo.com.

<sup>4</sup>Departement of Physiology, Pharmacology and Toxicology, Faculty of Veterinary Medicine, Institut Pertanian Bogor. Jl. Agatis, Kampus IPBDarmaga,  
Bogor 16680, West Java, Indonesia

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**Abstract.** Ahaddin AY, Budiarti S, Mustopa AZ, Darusman HS, Triratna L. 2021. Short Communication: Acute toxicity study of plantaricin from *Lactobacillus plantarum* S34 and its antibacterial activity. *Biodiversitas* 22: 227-232. *Lactobacillus plantarum* S34 belongs to the Lactobacilli class produces a bacteriocin called plantaricin. Bacteriocins are well known as active compounds that inhibit bacterial growth. This study was conducted to determine the antimicrobial activity of plantaricin S34 and its safety profile in the ddY mouse animal models. Plantaricin 34 from the crude extract was identified using Tricine SDS-PAGE. Antimicrobial activity was observed using disk diffusion against EPEC K1.1, *S. aureus*, *S. typhosa*, *S typhimurium*, and *Proteus* sp. The safety assessment showed that crude extract of plantaricin S34 did not cause any abnormalities to experimental mice even after being administrated with 5000 mg/kg BB. The identification of plantaricin S34 showed an active molecule at 7.34 kDa and had an activity to inhibit the pathogens used in this study. The blood analysis showed that the hematological and biochemical blood properties were in the normal range. Histopathological examination showed no damage to the intestine, liver, and kidneys. Thus, the crude extract of plantaricin S34 is active as an antimicrobial agent without any toxicity effects.

**Keywords:** Antibiotics, bacteriocin, *Lactobacillus plantarum*, plantaricin, toxicity

**Abbreviations:** AMPs: Antimicrobial peptides, AST: Aspartate transaminase, ALT: Alanine transaminase, LAB: Lactic Acid Bacteria, MDR: Multiple drug-resistant, PCV: Packed cell volume

### INTRODUCTION

Multiple drug-resistant (MDR) has been a major problem to date. MDR leads to a decrease in the efficacy of drugs against bacterial infections. According to WHO (2017), 12 types of bacteria are MDR. Several studies have been conducted to find alternative active compounds such as lytic phage (Budiarti et al. 2011) and natural compounds like antimicrobial peptide (Arief et al. 2015, Hanny et al. 2019). Antimicrobial peptides are naturally produced by individual organisms to kill threatened bacteria. The antimicrobial activity possessed by AMPs is almost the same as that of broad-spectrum antibiotics. It can be used against Gram-positive and Gram-negative bacteria, viruses, fungi, and parasites (Dosler and Mataraci 2013). This ability makes bacteriocin a potential alternative to substitute antibiotics.

Lactic acid bacteria (LAB) are a group of Gram-positive bacteria that produce a bacteriocin. Lactic acid bacteria are naturally found in processed milk or fermented food. Some of the LAB genera are known to have antimicrobial, antifungal, or antioxidant activity, i.e.,

*Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, *Bifidobacterium*, and *Propionibacterium* (Utami 2011). A study by Utami (2011) showed that *Lactobacillus plantarum* was the most developed bacteria reported to produce plantaricin with antimicrobial properties.

Plantaricin is an exoprotein produced by *Lactobacillus plantarum*, classified as class II bacteriocin. Class II bacteriocin is a non-lantibiotic peptide with low molecular weight, heat resistance, and extensive modification (Ekblad et al. 2016). Plantaricin inhibits *Candida albicans* (Sharma and Srivastava 2014), *Listeria innocua* NRRL B33314, *Micrococcus luteus* MTCC 106, *Enterococcus casseliflavus* NRRL B3502, *Lactococcus lactis* NRRL 1821 *Lactobacillus curvatus* NRRL B4562, and *Lactobacillus plantarum* NRRL B4496 (Pal et al. 2014). A study on plantaricin S34 in Indonesia was initiated by isolating *Lb. plantarum* S34 from bekasam, a traditional Indonesian fermented food (Mustopa 2013). On the genetic level, identification of the plantaricin gene showed that plantaricin S34 pln gene encodes plantaricin EF (Mustopa et al. 2016) and W (Umami et al. 2017). The encoded plantaricin gene transformed into *Lactococcus lactis*, and

the presence of each peptide was evaluated. The recombinant protein of plantaricin can inhibit Enteropathogen *Escherichia coli*, both in vitro and in vivo (Hanny et al. 2019). Hanny et al. (2019) recommended that *Lb. plantarum* S34 is a remarkable candidate for antibiotic replacement. Thus, to address those issues above, we evaluate the antimicrobial activity of plantaricin S34 in vitro and its safety in vivo using the animal model of the ddY mice.

## MATERIALS AND METHODS

### Materials

Materials used in this study were ammonium sulfate (Merck, Denmark), agarose (Himedia, India), de Man Rogosa Sharpe Broth (Himedia, India), Luria Bertani (Himedia, India), Hematoxylin-Eosin, formalin 4%, *ethanol absolute* (Merck, Denmark), xylol, paraffin, glycerin 99.5%, NaCl (Merck, Denmark), and TCA, TBA, BHT, Tris-HCl (Vivantis, USA).

### Microorganism

*Lactobacillus plantarum* S34 was obtained from Biotechnology Laboratory, Indonesian Institute of Sciences (LIPI), Cibinong, Bogor, West Java, Indonesia. The inoculum was stored in de Man Rogosa Sharpe broth media at 4°C. Isolate bacteria (*S. aureus*, *S. typhosa*, *S. typhimurium*, and *Proteus* sp.) were obtained from LIPI collection (Indonesia culture collection/Ina-CC) cultured in Luria Bertani media. EPEC K1.1 bacteria were obtained from the Animal Biotechnology Laboratory, Bogor Agricultural University, Bogor, Indonesia and were cultured in nutrient broth media.

### Procedures

This study is carried out in three steps; the first step is to identify plantaricin S34 in the LAB growth media using Tricine SDS-PAGE. The second step is to analyze the activity of identified plantaricin S34 against tested bacteria, such as EPEC K1.1, *S. aureus*, *S. typhosa*, *S. typhimurium*, and *Proteus* sp due to their MDR profile. The last step was to evaluate the safety of the crude extract of plantaricin S34 using the mouse animal model. As a new candidate for antibiotics, it is necessary to determine the safety of plantaricin S34 to ensure that no abnormalities will occur during treatment.

### Production of Plantaricin S34

*Lactobacillus plantarum* S34 was isolated from bekasam using serial dilution methods in saline solution and plated onto MRS agar (Oxoid, England). The plates were incubated at 37 °C for 2 d, and then a single confirmed colony was used afterward. Plantaricin S34 was produced following the method by Ekbal et al. (2016). *Lactobacillus plantarum* S34 was cultured in MRS medium and incubated overnight at 37°C. After the incubation, *Lb. plantarum* was centrifuged at 10000 × g at 4°C for 30 min. The supernatant was collected and adjusted with 0.1 N NaOH until pH 6.5. The supernatant was precipitated with

60% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> at 4°C overnight. The pellet was collected by centrifugation at 10000×g at 4°C for 30 minutes, then resuspended in Tris HCl buffer pH 7.4.

### Identification of Plantaricin S34 using Tricine SDS-PAGE and zymograms

The identification of plantaricin was performed in Tricine Sodium Dodecyl Sulfate Polyacrylamide gel (Tricine SDS-PAGE) at a concentration of 4% (stacking gel) and 16% (separating gel) (Haider et al. 2012). Polyacrylamide gel is made by mixing acrylamide: bis-acrylamide (29:1), aqua dest, glycerol, TEMED, and ammonium persulfate. The gel was stained using a silver stain (Thermo Fisher, USA). The zymograms were done by placing the gel above agar media, which contains 10<sup>6</sup> EPEC K1.1 bacteria. The gel was fixated using 25% ethanol and 5% formaldehyde for 30 minutes, then washed using sterilized water for three h. It was rewashed three times using Tween-80 for 40 min before placing it to pathogens media, then incubated at 37 °C for 24 h.

### Antibacterial activity test

Antibacterial activity was carried out by the agar diffusion method (Arief et al. 2015). The tested bacteria were cultured on LB for 16 h and diluted with physiological NaCl until the concentration reaches 10<sup>6</sup> CFU/mL. The bacteria were incubated on Luria Bertani agar (LA) at 37°C for 16 h, then a clear zone was measured.

### Experimental animals preparation

A total of 30 male ddY mice (15-30 g BW) were obtained from the Experimental Animal Laboratory Biofarma, Bandung. The adaptation period was seven days before the starting experiment. Mice were kept in plastic boxes with feed and drinking water available ad libitum. Light at the housing room was set to 12 h light-dark periods at 20°C. The experimental animals were divided into several groups using simple random sampling. Each group consists of five mice. This study has ethical approval from Bogor Agricultural University Ethics Commission with ethics number 80-2017IPB.

### Acute toxicity of Plantaricin S34 crude protein

The toxicity assay was done by following the method of Almeida Vaucher et al. (2011). Male ddY mice aged 4-8 weeks were kept in a group with standard food and drink. There are six treatment groups. Plantaricin S34 was administrated once orally to each group using 50, 100, 1000, and 5000 mg/kgBW. The clinical symptoms (skin and fur, eyes, lethargy, convulsions (seizures), tremors (trembling), diarrhea, and death) were observed at 2, 12, 24, and 48 h post-treatment. Bodyweight measurement was done every 24 h. After 48 h post-treatment, mice were euthanized (exsanguination), then the blood, liver, and kidney were collected for further analyses.

### Hematology analysis

The hematology analysis was done by following Aboderin and Oyetayo's (2016) methods. Hematology

parameters include PCV, hemoglobin, erythrocytes, leucocytes, and thrombocyte) using hematology analyzer Hemavet HV950FS (Drew Scientific Inc, German).

### Biochemical analysis

Biochemical analysis of the blood was carried out as follows: blood was centrifuged at  $6000 \times g$ . The serum was separated from the blood cells. The serum was tested for biochemical parameters (AST, ALT, urea, and creatinine) using hematology analyzer Hemavet HV950FS.

### Histopathological analysis

Histopathological analyses were performed using Schmitz et al. (2010) methods: the collected tissue was fixed in 10% buffer formalin and processed for paraffin embedding. The histological sections were stained with hematoxylin-eosin. The slides were coded and analyzed at the Primate Study Center, Bogor Agricultural University, Bogor, Indonesia.

### Data analysis

The results were expressed as mean  $\pm$  standard deviation of the groups and subjected to analysis of variance and Tukey's test using software Minitab 17.0. The differences were considered statistically significant when  $p < 0.05$ .

## RESULTS AND DISCUSSION

### Identification and antimicrobial activity of plantaricin S34

Plantaricin S34 was obtained using a 60% precipitation of ammonium sulfate. The concentration of crude plantaricin S34 was 66 mg/mL. The molecular identification using Tricine SDS-PAGE showed the presence of two bands lower than ten kDa at positions approximately 7.34 kDa (upper arrow) and 5.82 kDa (lower arrow) (Figure 1). The zymograms showed that the 7.34 kDa band has activity against EPEC K1.1 while the 5.82 kDa band has no activity.

Antimicrobial activity of crude plantaricin S34 against several pathogens was shown in Table 1. The crude plantaricin S34 has better antibacterial activity against *S. aureus* and EPEC K1.1 but has a different activity to the other pathogens.

### Plantaricin S34 Safety in ddY mice

The administration of plantaricin crude extract did not cause any changes in clinical observation (data not shown). The control group has slightly increased body weight while the treatment groups were decreased (Figure 2B). The bodyweight of treatment groups was not significantly different compared to the control group. The weight of organs (heart, kidney, brain, liver, spleen, and lungs) was also not significantly different from control (Figure 2A).

The treatment of plantaricin S34 resulted in decreased leucocyte and PCV levels. Plantaricin administration resulted in increased thrombocyte and erythrocyte. The decrease of leucocytes level increases with the higher the

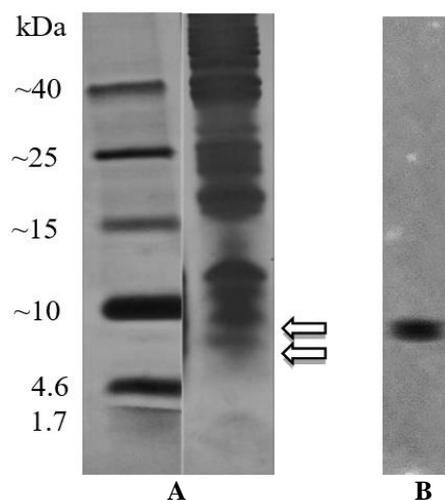
given concentration. The leucocyte was lowered from 3.10 thous/ $\mu$ L to 1.95 thous/ $\mu$ L. The decrease in leucocytes was contrasted to the PCV level, which shows a fluctuation depends on the given doses. The highest level of PCV was observed in plantaricin treatment at the concentration of 50 mg/kg BW, while the lowest PVC level was in the treatment of 100 mg/kg BW. The highest level of thrombocytes and erythrocytes were  $524.67 \pm 43.25$  thous/ $\mu$ L and  $8.93 \pm 0.03$  mill/ $\mu$ L, respectively. The lowest level of thrombocytes and erythrocytes were  $378.00 \pm 26.06$  thous/ $\mu$ L and  $35.03 \pm 1.97$  mill/ $\mu$ L, respectively. These values were significantly different compared to control (Table 2). The normal amount of leukocytes ranges from 1.5 to 4.8 thous/ $\mu$ L; thrombocytes range from 325 to 888 thous/ $\mu$ L, and erythrocytes range from 6.1 to 10.7 mill/ $\mu$ L, and PCV ranges from 33.5 to 47.8% (Santos et al. 2016).

Blood serum analysis was also performed to determine the effect of plantaricin S34 on liver and kidney performance. Table 3 showed that plantaricin treatment increased urea, creatinine, AST, and ALT levels. The increasing level of each component was not correlated. The administration of 1000 mg/kg BW plantaricin S34 increases most of the blood serum parameters. There was a significant difference in all observed components compared to control.

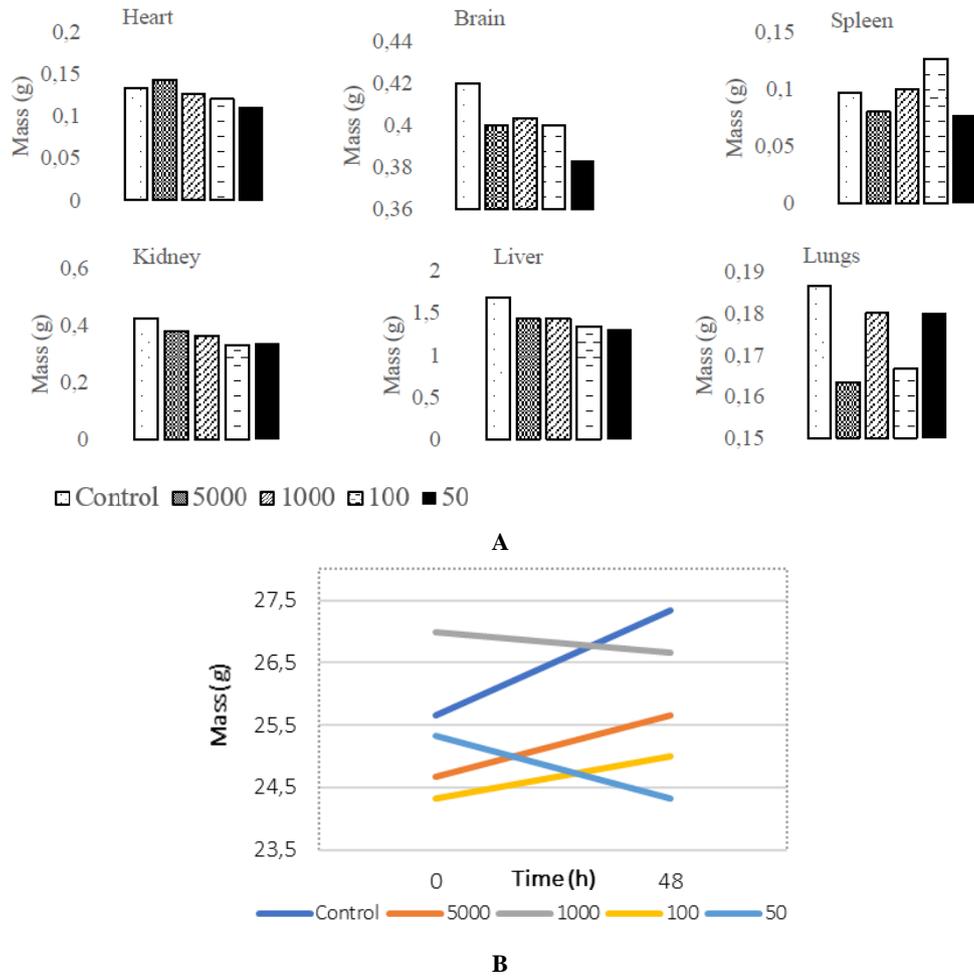
There were no abnormalities cause by plantaricin administration in hepatocytes and central venous cells (Figure 2F), kidney cells, tubules, and glomerulus (Figure 2E). Observation of mice intestinal sections showed no damage to the small villous intestine (Figure 2D).

**Table 1.** Antimicrobial activity of plantaricin S34

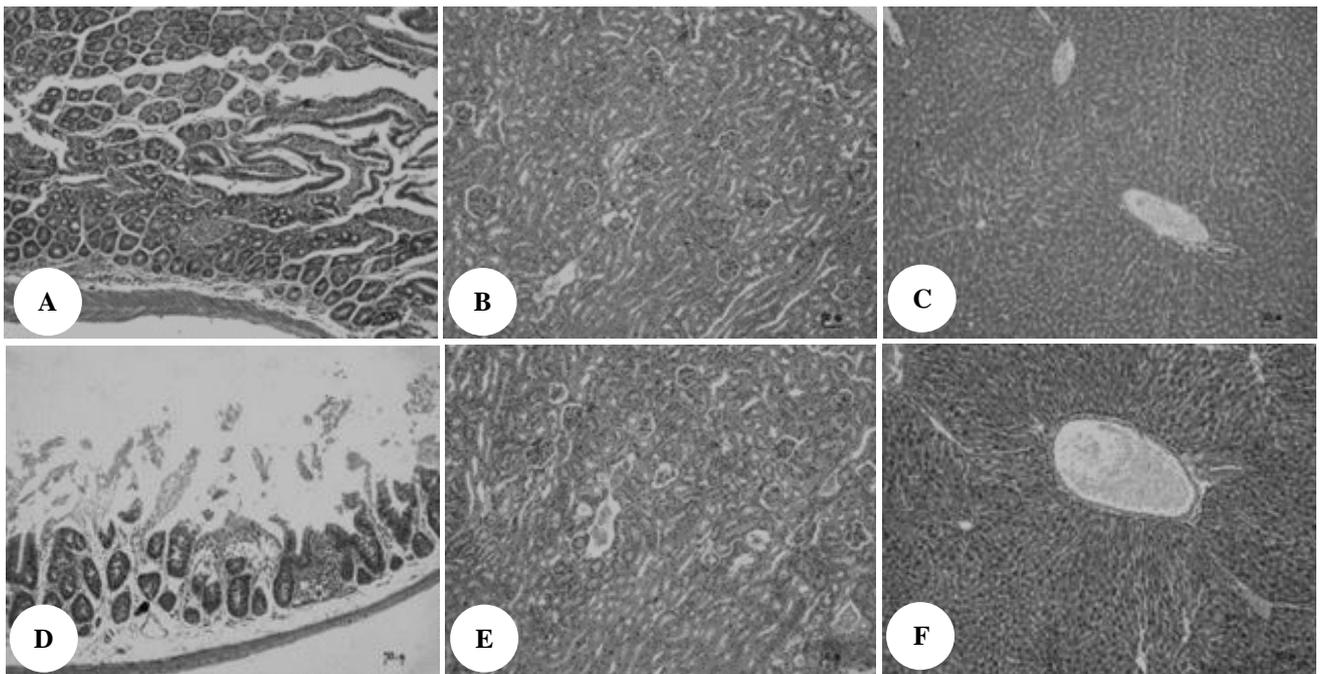
Pathogen	Clear zone (mm)	
	Ampicillin	Plantaricin S34
EPEC K1.1	$1.5 \pm 0.29$	$6 \pm 0.82$
<i>S. aureus</i>	$12 \pm 0.41$	$7 \pm 0.32$
<i>S. typhimurium</i>	$18 \pm 0.00$	$4 \pm 0.96$
<i>Proteus</i> sp.	$13.5 \pm 1.25$	$4 \pm 0.50$



**Figure 1.** Separation of plantaricin S34 using Tricine SDS-PAGE(A) and zymograms (B)



**Figure 2.** Effect of plantaricin S34 crude protein administration on the mass of: A. Several organs, B. Bodyweight



**Figure 3.** A. Photomicrographs of intestine section; B. Kidney section; and C. Liver section of control group. D. Histopathological observation of intestine section, E. Kidney section, and F. Liver section of mice treated with 5000 mg/kg BW.

**Table 2.** Hematological parameters of the ddY mice 48 h after plantaricin S34 administration

Groups	Hemoglobin (g/dL)	Leucocyte (thous/ $\mu$ L)	Thrombocyte (thous/ $\mu$ L)	Erythrocyte (mill/ $\mu$ L)	PCV (%)
Kontrol	12,73 $\pm$ 1.31 <sup>a</sup>	3,10 $\pm$ 0.10 <sup>a</sup>	473,33 $\pm$ 15.31 <sup>a</sup>	7,02 $\pm$ 0.64 <sup>a</sup>	34,93 $\pm$ 3.23 <sup>a</sup>
NaCl	11.05 $\pm$ 0.31 <sup>a</sup>	1.50 $\pm$ 0.20 <sup>b</sup>	451.00 $\pm$ 39.74 <sup>a</sup>	7.10 $\pm$ 0.47 <sup>a</sup>	34.13 $\pm$ 1.35 <sup>a</sup>
S34 5000	12.60 $\pm$ 0.10 <sup>a</sup>	1.95 $\pm$ 0.53 <sup>bc</sup>	378.00 $\pm$ 26.06 <sup>b</sup>	8.30 $\pm$ 0.22 <sup>b</sup>	40.73 $\pm$ 0.55 <sup>b</sup>
S34 1000	13.43 $\pm$ 0.59 <sup>a</sup>	2.20 $\pm$ 0.10 <sup>c</sup>	466.00 $\pm$ 8.54 <sup>a</sup>	8.58 $\pm$ 0.28 <sup>b</sup>	42.00 $\pm$ 2.19 <sup>b</sup>
S34 100	12.00 $\pm$ 1.35 <sup>a</sup>	2.10 $\pm$ 0.85 <sup>bc</sup>	524.67 $\pm$ 43.25 <sup>a</sup>	6.49 $\pm$ 0.13 <sup>a</sup>	35.03 $\pm$ 1.97 <sup>a</sup>
S34 50	13.13 $\pm$ 0.78 <sup>a</sup>	2.30 $\pm$ 0.10 <sup>c</sup>	436.67 $\pm$ 65.96 <sup>a</sup>	8.93 $\pm$ 0.03 <sup>b</sup>	44.63 $\pm$ 2.69 <sup>b</sup>

Note: \*The number shared the same character have no significant result with  $P < 0.05$

**Table 3.** Effect of plantaricin S34 administration on some serum biochemical parameters in ddY mice

Groups	Ureum (g/dL)	Creatinin (g/dL)	AST (U/L)	ALT (U/L)
Control	46.67 $\pm$ 8.14 <sup>a</sup>	0.39 $\pm$ 0.03 <sup>a</sup>	207.25 $\pm$ 74.43 <sup>a</sup>	52.33 $\pm$ 1.15 <sup>a</sup>
NaCl	85.33 $\pm$ 5.51 <sup>b</sup>	0.43 $\pm$ 0.02 <sup>a</sup>	250.00 $\pm$ 37.47 <sup>a</sup>	80.33 $\pm$ 7.77 <sup>b</sup>
S34 5000	80.67 $\pm$ 3.06 <sup>b</sup>	0.65 $\pm$ 0.06 <sup>b</sup>	230.67 $\pm$ 36.67 <sup>a</sup>	83.87 $\pm$ 7.76 <sup>b</sup>
S34 1000	95.33 $\pm$ 10.41 <sup>b</sup>	0.53 $\pm$ 0.08 <sup>b</sup>	395.00 $\pm$ 92.07 <sup>b</sup>	128.67 $\pm$ 26.10 <sup>c</sup>
S34 100	61.67 $\pm$ 1.15 <sup>d</sup>	0.45 $\pm$ 0.04 <sup>a</sup>	304.00 $\pm$ 31.19 <sup>a</sup>	64.33 $\pm$ 4.93 <sup>d</sup>
S34 50	90.67 $\pm$ 1.53 <sup>bc</sup>	0.41 $\pm$ 0.03 <sup>a</sup>	288.00 $\pm$ 42.58 <sup>a</sup>	131.33 $\pm$ 20.21 <sup>c</sup>

Note: \*The number shared the same character have no significant result with  $P < 0.05$

## Discussion

The identified protein in Tricine SDS-PAGE was predicted as two types of class II bacteriocin based on molecular weight. The low molecular weight determined from Tricine SDS-PAGE was suspected as plantaricin S34. Zymogram data described that plantaricin S34 only has one active band, which is 7.34 kDa.

Plantaricin can inhibit bacterial growth through the mechanism of electrolyte efflux, disrupt membrane potential (Zhang et al. 2015), and inhibit the 14- $\alpha$  demethylase enzyme (Omar and Yadav 2018). Plantaricin S34 crude protein has better activity than ampicillin in inhibiting EPEC K1.1. Budiarti (2011) reported that EPEC K1.1 has resistances against ampicillin compared to other bacteria. The presence of plantaricin S34 crude protein activity against pathogens makes this compound has potencies to substitute antibiotic.

All mice groups that received plantaricin S34 treatments showed significant differences in levels of leukocytes, platelet, erythrocytes, and PCV. However, these changes are still at the normal levels for each component. Plantaricin S34 is a protein that is easily degraded by proteolytic enzymes. The residue of degraded plantaricin S34 did not cause elevation of hematological profile to exceed the normal range.

Blood serum analysis was performed to determine the effect of plantaricin S34 on the liver and kidney. The increment of ureum level in the blood level indicating that plantaricin S34 can be digested by enzymes and converted to amino acids and ammonia. Besides, urea levels are influenced by protein consumption, protein digestion in the digestive tract, muscle protein degradation as well as creatinine levels that are related to muscle mass, body metabolism, infection, or inflammation (Martono and Satino 2014).

AST and ALT changes are related to the secretion of protease enzymes in the liver. It can be caused by protein consumption, activity, damage to body cells, or infection. Tissue damage or liver disorders can be indicated by an increase of 5-15 times from the normal level. The use of drugs that risk to generate liver disorders can increase the ALT/AST activity up to  $\geq 20$  times from normal activity (Aleya and Berawi 2015).

Observation of liver cells showed normal hepatocytes and central venous cells (Figure 2F). The observations on the kidney section did not show any abnormalities in kidney cells, tubules, or glomerulus (Figure 2E). Observation of mice intestinal sections showed no damage to the epithelium or small villous (Figure 2D). However, observation of intestinal sections showed a slight infiltration of mononuclear inflammatory cells. The low damage caused by the administration of plantaricin S34 at a dose of 5000 mg/kg BW indicates that this substance is safe to be used for short terms used.

We conclude that plantaricin S34 is an exoprotein with molecular weight of 7.34 kDa. Plantaricin S34 could inhibit the growth of EPEC K1.1, *S. typhi*, *S. typhosa*, *S. aureus*, and *Proteus* sp. Hematological and biochemical analysis of blood showed that plantaricin S34 administration increases those parameters but was still in the normal range. The histopathological analysis showed no abnormalities in the kidney, liver, and intestine sections. Thus, our results support that plantaricin, particularly plantaricin S34 is a candidate for substituting antibiotics.

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