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Cloning and Heterologous Expression of Extracellular Plantaricin F Produced by *Lactobacillus plantarum* S34 Isolated from "Bekasam" in *Lactococcus lactis*

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Plantaricin F (pln F) is bacteriocins produced by *Lactobacillus plantarum* are mostly applied in food to prevent microbial contamination. Biosynthesis of pln F is controlled by plantaricin A (pln A) which is primarily a peptide pheromone that controls the production of antimicrobial peptides in *L. plantarum.* Pre-mature pln A contains signal peptide and utilizes the general secretory pathway for export this peptide. The aim of this study was to construct a fusion of pln A signal peptide with mature pln F and to investigate the antimicrobial activity of pln F. Extracellular pln A- encoding the *plnA* gene were cloned into pGEM-Teasy vector to be used as a source for signal peptide SP_{pln}A. A polymerase chain reaction (PCR) overlaps technique has been used in the construction of fused gene with size of 171 bp while the individual gene obtained by this technique was 66 bp for pln A signal peptide and 105 bp for pln F. A gene encoding the pln A signal peptide (SP_{pln}A) fused to mature plantaricin F, fused gene were then cloned into pNZ8148 as expression vector under the control of the nisin promoter (Pnis A) to generate a pNZ8148 SP_{pln} A-plnF. Molecular expression study showed that recombinant *Lactococcus lactis* pln NZ3900 was able to express the mature pln F at transcription and translation level with size of 171 bp (by RT-PCR) and 3.8 kDa (by SDS-PAGE), respectively after 0.5-5 ng mL^{-1} nisin induction (OD_{600} 0,5). Furthermore, the supernatants of the recombinant *L. lactis* NZ3900 showed antimicrobial activity against *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6539, and *Listeria monocytogenes* BTCC B693. Collectively, the successfulness of expression of functional *pln F* gene under the control of nisin induction in *L. lactis* Nz3900 is the first publication.

Key words: bacteriocin, gene fusion, *Lactococcus lactis*, Plantaricin F

Plantaricin F (pln F) merupakan bakteriosin yang diproduksi dari *Lactobacillus plantarum* dan telah banyak digunakan dalam makanan untuk mencegah kontaminasi mikroba. Biosintesis dari plantarisin F dikendalikan plantaricin A (plnA) yang merupakan peptida feromon untuk memproduksi peptida antimikroba dari *Lactobacillus plantarum. Pre mature* pln A terdiri dari peptide signal dan secara umum memiliki peran mengarahkan sekresi agar mengeluarkan peptida tersebut. Penelitian ini bertujuan untuk mengkonstruksi gabungan sinyal peptida pln A dan pln F dan mempelajari aktifitas antimikroba dari plantarisin F. Ektraseluler pln A yang dikodekan oleh gen pln A kemudian diklon ke dalam vector kloning pGEMT untuk digunakan sebagai sumber sinyal peptida SP_{pln}A. Teknik polymerase chain reaction (PCR) *overlapping* telah digunakan untuk mengkonstruksi fusi gen dengan ukuran 171 bp dimana ukuran masing-masing gen adalah 66 bp untuk sinyal peptida plnA dan 105 bp untuk pln F. Gen yang mengkodekan sinyal peptida pln A (SP_{pln} A) di fusikan dengan plantarisin F, fusi gen telah diklon ke dalam vektor ekspresi pNZ8148 dibawah kontrol promoter nisin (Pnis A) untuk menghasilkan pNZ8148 SPA-plnF. Studi ekspresi secara molekuler ditunjukkan pada *Lactococcus lactis* plnNZ3900 rekombinan yang mengekspresikan protein matang dari plantarisin F pada level transkripsi dan translasi dengan ukuran 171 bp (RT-PCR) dan 3.8 kDa (SDS-PAGE, berturut-turut setelah induksi 0,5-5 ng/mL induksi nisin OD 0.5). Oleh karena itu, supernatan dari rekombinan *L.lactis* NZ3900 menunjukkan aktifitas antimicrobial terhadap *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6539, dan *Listeri monocytogen* BTCC B693. Penelitian ini merupakan penelitian yang dilakukan pertama kali untuk mengekspresikan gen plantarisin F di bawah kontrol induksi nisin pada *L.lactis* NZ3900.

Kata kunci: bacteriosin, fusi gen, *Lactococcus lactis*, Plantaricin F

Bacteriocins are gene encoded, ribosomally synthesized peptides with bactericidal or bacteriostatic mode of action against closely related species, spoilage and pathogenic bacteria. Bacteriocins produced by lactic acid bacteria (LAB) are investigated extensively due to their potential use as safe and non-toxic antimicrobial in biopreservatives food industries and

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the most interesting as potential alternatives to antibiotics as therapeutic agents in medical application (Lohans and Venderas 2011). Bacteriocin-producing LAB includes lactococci, lactobacilli, pediococci, and enterococci, the latter are of particular relevance to the current investigation.

Lactobacillus plantarum is one of the most widespread species of the genus *Lactobacillus* and is being widely used in food-related technologies. *L. plantarum* is the most important and versatile species of the group, and can be found as part of the microbiota in Indonesian fermented foods such as bekasam (fermented meat), tempoyak (fermented durian), dadih (bufallo fermented milk), and tape (fermented glutinous rice) (Mustopa 2013; Mustopa and Fatimah 2014). Moreover, this strain also showed broad adaptive response to environmental stressors (Margareta *et al*. 2015).

Previous study suggested that *L. plantarum* S31 and S34 isolated from bekasam (fermented meat) could produce bacteriocin named plantaricin (encoded by *pln* gene) which has the activity against several pathogenic bacteria (Mustopa and Fatimah 2014; Mustopa *et al*. 2014). *L. plantarum* S31 is a strain which is used as a source of pln A signal peptide ($SP_{pln}A$) DNA sequence, and this strain has also been well characterized about antimicrobial activity (Mustopa *et al*. 2014) and protease activity (Budiarto *et al*. 2015). While, *L. plantarum* S34 is a strain that use as a source of mature plantaricin F (pln F). This strain has also been well characterized about antimicrobial activity, stability of protein and mode of action (Mustopa 2013).

Plantaricin genes are spreaded into several loci which have different functions in biosynthesis system. There are five operons in plantaricin loci named *plnABCD*, *plnEFI*, *plnJKLR*, *plnMNOP*, and *plnGHSTUVW*. *plnABCD locus,* known as regulatory operon and the next three loci (*plnEFI*, *plnJKLR*, and *plnMNOP*) are related to plantaricin biosynthesis and their cognate immunity. *plnGHSTUVW locus* encodes the ATP-binding cassette (ABC) transporter and suggesting a putative membrane protein (Tsapieva *et al*. 2011).

Most bacteriocins, including those produced by *L. plantarum* and named plantaricin are synthesized as biologically inactive precursors or prepeptides containing an N-terminal extension of the so-called double-glycine type (leader sequence) that is cleaved concomitantly with export across the cytoplasmic membrane by dedicated ATP-binding cassette transporters (ABC transporters) and their accessory proteins (Håvarstein *et al*. 1995). However, many secreted prokaryotic proteins and a few class II bacteriocins, such as enterocin P (Cintas *et al*. 1997), hiracin JM79 (Sánchez *et al*. 2007) and plantaricin A (Diep *et al*. 1996) contain N-terminal extensions of the so-called sectype (signal peptide), which are proteolytically cleaved concomitantly with bacteriocin externalization by the general secretory pathway or sec-dependent pathway. Pln A a one-peptide bacteriocin without posttranslational modifications, is included in subclass IIc (Diep *et al*. 1996). Pln A is primarily a peptide pheromone that controls the production of antimicrobial peptides in *L. plantarum C11*, but it also has a direct, permeabilizing effect on certain bacterial strains (Sand *et al*. 2013).

Secretory proteins are equipped with an N-terminal signal peptide (SP) that functions as a target and recognition signal for signal peptidases that remove the SP from the translocated protein, resulting in the extracellular release of the mature protein or peptide (Natale *et al*. 2008). The signal peptide (SP) of secretory proteins and bacteriocins may drive fused mature bacteriocins to SPs for their secretion by recombinant LAB (Borrero *et al*. 2011).

Heterologous expression of several plantaricin peptides have been studied in *Escherichia coli* (Pal and Sheela 2013; Kusdianawati *et al*. 2015; Mustopa *et al.* 2016), *L. sakei* (Straume *et al*. 2006), *Saccharomyces cerevisiae* (Van Reenen *et al*. 2003) and *L. lactis* (Lages *et al*. 2015). It has been reported in previous study that full length of *Pln EF* gene from *L. plantarum* S34 was cloned into pGEM-Teasy. Furthermore, mature-pln F was subcloned in pET32a expression vector and expressed in *E. coli* BL21 (Mustopa *et al*. 2014) and exhibied synergistic effect against *E. coli*. Unfortunately, until now no studies have reported about expression of *Pln F* gene in a safe host with mature pln F fused to homologous signal peptides that act as secretion signals. Here, we report for the first time the cloning of *plnF* fusion using lactococcal Nisin Controlled Expression (NICE) system. The aim of this study was to construct a fusion of pln A signal peptide with mature pln F and to investigate the antimicrobial activity of pln F.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *L. plantarum* S31 was grown in deMan–Rogosa–Sharpe broth (Oxoid) at 37 ºC

without agitation. *Escherichia coli* TOP10 and MC1061 (Invitrogen and MoBiTec) were grown in LB broth (Oxoid) at 37 ºC with shaking. Lactococcal strains used in this study was *Lactococcus lactis* NZ3900 and was incubated statically in GM17B (M17 broth) (Oxoid) supplemented with 0.5% (w/v) glucose), regeneration medium to recombinant *L. lactis* were GM17B plus 0.5 M sucrose, 20 mM $CaCl_2$ and 20 mM $MgCl_2$ at 30 ºC. *L. lactis* NZ3900 (MG1363 derivative; standard host for a nisin-controlled expression system). Chloramphenicol was added to recombinant *E. coli* and *L. lactis* as selection marker. Pathogenic bacteria used were *E.coli* ATCC 8739, *S. aureus* ATCC 6539 and *L. monocytogenes* BTCC B693. All of pathogenic bacteria were grown in Nutrient broth (Oxoid) at 37 ºC. Agar plates were made by addition of 1.5% (w/v) agar (Oxoid) to the liquid media.

Basic Genetic Techniques and Enzymes. Total genomic deoxyribonucleic acid (DNA) from *L. plantarum* S31 was isolated using the CTAB methode with modification (Zhu *et al*. 1993). Plasmid DNA isolation from *L. lactis* strains was carried out using the Duan methode (Duan *et al*. 1999) and *E.coli* was carried out the *Presto Mini Plasmid Kit* (Geneaid). All DNA restriction enzymes such us *Nco*1 and *Pst*1 were supplied from New England Biolabs, Inc (NEB) and Thermo Fisher Scientific. Ligations were performed with the T4 DNA ligase (Kapa Biosystems). *E. coli* MC1061 competent cells were transformed as described by the (Sambrook *et al*. 1989). Electrocompetent *L. lactis* cells were transformed following the method of MoBiTec, with a Gene Pulser™ and Pulse Controller apparatus (Bio-Rad Laboratories, Hercules, CA).

Cloning of Plantaricin A Gene. The 300-bp fragment containing $SP_{pln}A$ and mature pln A gene was amplified from genomic DNA of *L. plantarum* S31 using primer PlnA_F/ PlnA_R (Table 2). PCR-amplifications were performed in 50 µL reaction mixtures containing 1 µL (10-100 ng) of purified DNA, 70 pmol of each primer, and 1 U of Kapa® DNA Polymerase. PCR was run under following conditions (Table 3). The PCR-generated fragments were purified by a *QIAquick® Gel Extraction Kit* (QIAGEN) before cloning into the vector. The PCR product was ligated into pGEM-T Easy vector (Promega, USA) produced pGEM-T pln A and introduced into *E. coli* TOP 10. The transformants were selected by blue-white colonies screening, then validated using PCR amplification from single transformant and its recombinant vector. Further confirmation of the correct nucleotide sequence of *plnA* was done using nucleotide sequencing of recombinant vector (Mustopa *et al*. 2016).

Construction of Recombinant Plasmids. The primers used for construction of the recombinant plasmids are listed in Table 2. A polymerase chain reaction (PCR) overlaps technique has been used in the construction, PCR was run under following conditions (Table 3). The primer pairs SP-plnA_F and Sp-plnA.F_R were used to amplify, a 66bp ($SP_{pln}A$) *NcoI* fragment (product 1) from plasmid of *E.coli* pGEM-T pln A. Primers pairs M-plnF_F and M-plnF_R were used to amplify mature pln F (105bp) containing *Pst1* frgament (product 2) from plasmid of *E.coli* pGEM-T pln EF. Mixtures of fragments (product 1 and product 2) were used as templates to amplify by PCR a 171bp *Nco1-Pst1* fragment using the primer pairs SP-plnA F and M-plnF_R. The fragments of gene fusion was digested with *Nco*1-*Pst*1 restriction enzymes and cloned into the pNZ8148 cutted vector with the same enzymes. The ligation mixtures were used to transform *E. coli* MC1061 according to the methods of (Sambrook and Russell 2001). The proper clones, containing pNZ8148 fusion were checked for PCR and sequencing of the inserts. The resultant plasmid pNZ8148 $SP_{pln}A$-plnF was isolated using *Presto Mini Plasmid Kit* (Geneaid) and was transformed into *L. lactis* NZ3900 by electroporation 200Ω and 2.5 µF in a Gene Pulser™ and Pulse Controller apparatus (Bio-Rad Laboratories, Hercules, CA) (MoBiTec). The transformants were selected by the addition of chloramphenicol. Positive clones from the *L. lactis* transformants were screened using primer Promoter8148 and Terminator8148.

DNA Sequencing and Analysis. The DNA sequencing was performed at the FirstBASE Laboratories (1st BASE Company, Malaysia). Similarity searches with sequences were perfomed by BLAST analysis in NCBI (http://www.ncbi.nlm.nih.gov/BLAST). Assembly of DNA sequences and translation were performed with DNAMAN version 4.0 (Lynnon BioSoft).

Expression of Plantaricin F Recombinant in *Lactococcus lactis*. To investigate the level of plantaricin F expression in *L. lactis*, cells were induced with nisin (MoBiTec) as follows. The *L. lactis* recombinant were grown in GM17 medium containing 10 µg mL^{-1} of chloramphenicol at 30 ºC. Cultures at an OD_{600} of 0.5 were induced by adding 0.5-5 ng mL^{-1} nisin and incubating for 5 h until OD_{600} of 1.0. Total RNA

Table 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Description	Source
Strain		
E. coli TOP 10	Host strain	Invitrogen
E. coli MC1061	Host strain, recA+	MoBiTec
L. plantarum S31	isolated from Indonesian fermented food bekasam, source of *pln A* gene	Laboratory collection*
L. lactis NZ3900	Host strain, Plasmid-free strain, LacF-:pepN::nisRK	MoBiTec
E.coli pGEM-T EF	source of *pln F* gene	Laboratory collection*
E.coli ATCC 8739	indicator strain	
S. aureus ATCC 6539		
L. monocytogenes BTCC B693		
Plasmid		
pNZ8148	Cm^r; inducible expression vector carrying the nisA promoter	MoBiTec
pGEM-T pln A	Amp^r, containing plantaricin A	This work
pNZ8148 Sp_{pln}A-F	Cm^r , pNZ8148 derivative carrying the PCR product of gene fusion, containing the SPplnA-pln F	This work

Cm^r, chloramphenicol resistance; Amp^r, Amphicilin resistance
*Laboratory collection Research Center for Biotechnology, LIPI

Table 2 Primers and PCR products used in this study

Primer	Nucleotide Sequences (5'->'3)	Purpose (Amplification of)	Expected size (bp)
PlnA _F	ATTTCATGGTGATTCACGTTTAAATT	plnA	300
PlnA _R	CTTACGCCATCTATACG		
SP-plnA _F	CAAC***CCATGG***CCATGAAAATTCAAATTA AAGGT	signal peptide plnA	66
SPplnA_F .R	TGGAAAACTCCACCTACT		
M-plnF_ F	AGTAGGTGGAGTTTTCCATG	plantaricin F	105
M-plnF _R	GATC***CTGCAG***CTATCCGTGGATGAATC		

Cleavage site for restriction enzymes is in italics in the primer sequences

Table 3 PCR reaction condition

Condition	Pln A	Signal peptide	Pln F and Fusion SP_{pln}A-F
Predenaturation	94 ºC ; 3 min	94 ºC ; 3'	94 ºC ; 3'
Denaturation	94 ºC ; 1 min	94 ºC ; 1'	94 ºC ; 1'
Annealing	54 ºC ; 1 min	58 ºC ; 1'	57 ºC ; 1'
Ekstention	72 ºC ; 30 sec	72 ºC ; 30 "	72 ºC ; 30 "
Final Ekstention	72 ºC ; 6 min	72 ºC ; 6'	72 ºC ; 6'
Cycle	35	35	35

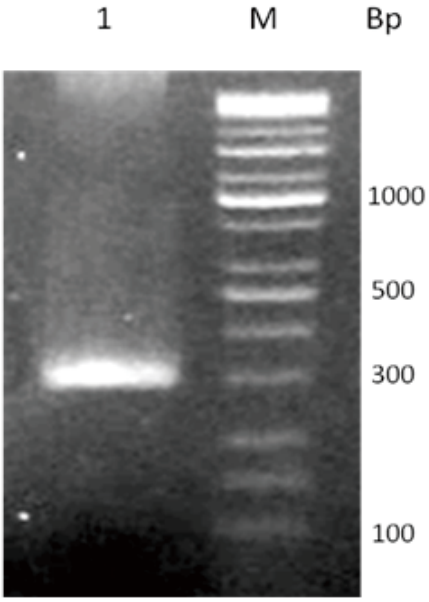

Fig 1 Results of PCR amplification of plantaricin A gene. M : 100 bp DNA ladder marker, 1: pln A 300 bp.

from *L.lactis* pNZ8148 fusion was isolated using the *Ambion®Totally RNA™ Total RNA Isolation Kit*. The cells were harvested by centrifugation at 13000xg. Furthermore, 150 ng of total RNA were used in a final volume of 25 µL for the Reverse Transcript -PCR experiments, to evaluate the expression of gene fusion at transcription level. The RT-PCR program was as follow: 45 ºC, 30 min (reverse transcriptase reaction); 94 ºC, 3 min; 94 ºC, 30 s (denaturation); 55 ºC, 1 min (annealing); 72 ºC, 1 min; 72 ºC, 5 min (extention) (Mustopa 2013). The PCR fragments were visualized on 2% agarose gel.

Supernatant of *L. lactis* recombinant used for SDS-PAGE analysis. Protein concentration was determined by the Pierce BCA Protein Assay kit using bovine serum albumin as a standard. For protein analysis, the supernatant was mixed with a fivefold-concentrated LaemmLi buffer, and after being heated at 97 ºC for 10 min, 15 µL of each sample was subjected to 16% (wt/v) SDS–PAGE.

Antimicrobial Activity Assays. Recombinant LAB cultures, transformed with either pNZ8148 derivatives, were induced for production of plantaricin F at an optical density at 600 nm (OD_{600}) of 0.5, using nisin at a final concentration of 0,5-5 ng mL^{-1}. The induced cultures and control cultures were grown at 30 ºC for 6 h. Cell-free supernatants were obtained by centrifugation of cultures at 12000×g at 4 ºC for 10 min, freeze dried and stored at −20 ºC until used. Bacteriocin activity was determined by the well-diffusion assay. Cell-free supernatants adjusted to pH 6.5 by the addition of sterile 1N NaOH using a digital pH meter (Eutech pH510). Amount of 50 µL supernatants were then spotted onto paper discs (diameter 6 mm; Filtres Fioroni, France) and loaded onto soft agar plates. Soft agar media containing the pathogenic bacteria were then poured into the plates. These plates were incubated at 37 ºC and examined for inhibition zones (Arief *et al*. 2013).

RESULTS

Isolation of the Partial Gene Encoding Plantaricin A from *L. plantarum* S31. We were interested to identifying plantaricin A, primarily a peptide pheromone that controls the production of antimicrobial peptides in *L. plantarum* S31 but it also has a direct, permeabilizing effect on certain bacterial strains (Sand *et al*. 2010). For this, a 300 bp of plantaricin A from *L. plantarum* S31 was amplified using specific primer (Fig 1). Plantaricin A from *L. plantarum* S31 was succesfully cloned into cloning vector pGEM-Teasy vector and the schematic procedure construction show in Fig 3. Sequencing analysis result showed 100% similarity of *L. plantarum* S31's plnA with *L. plantarum C11* as reference sequence (data not shown). This result indicated that mutations in plnA's sequence was not found and this DNA fragment could be used as a source for signal peptide pln A ($SP_{pln}A$) gene to be applied in the next experiment.

Based on the alligment result amino-terminal sequence analysis revealed that pln A consist of a 22 amino acid leader peptide, the mature pln A protein

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ATTTCATGGTGATTCACGTTTAAATTTAAAAAATGTACGTTAATAGAAAT
                 -35                        -10

AATTCCTCCGTACATCAAAAACACATTATCCTAAAAGCGAGGTGATTATT
rpoD16

ATGAAAATTCAAATTAAAGGTATGAAGCAACTTAGTAATAAGGAAATGCAAAAAATAGTA
 1           5           10          15          20
 M  K  I  Q  I  K  G  M  K  Q  L  S  N  K  E  M  Q  K  I  V

GGTGGAAAGAGTAGTGCGTATTCTTTGCAGATGGGGGCAACTGCAATTAAACAGGTAAAG
            25          30          35          40
 G  G  K  S  S  A  Y  S  L  Q  M  G  A  T  A  I  K  Q  V  K

AAACTGTTTAAAAAATGGGGATGGTAATTGATTTATTGAATAACTGTTTTTTAAGTTATA

 K  L  F  K  K  W  G  W  *

TTTCGTATAGATGGCGTAAG
```

Fig 2 The nucleotide sequence encoding the N-terminal part of pln A, its translation, and upstream sequences. The - 35 and – 10 promoter sequences and the start of transcription are *underlined.* Relevant restriction sites are indicated. Predicted signal peptidase I cleavage sites are indicated by *vertical arrowheads.*

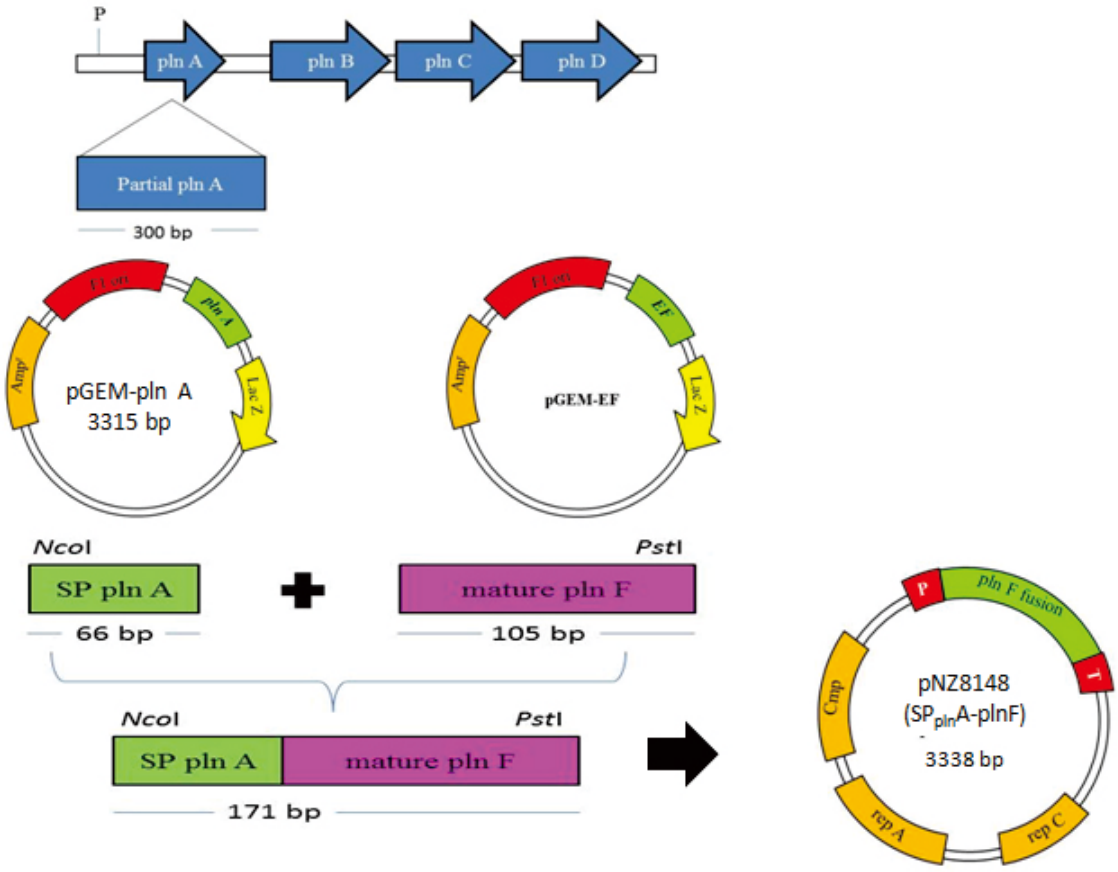

Fig 3 Schematic overview of the construction procedure of the LAB expression vector pNZ8148 SP_{pln}A-plnF. A polymerase chain reaction (PCR) overlaps technique has been used in the construction.

starts at residue 23 in the primary sequence. Leader peptide preceding the amino-terminus of mature Pln A contain two putative signal peptidase cleavage sites after glysin21 and glysin22 (Fig 2).

Construction of Recombinant Plasmid for Production of Plantaricin F. The vector construction was done containing the SPplnA fused to mature plantaricin F resulted in plasmid pNZ8148 SP_{pln}A-plnF. Schematic of the construction of the LAB expression vector pNZ8148 SP_{pln}A-plnF was shown in Fig 3. An overlapping PCR fragment containing both leader peptide pln A (66 bp) and mature pln F (105 bp)

was fused to generate 171 bp gene fusion (Fig 4a). It was inserted into pNZ8148 between the *Nco*1-*Pst*1 cleavage sites to generae the expression vector pNZ8148 SP_{pln}A-plnF as described in Fig 4b. The gene of interest that would be cloned into this vector should be adapted and two base pair CC were added to allow translation fusion at *Nco*1 (CC**ATG**G) site. Derivatives of plasmid pNZ8148 were used to transform *E. coli* MC1061 to generate cloning vector and to get stable plasmid before inserting into *L. lactis* host. The pNZ8148 is a shuttle vector that have *rep A* and *rep C* where can be used to *E. coli* MC1061 and *L.lactis*. This was made possible to construct in *E. coli*, and the system should work in *L. lactis* (de Ruyter *et a*l. 1996). Furthermore, derivatives of pNZ8148 were used to transform *L.lactis* NZ3900 as a for recombinant production of plantaricin. The fragment inserted was verified by PCR and sequencing. The 627 bp of PCR result contained 171 bp of plnF gene (Fig 4b). This size of fragment in the plasmid pNZ8148 from promoter and terminator. This result indicated that the gene fusion was successfully inserted in expression vector pNZ8148. The DNA sequence of *plnF* fusion was analyzed to determine start-end position of transcription and translation (Fig 5).

Heterologous Expression of Plantaricin F in *L. Latis*. To further investigate expression of plantaricin F produced by this gene fusion the expression in the transcriptional level of *L.lactis* recombinant strain, a reverse transcript PCR was performed. Total RNA from recombinant strain and control strain was successfully isolated after 5 ng mL^{-1} nisin induction (OD_{600} 0,5) and it is used as template for the reverse transcriptase process. A reverse transcriptase-PCR *L.lactis* pNZ8148 SP_{pln}A-plnF resulted 171 bp (Fig 6). This result indicated that pln F fusion in encoded by derivatives pNZ8148 was succesfully induced by the nisin.

The soluble and insoluble protein fraction prepared from recombinant and non-recombinant cells were subjected to SDS-PAGE analysis. As shown in Fig 7a, secreted plantaricin F protein from *L.lactis* pNZ8148 harboring SP_{pln}A-plnF produced protein band wih 3.8 kDa in size after nisin induction a 0.5-5 ng mL^{-1}. Its size was consistent with the molecular weight of the mature plantaricin F (3.8 kDa) encoded by 105 bp *pln F* gene which was inserted into pNZ8148. The expected molecular weight bands could not be observed in blank control (host) and non induced strain. Furthermore, compared with pellet fraction (A cell extract of strain NZ3900 harboring SP_{pln}A-plnF) in Fig 7b there is no protein band could be detected under 4.6 kDa of protein marker.

Antimicrobial Activity of Supernatants from Recombinant *L. Lactis*. The optimum induction time and nisin concentration on recombinant pln F have been investigated to known those effect on antibacterial activity of pln F againts certain pathogenic bacteria. The supernatants of recombinant *L. lactis* showed a observable antagonistic activity against *E. coli* ATCC 8739 (Fig 8). The cultures were induced at the (OD_{600} 0,5), protein expression was induced by the addition of nisin in the final concentration of 0.5 ng mL^{-1}, 1 ng mL^{-1}, 5 ng mL^{-1}. The clear zone of inhibition produced by *L. lactis* recombinant carrying pNZ8148-SP_{pln}A-plnF presents in all 0.5, 1 and 5 ng mL^{-1} addition nisin induction (Fig 8). However, the clear zone produced by 5 ng mL^{-1} nisin induction is larger than 0.5 ng mL^{-1} and 1 ng mL^{-1}. But of the three pathogens, the biggest inhibition is present in *E. coli* ATCC 8739 (Table 4).

DISCUSSION

Production of bacteriocins by heterologous hosts may be based on the expression of native biosynthetic genes, by exchanging leader peptides and/or dedicated secretion and processing systems (ABC-transporters) or by fusion to signal peptides that act as secretion signals (Gutiérrez *et al*. 2006). Plantaricin F is class IIb of bacteriocins produced by *L. plantarum* are mostly applied in food to prevent microbial contamination due to their strong antibacterial activity contained. These unique peptides have a conserved N-terminal, named GxxxG motifs in its mature peptide form (Pal and Sheela 2014). Pln F is placed in plnEFI locus and was induced by pln A. Naturally in the synthesis of pln F has signal peptide that functions for secretion pln F. But there has been no report avalable that shows the replacement of signal peptide pln F with other signal peptide and no one has expressed on a safe host like *L. lactis*.

The use of a vector which is compatible with the host lactis is also noteworthy. Previous study has shown that pNZ8048 is the most efficient vector for expressing bacteriocins in *L. lactis* NZ9000 (Borrero *et al*. 2011) therefore we use a derivative of the vector that is pNZ8148. pNZ8148 is a shuttle vector that can replicate in *E. coli* and LAB. Plasmid pNZ8148 contains the high-copy number heterogramic replicon of the lactococcal plasmid pSH71 with a unique *NcoI* cleavage site, downstream of the nisA ribosomal

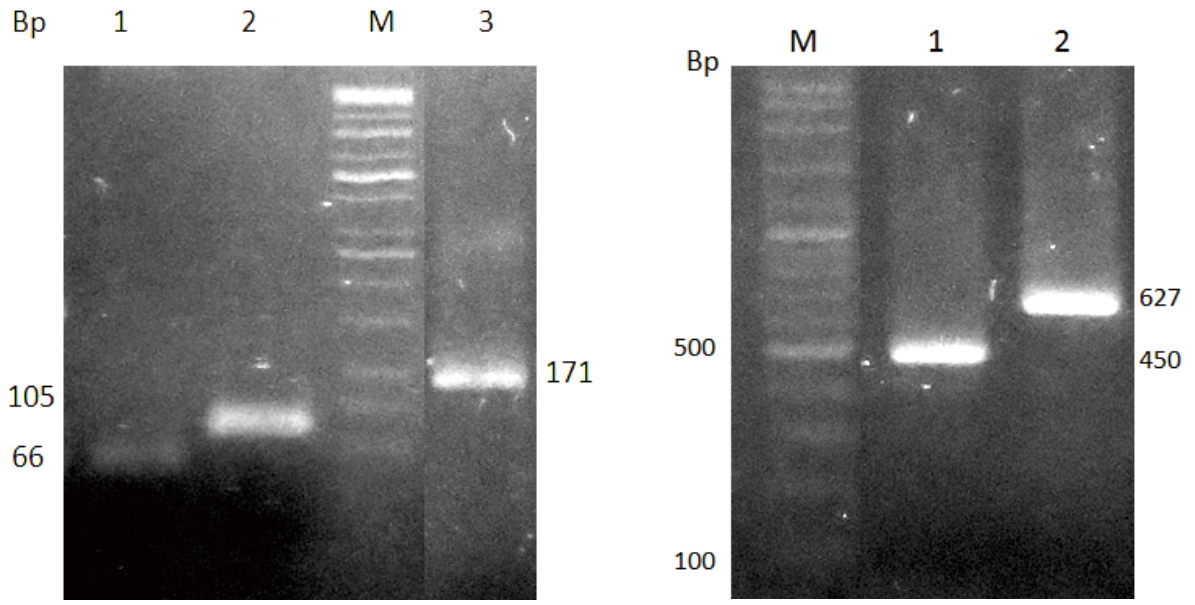

Fig 4 Results of PCR amplification gene fusion (a) PCR product of 1: signal peptide pln A, 2: plantaricin F 3: fusion fragment containing the SPpln A and mature plantaricin F (b) Identification of plasmid *L. lactis* NZ3900 (pNZ8148-SP_{pln}A-plnF) by PCR, 1: negative control (*L. lactis* pNZ8148), 2: PCR product of gene fusion.

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  1  AGATCTAGTCTTATAACTATACTGACAATAGAAACATTAACAAATCTAAAACAGTCTTAATTCTATCTTGAGA
                                                          -35
 74  AAGTATTGGTAATAATATTATTGTCGATAACGCGAGCATAATAAACGGCTCTGATTAAATTCTGAAGTTTGTT
       -10          1                          RBS        NcoI
147  AGATACAATGATTTCGTTCGAAGGAACTACAAAATAAATTATAAGGAGGCACTCACCATGGCCATGAAAATTC
  1                                                        M  A  M  K  I

220  AAATTAAAGGTATGAAGCAACTTAGTAATAAGGAAATGCAAAAAATAGTAGGTGGAGTTTTCCATGCCTATAG
  6  Q  I  K  G  M  K  Q  L  S  N  K  E  M  Q  K  I  V  G  G  V  F  H  A  Y  S

293  CGCGCGTGGCGTTCGGAATAATTATAAAAGTGCTGTTGGGCCTGCCGATTGGATCATTAGCGCTGTCCGAGGA
 31    A  R  G  V  R  N  N  Y  K  S  A  V  G  P  A  D  W  I  I  S  A  V  R  G
                  PstI
366  TTCATCCACGGATAGCTGCAGGCATGCGGTACCACTAGTTCTAGAGAGCTCAAGCTTTCTTTGAACCAAAATT
 55   F  I  H  G  *

439  AGAAAACCAAGGCTTGAAACGTTCAATTGAAATGGCAATTAAACAAATTACAGCACGTGTTGCTTTGATTGAT


512  AGCCAAAAAGCAGCAGTTGATAAAGCAATTACTGATATTGCTGAAAAATTGTAATTTATAAATAAAAATCACC

       Terminator
585  TTTTAGAGGTGGTTTTTTTATTTATAA
```

Fig 5 Analysis of DNA construct pNZ8148 SP_{pln}A-plnF: Sequence analysis of *pln F*, RBS: ribosome binding site, *: stop codon, +1: transcription start position.

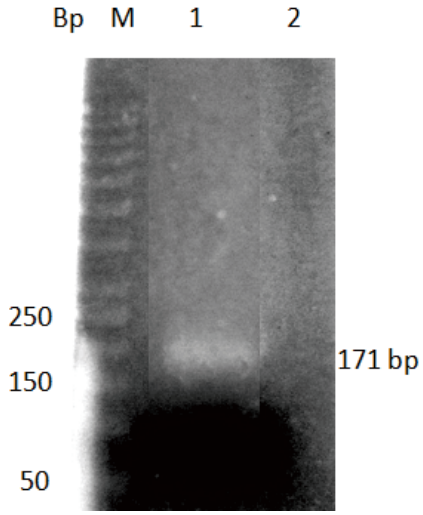

Fig 6 Reverse transcription of mRNA *L.lactis* pNZ8148 fusion to examine transcription level. Lane 1: 5 ng mL^{-1} nisin induction, lane 2: blank control (host/*L.lactis*).

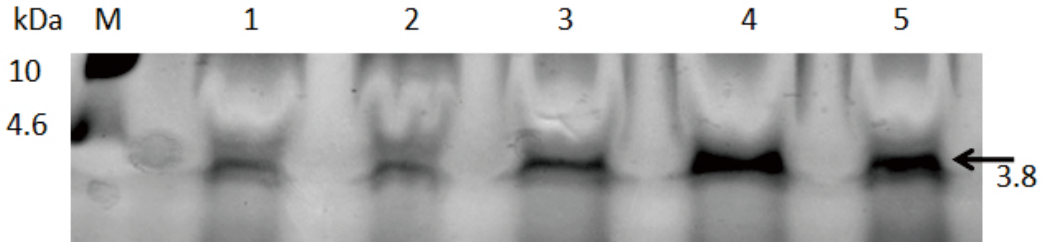

Fig 7 SDS–PAGE analysis of the expression of the plantaricin F using NICE system in *L. lactis*. a) the supernatan from *L.lactis*, lane 1: host, lane 2: non induced recombinant *L. lactis,* lane 3: 0.5 ng mL^{-1} induced, lane 4: 1 ng mL^{-1} induced, lane 5: 5 ng mL^{-1}. b) pellet from *L.lactis,* Lane 1:non induced, lane 2: host, lane 3: induced 5 ng. The locations of the overproduced proteins are indicated (arrows).

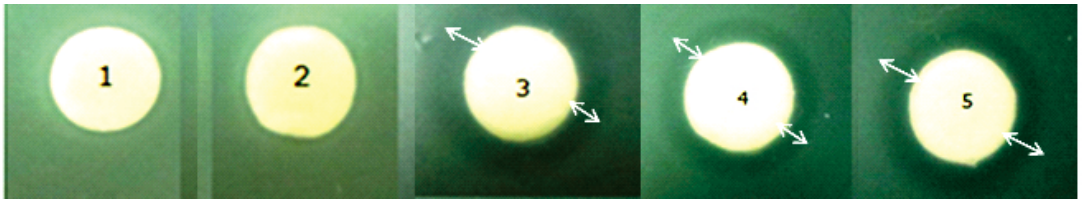

Fig 8 Antimicrobial activity of *L. lactis* and *L. plantarum* in *E. coli* ATCC 8739 as determined by the agar well diffusion test. Supernatants of: (1) *L. lactis* NZ3900 (2) *L. lactis* recombinant non induced (3) *L. lactis* recombinant 5 ng mL^{-1} nisin induction (4) *L. lactis* recombinant 1 ng mL^{-1} nisin induction (5) *L. lactis* recombinant 0.5 ng mL^{-1} nisin induction.

Table 4 Bacterial strains and plasmids used in this study

Strain Indicator	Gram (+/-)	Diameter (mm) of zone of inhibition*		
		Nisin concentration		
		0.5 ng mL^{-1}	1 ng mL^{-1}	5 ng mL^{-1}
E. coli ATCC 8739	-	10±0.5	10±0.8	11±0.5
S. aureus ATCC 6539	+	8±0.5	8±0.5	9±0.3
L. monocytogenes BTCC B693	+	7±0.3	7±0.8	9.5±0.7

ATCC, American Type Culture Collection; BTCC, Biotechnology Type Culture Collection
*Wells (6 mm in diameter) were filled with 25 µL samples cell-free supernatant which pH neutralized to 6.5; mean counts of tri-trials (mean ± SD); "–"

binding sequence (RBS), used for translational fusions inducible by nisin A (NisA) (Kuipers *et al*. 1998; Douillard *et al*. 2011; Mirończuk *et al*. 2012).

We use pNZ8148 NICE vectors and we compared different ranges of nisin concentration for induction. For that purpose, the *pln F* gene encoding *L. plantarum* S34 extracellular plantaricin F was cloned downstream of the strong inducible promoter nisA. In this system, nisin induces the regulatory cascade starting with binding to the membrane-bound receptor NisK. Next, the phosphate group from the activated NisK is transferred to the intracellular response regulator NisR, activating this regulator. Subsequently, NisR, induces the nisin operon at the promoter nisA. The NisA promoter controls the expression of the genes involved in the pln F biosynthesis. We compared different nisin concentrations for *L. lactis* induction. The studied nisin concentration range was from 0.5 to 5 ng mL^{-1} and the highest antibacteria activity was obtained when 5 ng mL^{-1} of nisin. In comparison, nisin concentration in published data varies from 0.1 to 40 ng mL^{-1} (Mierau *et al*. 2005). When the nisin concentration is too low, it is not able to activate nis K in host, but if inducer concentrations that are too high can inhibit the growth of the *L. lactis* host. In this study with induction of 5 ng/ml nisin was able to activate the cascade reaction NisK / NisR so mRNA is formed with a size of 171 bp and produce 3.8 kDa protein.

Previous studies have shown that the heterologous expression of pln F from *L. plantarum* S34 by cloning and expression of the mature and pre-mature pln F under the control of an inducible promoter in *E. coli* BL21. It showed a very low-level of antimrobial activity (Mustopa *et al*. 2014; Kusdianawati *et al*. 2015). Based on the results we make a replacement not only of signal peptide from pln F to signal peptide pln A, but also host of expression from *E. coli* to *L lactis*. Gene fusion PCR overlapping was developped to obtain this construct. Signal peptide from pln A *L. plantarum* S31 was used to replace signal peptide from pln F. The structural characteristics of signal peptides of procaryotic origin are highly conserved. The most common type of signal peptide consists of a positively charged N-terminus, a central hydrophobic core and a C-terminal cleavage region (Asseldonk *et al*. 1993). Furthermore, in *L.lactis* this signal peptide pln A is processed by the signal peptidase of the sec-dependent pathway during translocation to the surrounding growth medium. In that condition pln F was activated, so that in this research has antimicrobial activity. The importance of replacing the signal peptide also has been done by previous researchers about bacteriocins carnobacteriocin B2 with divergicin A (McCormick *et al*. 1996), enterocin A with enterocin P (Borrero *et al*. 2011; Martin *et al*. 2007). Antimicrobial activity produced after the replacement is higher than ever before.

In this study, recombinant plantaricin F have inhibitory activity against *E. coli* ATCC 8739, *S. aureus* ATCC 6539 and *L. monocytogene* BTCC B693. Previous research has described the inhibition of pln F against *E. coli* but there must be addition of EDTA (Pal and Sheela 2014). In this study, pln F recombinant is able to inhibit pathogens without the addition of EDTA. In particular, these mechanisms can promote a bactericidal effect, with or without cell lysis, or bacteriostatic, inhibiting cell growth. This mechanism is triggered when bacteriocin binds to a protein-receptor on the cell membrane of the target bacteria. Pore formation, which results in the variation of the cytoplasm membrane potential due to the hydroniumion exchanging between the inner and outer membrane surfaces., is the main mechanism by plantaricin from LAB exert their antibacterial effect (Sabo *et al*. 2014). Of the three pathogens were tested, the inhibition of *E. coli* has the greatest value than the others pathogens. This is because *E. coli* is a gram-negative that have peptidoglycan layer is thinner than the gram-positive.

The pln A signal peptide was succesfully fused with mature pln F with size 171 bp. Plantaricin F gene fusion was succesfully cloned in to pNZ8148 and expressed in *L. lactis* NZ3900 host at transcription and translation level at 171 bp and 3.8 kDa. The optimum of nisin induction concentration to production and to inhibit indicator strain is 5 ng mL^{-1}. This study found that replacement of pln F signal peptide by pln A signal peptide permits the production, secretion, and functional expression of pln F by *L. lactis* NZ3900.

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