

# The detection of plantaricin-encoding genes and their amino acid profiles in *Lactobacillus plantarum* AKK30 isolated from Indonesian native chicken

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**Abstract.** Elisabeth LUB, Abinawanto, Sophian A, Julendra H, Sofyan A. 2020. The detection of plantaricin-encoding genes and amino acid profile in *Lactobacillus plantarum* AKK30 isolated from Indonesian native chicken. *Biodiversitas* 21: 5792-5799. Plantaricin-encoding genes of lactic acid bacteria (LAB) *Lactobacillus plantarum* AKK30 which was isolated from Indonesian native chicken has been characterized using DNA sequencing technology. In order to substitute antibiotics for chickens and enhance probiotics in their gut, characterization of the indigenous probiotics in the chicken should be conducted. A potential probiotic in native chicken gut is *Lactobacillus plantarum* AKK30. Genes that harbor the bacteriocin biosynthesis of the strain were reported for the first time. The bacterium has diverse plantaricin genes, i.e., *plnA*, *plnEF*, *plnJ*, *plnK* and *plnO*. According to the dendrogram, the five plantaricin genes were closely related to *plnA*, *plnE*, *plnF*, *plnJ*, *plnK* and *plnO* genes from *L. plantarum* WCFS1, *L. plantarum* C11, and *L. plantarum* V90. Intriguingly, besides various plantaricin-encoding genes, another important probiotic property in *L. plantarum* AKK30 is cholesterol-lowering activity. Amino acid profiling also indicated proteolysis activity and probiotic potential, for instance, GABA production. Thus, *L. plantarum* AKK30 may be added in chicken broiler's diet to provide probiotic benefits.

**Keywords:** Chromatography, DNA sequencing, *Lactobacillus plantarum* AKK30, native chicken, plantaricin

**Abbreviations:** Ala: Alanine, CLA: Conjugated linoleic acid, DNA: deoxyribonucleic acid, MRS: de Man, Rogosa and Sharpe, DMSO: dimethyl sulfoxide, GRAS: generally recognized as safe, Glu: Glutamic acid, Gly: Glycine, HPLC: high-performance liquid chromatography, LAB: lactic acid bacteria, PCR: polymerase chain reaction, Pro: Proline, UPLC: ultraperformance liquid chromatography

## INTRODUCTION

Based on the Indonesian authorities' goals (Ministry of Agriculture Republic Indonesia), it is suggested that antibiotic treatment should be minimized. Therefore, probiotics could substitute any medicine in maintaining poultries' health. In addition, alternating antibiotics is useful to minimize residual consequences from the antibiotics administered in either poultries' or beef's meat. Furthermore, den Bogaard et al. (2001) and Kirbiš (2007) found that some microbes are resistant to antibiotic residues, which are either from meat or food industries. Consequently, it is dangerous when meat containing antibiotic residue is consumed by humans as the antibiotic residue may cause toxicity.

*Lactobacillus* is known for its probiotic potential. *Lactobacillus plantarum*, lactic acid bacteria (LAB), is a member of the genus *Lactobacillus* bacterium, a Gram-positive facultative anaerobic (Menconi et al. 2014; Arasu et al. 2016) and a versatile species (de Vries et al. 2006). These special characteristics make the species a safe probiotic, known as GRAS or generally recognized as safe

(Deegan et al. 2006; Settanni and Corsetti 2007). In addition, *Lactobacillus* is also known for its conjugated linoleic acid (CLA) production ability (Wen Jiang et al. 2014; Linares et al. 2017). Moreover, probiotic potential from *Lactobacillus* is beneficial for animals and humans. One reason is the cholesterol-lowering activity of the *Lactobacillus* (Linares et al. 2017). Hence, ability of the *Lactobacillus* to lower cholesterol is important to be investigated besides its probiotic production.

This paper focuses on the probiotics used for poultry. Huang et al. (2004) and Kabir (2009) found that probiotics are beneficial for the chickens as they found that provision of prebiotic caused significantly positive effect on the poultries' immune systems and meat quality. In addition, *Lactobacillus* spp. is known to have an effective anticancer ability in human intestines for good human health (Kieun Lee and Yeonhee Lee 2009; Masood et al. 2011).

An indigenous LAB strain of *L. plantarum* in Indonesia, namely *L. plantarum* AKK30 (Figure 1) was first reported by Julendra et al. (2017). The bacteria were isolated from the colons of native Indonesian chicken. Intriguingly, *L. Plantarum* AKK30 has the highest *in vitro*

test by using microplate reader (ppb) percentage cholesterol-lowering activity for decreasing cholesterol among the bacteria isolated from Indonesian chicken intestinal tracts as conducted by Julendra et al. (2017) with formula as: Cholesterol-lowering Percentage = [(cholesterol-lowering ( $\mu\text{g/mL}$ )]\*100% (Table 1). Therefore, it is interesting to carry out research about *L. plantarum* AKK30, since it is isolated from Indonesian native chicken and has beneficial properties.

Bacteria from the genera *Lactobacillus*, *Lactococcus*, *Pediococcus*, and *Streptococcus* are well known for their bacteriocin, a complex protein that can be an effective antibacterial protein (Deegan et al. 2006; Todorov 2009). Bacteriocin isolated from *L. Plantarum* is known as plantaricin. For instance, plantaricin SLG1 from *L. plantarum* SLG1 was reported for its bacteriocin biosynthesis (Jinjin Pei et al. 2018). This plantaricin was found to have activity against Gram-negative bacteria and fungi. Particularly, it inhibits the growth of *Listeria monocytogenes*, which is a known culprit in food-borne disease (Farber and Peterkin 1991). Therefore, research about *plantaricin* genes in *L. plantarum* is interesting to be conducted.

Plantaricin characterization has been usually conducted by using chromatography and sequencing (Jiménez-Díaz et al. 1995; Holo et al. 2001; Maldonado et al. 2003; Sabo et al. 2014; Jinjin Pei et al. 2018). One of the advantages of *plantaricin* gene characterization is to provide useful information about a particular LAB strain. However, study about *L. plantarum* AKK 30, especially about plantaricin is still limited. Here, this paper described the five *pln* genes, namely *plnA*, *plnEF*, *plnJ*, *plnK*, and *plnO* in *L. plantarum* AKK30 characterized by using DNA sequencing technology. This information hopefully could be utilized to suggest alternative antibiotics for poultries.

## MATERIALS AND METHODS

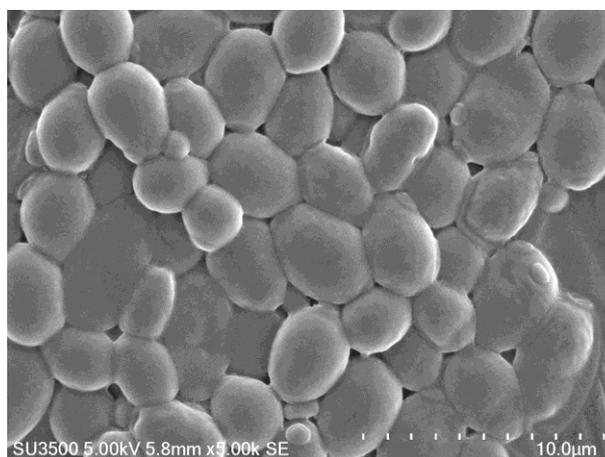
### *Lactobacillus plantarum* AKK30 and DNA extraction

*Lactobacillus plantarum* strain AKK30 isolates were obtained from Research Center for Natural Product Technology (BPTBA), Indonesian Institute of Sciences (LIPI), Gunungkidul, Yogyakarta, Indonesia. The bacteria culture was incubated in de Man, Rogosa, and Sharpe (MRS) broth at 37 °C and then kept at 4 °C. The genomic

DNA of bacteria was extracted by using gDNA wizard extraction kit (Promega). Micro-volume spectrophotometer (BioDrop) was used to calculate the gDNA concentration.

### Plantaricin genes amplification and DNA sequencing

*Plantaricin* genes were amplified by using polymerase chain reaction (PCR) with primers are included (Table 2). Two polymerase enzyme kits were used, i.e., 3 uM MgCl<sub>2</sub> Pol MasterMix and TaqPol kit (ProMega) for amplification due to optimized concentration of MgCl<sub>2</sub>. In addition, 5% dimethyl sulfoxide (DMSO) was also used for each amplification reaction in the total mixture volume 25  $\mu\text{L}$ . Then the mixture was added Followed by 0.75  $\mu\text{M}$  of MgCl<sub>2</sub>, specifically while using TaqPol kit for *plnA* and *plnEF* genes amplification. The final concentration from each primer was 0.35  $\mu\text{M}$ . The PCR conditions are described in Table 3.



**Figure 1.** Image of *Lactobacillus plantarum* (each rod-shaped figure) AKK30 from BPTBA-LIPI picture was taken by using scanning electron microscope (Hitachi SU3 500)

**Table 1.** *In vitro* test of cholesterol-lowering activity results done by Julendra et al. (2017)

No.	LAB isolates	%
1	AKK30	17.43%
2	AKU-4	15.24%
3	AKS-16	15.02%
4	AKS-19	8.41%

**Table 2.** Primers sequence of *plantaricin* genes amplification

Genes		Sequences (5'–3')	References
<i>PlnA</i>	F	GTA CAG TAC TAA TGG GAG	Diep et al. (1996), Remiger et al. (1996), Ben-Omar et al. (2006), Arief et al. (2015)
	R	CTT ACG CCA ATC TAT ACG	
<i>PlnEF</i>	F	GGC ATA GTT AAA ATT CCC CCC	Diep et al. (1996), Ben-Omar et al. (2006), Arief et al. (2015), Tai et al. (2015)
	R	CAG GTT GCC GCA AAA AAA G	
<i>PlnJ</i>	F	TAA CGA CGG ATT GCT CTG	Diep et al. (1996), Ben-Omar et al. (2006), Arief et al. (2015), Tai et al. (2015)
	R	AAT CAA GGA ATT ATC ACA TTA GTC	
<i>PlnK</i>	F	CTG TAA GCA TTG CTA ACC AAT C	Diep et al. (1996), Ben-Omar et al. (2006), Arief et al. (2015), Tai et al. (2015)
	R	ACT GCT GAC GCT GAA AAG	
<i>PlnO</i>	F	GTT CGT CCG GAA CGT TTT GT	Kleerebezem et al. (2003), <i>This paper</i>
	R	CGG ACC CCT CTG ATT CAC TG	

**Table 3.** PCR conditions for plantaricin genes amplification

Stage	Cycle (times)	Temp (°C)	Duration (min:sec)
Predenaturation	1	95	5:00
Denaturation		95	1:00
Annealing	35	60	1:00
Extention		72	1:00
Postextention	1	72	5:00
Hold		8	∞

### Liquid-phase based chromatography

The culture was divided into two tubes with two different concentrations of inulin (I), i.e., 0% and 0.5%. The inulin with 0.5% concentration was used based on Julendra et al. (2019) who found that addition of 0.5% inulin helped the fermentation process of the bacteria in *L. plantarum* AKK30 medium. Amino acid composition was determined by using ultra-performance liquid chromatography (UPLC) and high-performance liquid chromatography (HPLC) which completed with column chromatography of AccQ. Tag Ultra C18 1.7  $\mu\text{m}$  (2.1 x 100 mm) and Merck Lichospher 25 cm x 4.0 mm, 5  $\mu\text{m}$ , respectively.

### Data analysis

Capillary electrophoresis sequencing (CES) from MacroGen (South Korea) DNA sequencing service was used for sequencing the PCR amplicons. The DNA sequencing results were compared with the existing data in the NCBI by using a basic local alignment search tool (BLAST) alignment. Dendrogram for phylogenetic analysis was constructed using NJplot and ClustalX. Measurement which was used for grouping was 1000 bootstrap replicates (Perrière and Gouy 1996).

## RESULTS AND DISCUSSION

### *Lactobacillus plantarum* AKK30 DNA concentration

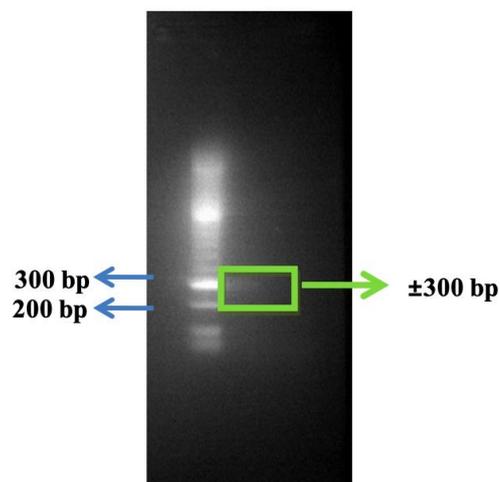
Culture of *L. plantarum* AKK30 was obtained from Research Division for Natural Product Technology (BPTBA), Indonesian Institute of Sciences (LIPI), Gunungkidul, Yogyakarta, Indonesia. The extracted DNA concentration was quantified by using a spectrophotometer in micro-volume. Result of the extracted DNA concentration was 116.6 ng  $\mu\text{L}^{-1}$ . Proportion of the results of UV absorbance for A260/A230 and A260/A280 were 2.216 and 2.024, respectively.

### *Lactobacillus plantarum* AKK30 DNA sequencing

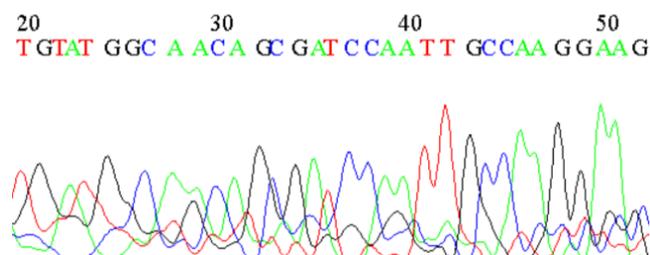
The first characterization of diversity of *plantaricin* genes in *L. plantarum* AKK30 was successfully described. The genes were *plnA*, *plnEF*, *plnJ*, *plnK*, and *plnO*. The PCR products were separated using electrophoresis (Figure 2) and the nucleotide sequence of the amplified PCR products was sequenced by using a CES and presented in an electropherogram (Figures 3).

Based on the DNA sequencing results, a BLASTn alignment analysis was done against NCBI data. However, there was no significant alignment of the NCBI database with our DNA sequence data. Then, a phylogenetic tree was constructed to look for the similarity between *pln* genes (*plnA*, *plnEF*, *plnJ*, *plnK*, and *plnO*). The result of cluster analysis showed that bootstrap of 1000 indicates the highest similarity (100%) between the genes at the same sub-cluster. The scale bar indicates 0.05 nucleotide substitution per nucleotide position of *plantaricin* gene (Figure 4).

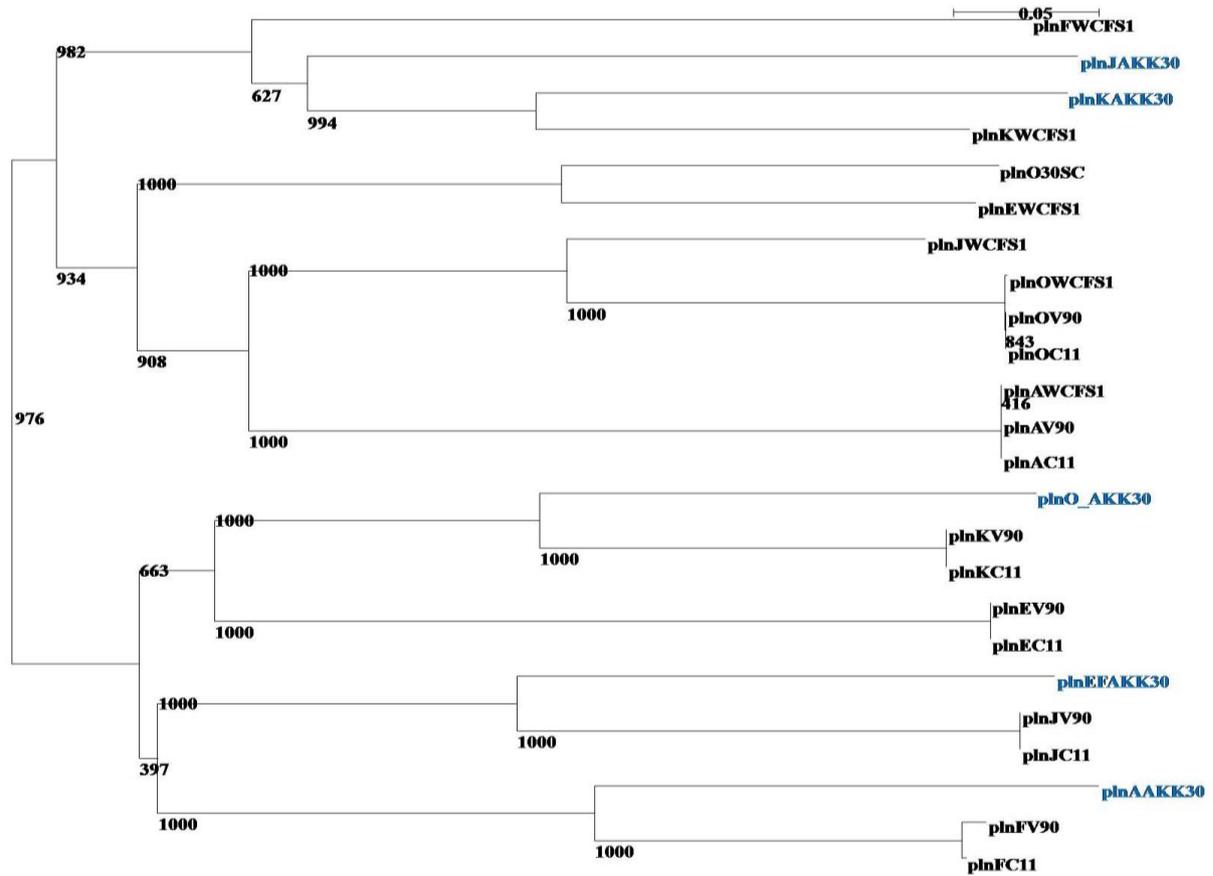
In this study, *pln* genes in the database are *L. plantarum* WCFS1 (accession number: NC\_004567.2), *L. plantarum* *pln* gene locus, strain C11 (accession number: X94434.2), and *L. plantarum* strain V90 *pln* gene locus, partial sequence (accession number: FJ809773.1). These strains were selected for phylogenetic analysis because *L. plantarum* WCFS1 is the first strain to be completely sequenced (Kleerebezem et al. 2003). Additionally, *plantaricin* genes in *L. plantarum* C11 have been characterized and reported by Diep et al. (1996). Moreover, *L. plantarum* V90 is the novel *pln* locus which is identical to those two aforementioned strains and reviewed by Diep et al. (2009).



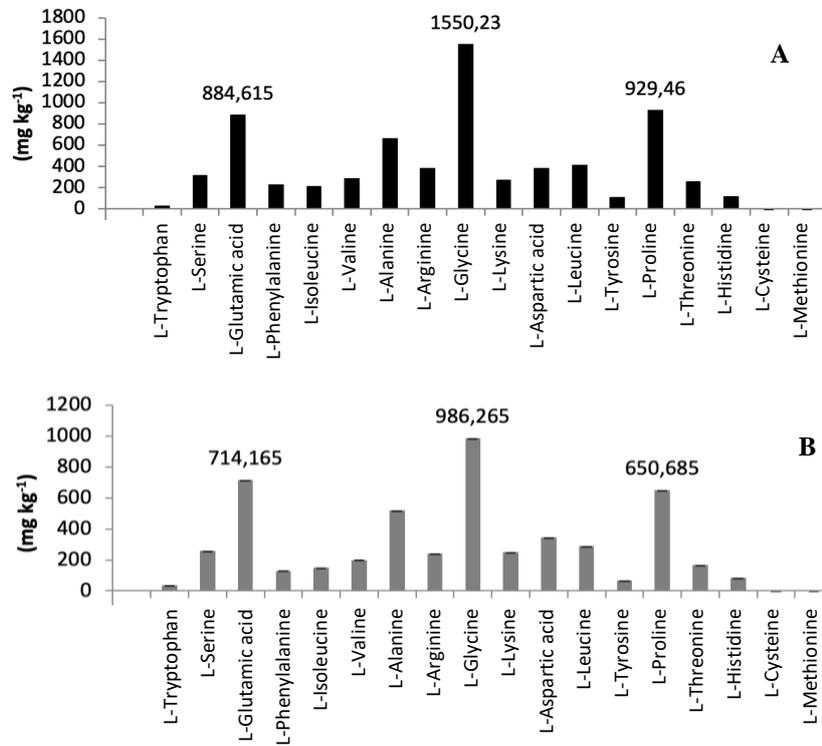
**Figure 2.** The electrophoresis of PCR product of *plnO* gene in *Lactobacillus plantarum* AKK30. The pointed green box and arrow indicated the expected band of 300bp



**Figure 3.** Electropherogram of *plnO* gene in *Lactobacillus plantarum* AKK30



**Figure 4.** The dendrogram of *Lactobacillus plantarum* AKK30 (blue) plantaricin genes analysis



**Figure 5.** A. Amino acid profile of *Lactobacillus plantarum* AKK30 without Inulin (Control group, 0% inulin); B. Amino acid profile of *L. plantarum* AKK30 with 0.5% Inulin

**Table 4.** Three amino acids found in high amount (mg kg<sup>-1</sup>) in the culture with (0%) and (0.5%) inulin

Amino acid	Oligosaccharides (inulin)	
	0%	0.5%
Glycine	1550.23	986.265
Proline	929.46	650.685
Glutamic acid	884.615	714.165

Amino acid sequence in *L. plantarum* AKK30 has been identified and Glycine (Gly) is the most prevalent amino acid (Figure 5a, 5b and Table 4). However, amino acid compound in *L. plantarum* AKK30 with additional 0.5% was 36% inulin was less than glycine in control group or with 0% inulin. Inulin was added because it is indispensable for *L. plantarum* in its growth and viability (Gänzle and Follador 2012; Valero-Cases and Frutos 2017). Our result showed that Gly was the amino acid most abundantly found in our sample, i.e., 1550.23 mg kg<sup>-1</sup> and 986.265 mg kg<sup>-1</sup> in 0% and 0.5% inulin concentration, respectively. Moreover, based on the results, Alanine (Ala) concentration level was higher than that of other types of amino acids, besides Proline (Pro) and Glutamic acid (Glu), 664.175 mg kg<sup>-1</sup> and 519.87 mg kg<sup>-1</sup> in 0% and 0.5% inulin concentration, respectively. Thus, *L. plantarum* AKK30 may produce plantaricin based on the result, as indicated by the Gly and Ala levels.

## Discussion

Results of BLASTn alignment showed no significant similarities between our nucleotide sequences with the database, consequently, the phylogenetic tree analysis was done in order to characterize the *pln* genes of *L. plantarum* AKK30. The dendrogram showed that AKK30 *pln* genes were divided into two clusters. In this paper, *pln* genes in the sample, i.e., *L. plantarum* strain AKK30, were explained first. In general, it is interesting that according to the phylogenetic tree, *pln* genes from AKK30 are closely related to the *pln* genes in the database with bootstrap of around 1000 indicating close similarity between genes in the clade (Holmes 2003).

Firstly, *plnK* gene from *L. plantarum* AKK30 was found to be closely related in one cluster with *plnK* gene from *L. plantarum* strain WCFS1, the first strain of *L. plantarum* which has been completely sequenced (Kleerebezem et al. 2003). In addition, the cluster has bootstrap value of 994 with *plnJ* gene from *L. plantarum* AKK30. The results showed that *plnJ* and *plnK* genes from *L. plantarum* AKK30 are closely related. Moreover, it is a significant finding that these genes were closely related and hypothesized as a group in the *plnJK* bacteriocin. Bacteriocin, namely *plnJK* is a member of class IIb two-peptide bacteriocins whose activity is dependent on the action of two different peptides (Diep et al. 2009). Also, analysis of immunity function of *plnK* in *L. plantarum* strain WCFS1 as supported by the phylogenetic analysis result, showed that they had closely related one another.

Secondly, other *pln* genes, namely *plnA*, *plnEF*, and *plnO* from *L. plantarum* strain AKK30 according to another cluster showed an interesting result. The *pln* genes tended to be in a group with *pln* genes from *L. plantarum* strain C11 and *L. plantarum* strain V90. This was particularly true for *plnK*, *plnJ*, and *plnF* genes. On the other hand, *plnO* and *plnA* genes from *L. plantarum* strain WCFS1, *L. plantarum* strain C11, and *L. plantarum* strain V90 tended to be in one group and were analyzed as in one node with *plnJ* gene from *L. plantarum* strain WCFS1.

The analysis results showed that *plnEFL. plantarum* AKK30 has quite similar characteristics as *plnJ* from *L. plantarum* strain C11 and V90. In addition, *plnEF* and *plnJK* are bacteriocins that consist of two peptides (Diep et al. 1996). Moreover, it is hypothesized that the *plnA* gene in the sample might be as strong as *plnF* of *L. plantarum* C11 and V90 in immunity level. This was supported by the results that *plnAL. plantarum* AKK30 as depicted by the phylogenetic tree, *plnA* was closely related to *plnF* from *L. plantarum* strain C11 and V90. Furthermore, it is intriguing that *plnABCD* operon in *L. plantarum* AKK30 is important in coding for a quorum-sensing network for expression of all the *pln* genes (Diep et al. 2009). The statement that plantaricin A, which was matured from *plnA* gene, is impedance as induction factor in bacteriocin biosynthesis is supported by Diep et al. (1996) study findings.

*Pln* genes reported in this paper were isolated from LAB found in indigenous chicken and has cholesterol-lowering activity. The genes reported here are also responsible for plantaricin biosynthesis. The phylogenetic tree showed that *L. plantarum* AKK30 has important *plantaricin* genes and operons. In addition, bacteriocin and antibiotics are quite similar to bactericidal compounds, but bacteriocin is synthesized in ribosome, whereas antibiotics are secondary metabolites (Deegan et al. 2006). Bacteriocins may be originated or encoded either from the chromosome or plasmid (Todorov 2009; Silva Sabo et al. 2014). Most bactericidal bacteriocins usually display modes of action towards closely related species (Deegan et al. 2006; Todorov 2009). Moreover, genes that play important roles in immunity and production are usually found to cluster in operons, i.e., *plnABCD*, *plnEFI*, *plnJKLR*, *plnGHSTUVW*, and *plnMNOP* (Diep et al. 2009). Therefore, plantaricin biosynthesis is also an important preference in producing probiotic.

Operons that have been reported are *plnABCD* operon for regulatory operon which is important for quorum sensing. In addition, plantaricin A from *plnA* also has immunity properties, even though peptides expressed by *plnEF* are still stronger in immunity level. *plnEFI* and *plnJKLR* operons are reported to involve in immunity. The transport operon consists of *plnGHSTUVW*, which helps coding for an ABC-transporter and an accessory protein in secretion of peptides employing a leader of double-glycine. Double-glycine is important to mark a plantaricin biosynthesis in a particular *L. plantarum* (Diep et al. 1996; Anderssen et al. 1998; Diep et al. 2009). Operon *plnOP* is reported to be responsible for coding proteins, namely *plnO* and *plnP* with significant homology to family 2-glycosyl

transferases and type II CAAX amino proteases, respectively (Diep et al. 2009).

For profiling amino acid in *L. plantarum* AKK30, it is interesting that inulin addition did not promote any synthesis, whereas it decreased the amino acid biosynthesis. The *L. plantarum* AKK30 has *pln* genes based on the glycine content described in this paper. However, additional inulin in the culture media did not affect the bacteria. Liquid chromatography assay, in particular UPLC, is recommended for amino acid analysis for this study.

Characterization of amino acid compounds in *L. plantarum* AKK30 was done by using UPLC and HPLC. Liquid chromatography, like UPLC, was used due to its sensitivity (Delavenne et al. 2011; Meussen et al. 2014; Galba et al. 2016). In this paper, the focus of discussion is on three amino acids with the highest concentration levels (exceeds 500 mg kg<sup>-1</sup>), i.e., Gly, Pro, and Glu. The amino acid which may indicate bacteriocin biosynthesis was Glycine, due to the double-Glycine found in plantaricin (Diep et al. 1996; Anderssen et al. 1998; Diep et al. 2009; Todorov 2009). In addition, high concentration levels of either Gly or Ala may indicate that there is biosynthesis of plantaricin (Jiménez-Díaz et al. 1995; Barbosa et al. 2016).

*Lactobacillus plantarum* was known for proteolytic ability by secreting proline iminopeptidase (PIP) (Chanalía et al. 2018). Our result showed that *L. plantarum* AKK30 may produce PIP indicated by Pro concentration level, 929.46 mg kg<sup>-1</sup> and 650.685 mg kg<sup>-1</sup> in 0% and 0.5% inulin concentrations, respectively. In addition, it was proven that LAB harbors the gene responsible for Glu expression (Tanous et al. 2005; Zareian et al. 2013). This is indispensable as a precursor in  $\gamma$ -aminobutyric acid (GABA) production (Li and Cao 2010; Rongzhen Zhang et al. 2014; Yunes et al. 2016). Chromatography results indicated that *L. plantarum* AKK30 may produce GABA as Glu concentration level exceeded 500 mg kg<sup>-1</sup>, i.e., 884.615 mg kg<sup>-1</sup> and 714.165 mg kg<sup>-1</sup> in 0% and 0.5% inulin concentrations, respectively.

Interestingly, the results showed that oligosaccharide did not oblige *L. plantarum* AKK30 in increasing amino acid level, even though, oligosaccharide is important in the *L. plantarum* AKK30 fermentation process (Julendra et al. 2019). We speculated that inulin has a negative-inducing lactose effect and the oligosaccharide was utilized for growth. This speculation was supported by study about operon in *L. plantarum* by Buntin et al. (2016) which showed that inulin is utilized as carbon source and indispensable for growth and viability of *L. plantarum*.

The present study results revealed five important plantaricin gene sequences in *L. plantarum* AKK30, a strain isolated from indigenous chicken. The amino acid profiling conducted by Cui Jin Toe et al. (2019) supports the chromatographic results of *L. plantarum* AKK30 and the suspected proteolytic activity of this strain. The data show proline levels above 500 mg kg<sup>-1</sup>. Lactic acid bacteria are known to have a gene for the production of glutamic acid (Glu) (Tanous et al. 2005; Zareian et al. 2013) which is important as a precursor for  $\gamma$ -aminobutyric acid (GABA) production (Li and Cao 2010; Rongzhen Zhang et

al. 2014; Yunes et al. 2016). Based on the chromatographic data of *L. plantarum* AKK30, Glu is an amino acid found in high amounts (884.615 mg kg<sup>-1</sup> and 714.165 mg kg<sup>-1</sup> for both inulin concentrations) in the sample. The data is supported by amino acid research in LAB by Cui Jin Toe et al. (2019), which shows a high Glu production of the eight samples (331.74 mg L<sup>-1</sup>). Alleged GABA production, based on Glu amino acid data from *L. plantarum* AKK30, is supported by several research results related to the production of Glu amino acids and the ability of LAB to produce amino nonpeptides (Tanous et al. 2005; Li and Cao 2010; Zareian et al. 2013; Rongzhen Zhang et al. 2014; Yunes et al. 2016). Interestingly, the Gly is not among the top three amino acids of the strain (94.90 mg L<sup>-1</sup>). However, these strains are included in the five sample strains yielding the amino acid Gly with a value approaching 100 mg L<sup>-1</sup>. Pro amino acids are mostly found in *P. pentosaceus* strain UL-6, amounting to 169.53 mg L<sup>-1</sup>. One of the LAB samples tested was from the genus *Lactobacillus* is *L. plantarum* UL-4. Based on the strain data, the three amino acids with the highest levels were Lysine (Lys), Glu, and Leucine (Leu). The strain is also not included in the strains discussed were related to the significance of the amino acid values of the eight LAB test strains. In conclusion, here we reported for the first time about five plantaricin genes in *Lactobacillus plantarum* AKK30, i.e., *plnA*, *plnEF*, *plnJ*, *plnK*, and *plnO*. The strain has diverse plantaricin genes, and it is interesting that *L. plantarum* strain from indigenous chicken in Indonesia is distinct. *L. plantarum* AKK30 is also a potential probiotic based on the amino acid profiling results.

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