Media Peternakan, August 2013, pp. 91-100
ISSN 0126-0472 EISSN 2087-4634
Accredited by DGHE No: 66b/DIKTI/Kep/2011

Online version:
http://medpet.journal.ipb.ac.id/
DOI: 10.5398/medpet.2013.36.2.91

Isolation and Characterization of Plantaricin Produced by *Lactobacillus plantarum* Strains (IIA-1A5, IIA-1B1, IIA-2B2)

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(Received 01-04-2013; Reviewed 03-05-2013; Accepted 08-07-2013)

ABSTRACT

Bacteriocins produced by Indonesian lactic acid bacteria *Lactobacillus plantarum* IIA-1A5, IIA-1B1, IIA-2B2 were purified and characterized. Plantaricin W gene had been successfully amplified from all strains. This amplicon showed the expected 200 bp size of plantaricin W gene. This bacteriocins purified from *L. plantarum* IIA-1A5, IIA-1B1, and IIA-2B2 were named plantaricin IIA-1A5, IIA-1B1, and IIA-2B2. Purification by cation exchange chromatography increased the purity (fold) and activity of plantaricins. Purity of plantaricin IIA-1A5 was increased by 3.13 fold with specific activity 13.40 AU/mg. Plantaricin IIA-1B1 had 2.98 fold purity with specific activity 5.12 AU/mg, while purity of plantaricin IIA-2B2 was 1.37 fold with specific activity 7.70 AU/mg. All plantaricins could inhibit the growth of pathogenic bacteria, such as *Escherichia coli, Salmonella typhimurium, Bacillus cereus,* and *Staphylococcus aureus*. Plantaricins could be digested by trypsin. Stability of plantaricins at 80 °C for 30 min and at 121 °C for 15 min were affected by type of plantaricin and species of pathogenic bacteria. Generally, plantaricin IIA-1A5 was better as antimicrobial agent than plantaricin IIA-1B1 and plantaricin IIA-2B2.

Key words: characterization, Lactobacillus plantarum IIA-1A5, L. plantarum IIA-1B1, L. plantarum IIA-2B2, plantaricin

ABSTRAK

Bakteriosin dihasilkan oleh *Lactobacillus plantarum* IIA-1A5, IIA-1B1, IIA-2B2, bakteri asam laktat (BAL) asal Indonesia, yang dipurifikasi dan dikarakterisasi. Gen plantarisin W berhasil diamplifikasi dari semua strain dengan ukuran basa 200 pb. Bakteriosin yang dipurifikasi dari *L. plantarum* IIA-1A5, IIA-1B1, dan IIA-2B2 dinamakan plantarisin IIA-1A5, IIA-1B1, dan IIA-2B2. Purifikasi dengan menggunakan kromatografi pertukaran kation mampu meningkatkan aktivitas plantarisin tersebut. Kemurnian plantarisin IIA-1A5 meningkat sebesar 312.65% dengan aktivitas spesifik 13.40 AU/mg. Plantarisin IIA-1B1 mempunyai tingkat kemurnian sebesar 297.72% dengan aktivitas spesifik 5.12 AU/mg, sedangkan peningkatan kemurnian produk plantarisin IIA-2B2 sebesar 137.48% dengan aktivitas spesifik 7.70 AU/mg. Karakterisasi plantarisin IIA-1A5, IIA-1B1 dan IIA-2B2 menunjukkan bahwa plantarisin tersebut mampu menghambat pertumbuhan bakteri patogen *Escherichia coli, Salmonella typhimurium, Bacillus cereus,* dan *Staphylococcus aureus* dan mampu didegradasi oleh tripsin. Stabilitas terhadap perlakuan pemanasan pada suhu pasteurisasi (80 °C selama 30 menit) dan sterilisasi (121 °C selama 15 menit) dipengaruhi oleh jenis plantarisin serta jenis bakteri patogen yang dihambat pertumbuhannya. Berdasarkan karakteristiknya secara umum, plantaricin IIA-1A5 mempunyai sifat antimikroba yang lebih baik dibandingkan dengan plantaricin IIA-1B1 dan plantaricin IIA-2B2.

Kata kunci: karakterisasi, Lactobacillus plantarum IIA-1A5, L. plantarum IIA-1B1, L. plantarum IIA-2B2, plantarisin

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INTRODUCTION

A wide variety of bacteriocins produced by Lactic Acid Bacteria (LAB) has been reported. Bacteriocins are ribosomally synthesized antimicrobial peptides or protein that can inhibit growth of bacteria by causing disruption of the cell membran and make a pore leading to cell death (Diep *et al*., 2009). The bacteriocins from LAB have attracted significant attention because of their potential use as non-toxic and safe additives for food preservation and prevention of food spoilage by food borne pathogenic bacteria (Hata *et al*., 2010; Savadogo *et al*., 2006).

The bacteriocins of LAB have been classified into four classes. Most of them belong to class I or II. Class I bacteriocins named as lantibiotics, are small (< 5 kDa) membrane-active peptides, which contain post-translationally modified amino acid residues like lanthionine. Class II bacteriocins are small, heat stable, non-lanthionine-containing peptides. Class III bacteriocins consist of large heat labile bacteriocin (> 10k Da) whereas Class IV bacterocins consist of a protein moiety with one or more other chemical moieties (e.g. carbohydrate and lipid) (Savadogo *et al*., 2006; Tiwari & Srivastava, 2008).

L. plantarum is LAB which produces bacteriocin, called plantaricin. Plantaricin A, plantaricin EF, and plantaricin JK are included into Class II bacteriocin (Diep *et al*., 2009). Whereas plantaricin W consisting two peptides plantaricin W-alpha and plantaricin W-beta from *L. plantarum* LMG 2379 belongs to a new family of two-peptide class I bacteriocin (Holo *et al*., 2001).

Our previous research showed that Indonesian indigenous strains of *L. plantarum* was isolated succesfully from Indonesian beef e g. *L. plantarum* IIA-1A5, *L. plantarum* IIA-1B1, and *L. plantarum* IIA-2B2. They have been identified as species and strains by molecular technique using PCR and 16S rRNA sequencing. *L. plantarum* IIA-1A5, *L. plantarum* IIA-1B1, and *L. plantarum* IIA-2B2 were in the same cluster with *L. plantarum* JDM 1 and *L. plantarum* ATCC 14917 by phylogenetic tree analysis using Kimura model and they were 97% similar identity with *L. plantarum* JDM 1 using BLAST analysis (Arief, 2010). Another strain of *L. plantarum* isolated from Indonesian beef, namely *L. plantarum* 2C12, which had probiotic characteristic as antidhiarrea againts enteropathogenic *Escherichia coli* was reported by Arief *et al.* (2010).

Exploration about their functions have been done, such as antibacterial activities against pathogenic bacteria (*E. coli, Staphylococcus aureus,* and *Salmonella typhimurium*). It was reported that all strains produced antibacterial substances that could inhibit growth of pathogenic bacteria (Arief, 2011). Purification of the plantaricin as bacteriocin from indigenous *L. plantarum* is very important to know their function. Purification and biochemichal characterization of plantaricins in terms of their stability are essential to evaluate their potential for various applications. Plantaricin W is interesting to be explored because it is unique lantibiotic beside nisin as preservatives. The objectives of the research were to isolate plantaricin W gene from three strains of Indonesian LAB *L. plantarum*, and to characterize the plantaricin produced by *L. plantarum* strains exhibiting antibacterial activity with a view to elucidate its potential applications as preservative agents.

MATERIALS AND METHODS

Experiment 1. Isolation and Amplification of Plantaricin W Genes

Isolation of genomic DNA from bacteria. The total genomic DNA of *L. plantarum* strains was isolated in small scale preparations from 10 mL of overnight culture grown at 24 °C in deMan Rogosa Sharp (MRS) broth (Oxoid) medium. The instruction of genomic DNA purification followed by Sambrook *et al.* (1989) for extraction and purification of plasmid DNA.

Amplification of plantaricin W genes by PCR. Plantaricin genes were amplified in 25 μL volumes each containing 100 ng template DNA, 5 unit/μL Taq DNA polymerase 0.1 μL, 10x buffer 0.1 μL, 10 mM dNTP 0.5 μL, 20 pM of each forward and reverse primer @0.3 μL, 25 mM MgCl2 2.0 μL PCR amplification of the bacteriocin genes was carried out using the primers for all described plantaricin genes. Forward primer was MY42F 5′-GATCAGCCACGATACCAAC-3′ and reverse primer was MY42R 5′-CTAAAGAAAAAGCCCCTGAAAC-3′ (Saenz *et al.,* 2009). The PCR reactions were performed with an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C primer annealing temperature for 45 s and 72 °C extension for 1 min, followed by a final extension step at 72 °C for 5 min. PCR products were separated by electrophoresis using a 2% (w/v) agarose gel, which was stained with ethidium bromide and visualized using UV light source.

Plantaricin W gene sequencing. Plantaricin W gene sequencing was made by sending a sample to 1st BASE Pte Ltd (Singapore). Sequence similarity searches were performed in the GenBank data library using the BLAST program. The sequence information was then imported into Genetic software program for assembly and CLUSTAL W software program for alignment. Phylogenetic trees were constructed by the neighbor-joining method using MEGA 4 software program.

Experiment 2. Purification and Characterization of Plantaricin

The experiment was carried out according to Tiwari & Srivastava (2008); Hata *et al*. (2010).

Bacterial strains and growth conditions. *L. plantarum* strains were grown in MRS broth and agar media. Pathogenic bacteria used were *E. coli* ATCC 25922, *Bacillus cereus* (collection of Animal Product Technology Laboratory, Faculty of Animal Science, Bogor Agricultural University), *Salmonella* Typhimurium ATCC 14028 and *S. aureus* ATCC 25923. All of pathogenic bacteria were grown in Nutrient agar as slab cultured stock. Stock cultures were stored in media at -20 °C, subcultured twice in the same media and incubated at 37 °C for 24 h before use.

Purification of bacteriocin. *L. plantarum* IIA-1A5, IIA-1B1, and IIA-2B2 were grown in MRS broth (Oxoid) medium supplemented by 3% yeast extract at 37 °C without agitation for 20 h for the production of bacteriocin. The cell were removed by cold centrifugation (20,000 g for 20 min, 4 °C), followed by filter-sterilized (0.2 μm Sartorius filter membrane). The plantaricins were purified from the cell free supernatant by ammonium sulphate precipitation and cation-exchange chromatography.

Ammonium sulphate precipitation. Ammonium sulphate was added until 80% saturation at 4 °C, followed by stirring for 2 h at 4 °C. The precipitated protein was centrifugated at 20,000 g for 30 min at 4 °C. The resulting pellets were solubilized by sodium phosphate buffer, pH 6.0 (buffer A). The sample was desalted by dialyzing (2.0 kDa cut-off membrane) against buffer A.

Cation-exchange chromatography. Samples were applied at 1 mL/3 min of flow rate to SP sepharose fast flow cation-exchange column equilibrated with buffer A. The column was washed with 4 bed volumes of buffer A. Fractions were then collected. The proteins were monitored at 280 nm with UV VIS spectrophotometer. Protein concentration of fractions were analyzed using Lowry method and tested for bacteriocin activity.

Bacteriocin activity and Minimum Inhibitory Concentration (MIC). The agar well diffusion method was used to detect the antibacterial spectrum of plantaricins, according to Hata *et al.* (2010). The test pathogenic bacteria (approximately 10^6 cfu/mL) were spread onto Muller Hinton Agar (MHA/ Difco) media. Six millimeter diamaters wells were made in MHA and 50 μL of the plantaricins were placed into each well. Plate was incubated at 37 °C for 24 h. The area of inhibition was calculated from the diameters of the inhibition zones. These experiments were done in triplicate.

MIC was determined by serial dilution methods. The tested pathogenic bacteria were grown in Nutrient Broth (Difco) media and standardized by 0.5 Mc.Farland to have population 10^6 cfu/mL. Plantaricins were diluted in 9 serial number dilution (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90%) in different tubes. Each dilution tube of plantaricin was confronted with pathogenic bacteria until volume 100% in the same tube. The tubes were incubated at 37 °C for 24 h. After incubation, they were observed for bacterial growth by plate count analysis. MIC was the lowest concentration of plantaricin that could inhibit 90% of growth of the tested pathogenic bacteria.

Stability against proteolytic enzyme and heat conditions. To test the sensitivity of plantaricins to proteolytic enzyme, the samples were incubated with trypsin at a final concentration of 0.5 mg/mL at 37 °C overnight. Samples were heated at 80 °C for 30 min (pasteurization temperature) and 121 °C for 15 min (sterilization temperature) at pH 7.0 to test the heat stability, considering pasteurization and sterilization methods. After each treatment, the remaining activities were determined by agar diffusion method. These experiments were done in triplicate. The antimicrobial activities were expressed as activity unit.

Statistical Analysis

For antibacterial activities of plantaricins, MIC and stability on heat treatment, experimental design used were Factorial Completely Randomized Design. For stability on trypsin experiments, and heat conditions experiments, experimental design used were Completely Randomized Design. Data were analyzed by ANOVA (Analysis of variance) or T test for stability on trypsin experiment (Steel & Torie, 1995). If significantly difference, Tukey test was used.

RESULTS AND DISCUSSION

Experiment 1. Isolation and Amplification of Plantaricin W Genes

Amplification of plantaricin W gene. PCR study was performed to determine the presence of the plantaricin W gene described for *L. plantarum* IIA-1B1, IIA-1A5, and IIA-2B2. PCR reaction resulted in amplification of DNA fragment from all *L. plantarum* strains. This amplicon showed the expected 200 bp size (Figure 1). It indicated that *L. plantarum* IIA-1B1, IIA-1A5, and IIA-2B2 had plantaricin W gene, expressed plantaricin W as protein. The primer used was deduced from the genome *L. plantarum* WCFS1 with sequence number AL93262. Twenty strains of *L. plantarum* isolated from grape must and wine during alcoholic and malolactic fermentation also had plantaricin W gene (Saenz *et al*., 2009). The sequence of the region of plantaricin W gene in class lantibiotic revealed that two genes (plantaricin W-alpha and plantaricin W-beta) encoding the plantaricin (Holo *et al*., 2001). The other bacteria produced plantaricin W that encoded by plantaricin W-alpha and plantaricin W-beta was strain *L. plantarum* LMG 2379, originally isolated from fermenting Pinot Noir wine in Oregon (Holo *et al*., 2001)

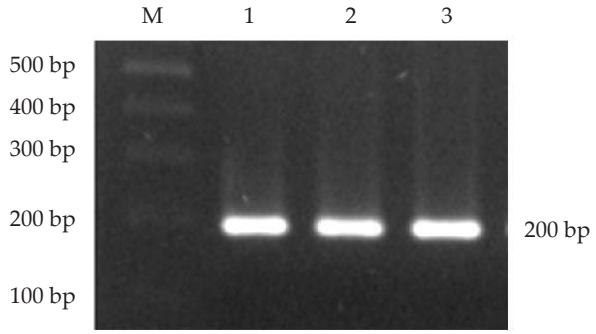

Figure 1. Amplification with plantaricin W-beta primer yielded a 200 bp fragment characteristic for *L. plantarum*. M= Marker 100 bp, lane 1: *L. plantarum* IIA-1A5, lane 2: *L. plantarum* IIA-1B1, lane 3: *L. plantarum* IIA-2B2.

Alignment of plantaricin gene sequencing. Plantaricin gene from Indonesian *L. plantarum* strains had different sequences with plantaricin W beta from *L. plantarum*

LMG 2379 and plantaricin from Malaysian *L. plantarum* strains, but it was similar with plantaricin W gene from *L. plantarum* WCFS1, *L. plantarum* ST III and *L. plantarum* JDM1 (Figure 2).

Analysis of individual genetic distances of plantaricin showed that plantaricin W beta did not differ with plantaricin from Malaysian *L. plantarum*, but had differences with plantaricin from *L. plantarum* IIA-1A5, IIA-

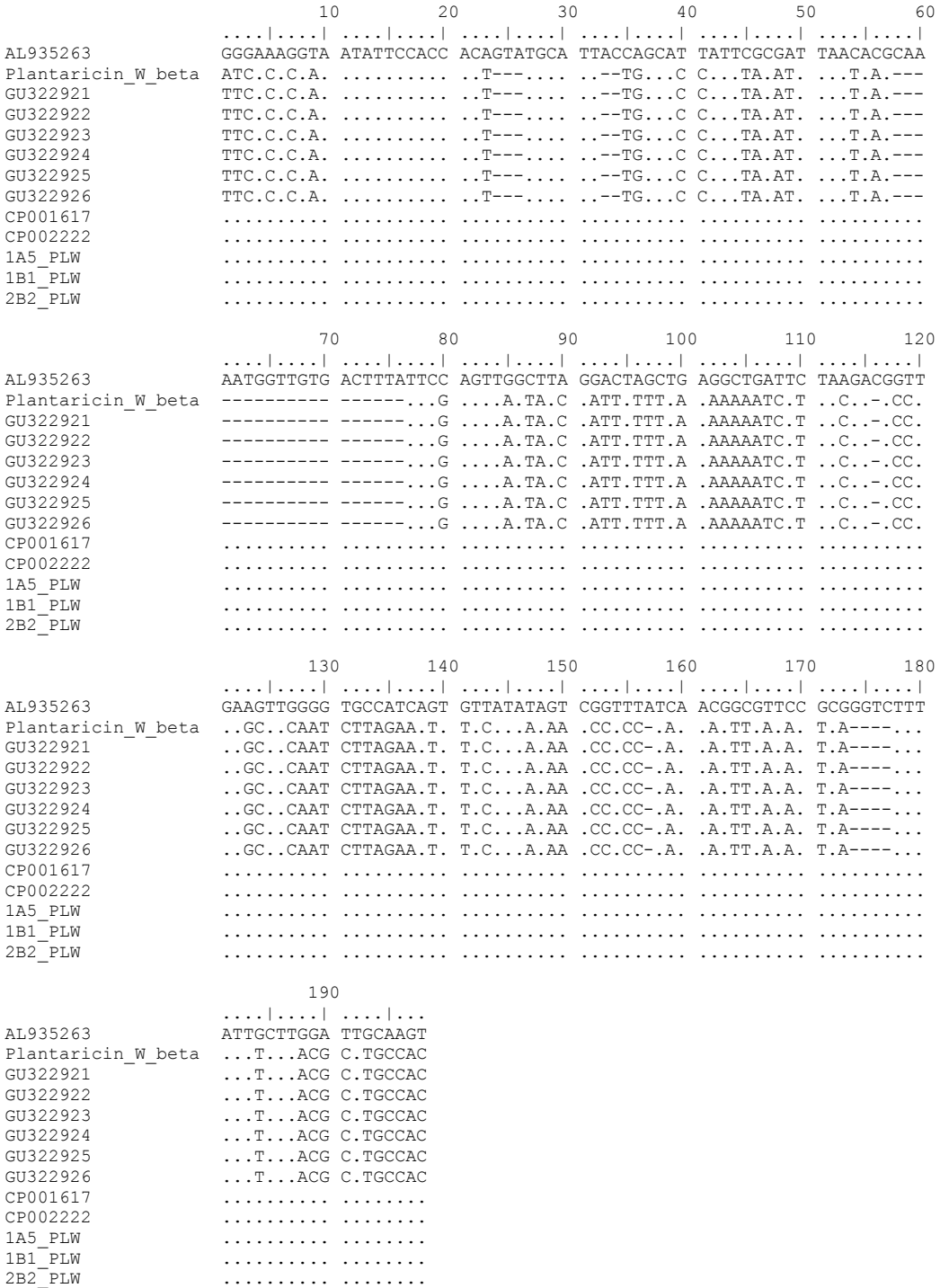

Figure 2. Alignment of plantaricins. AL935263 is NCBI accesion number of *L. plantarum* WCFS1, plantaricin W- beta from *L. plantarum* LMG 2379 (Holo *et al*., 2001); GU322926 is NCBI accesion number of *L. plantarum* strain UL4 isolated from Malaysian food; GU322922 is NCBI accesion number of *L. plantarum* strain TL1 isolated from Malaysian food; GU322923 is NCBI accesion number *L. plantarum* strain RG14 isolated from Malaysian food; GU322924 is NCBI accesion number of *L. plantarum* strain RG11 isolated from Malaysian food; GU322925 is NCBI accesion number of *L. plantarum* strain RI11 isolated from Malaysian food; GU322926 is NCBI accesion number of *L. plantarum* strain RS5 isolated from Malaysian food; CP001617 is NCBI accesion number of *L. plantarum* JDM1; CP002222 is NCBI accesion number of *L. plantarum* ST-III; 1A5, 1B1, and 2B2 were plantaricins produced by *L. plantarum* IIA-1A5; IIA-1B1 and IIA-2B2.

1B1 and IIA-2B2 with value of genetic distance was 0.54 (Table 1). It means that the similarity between plantaricin W gene sequences from *L. plantarum* IIA-1A5, IIA-1B1, and IIA-2B2 and plantaricin W beta from *L. plantarum* LMG 2379 and plantaricin W gene from Malaysian *L. plantarum* was low, only 46%. Plantaricin W gene from *L. plantarum* IIA-1A5, IIA-1B1 and IIA-2B2 were 100% identical to plantaricin W gene from *L. plantarum* WCFS1, *L. plantarum* ST III and *L. plantarum* JDM1. *L. plantarum* WCFS1 was isolated from human saliva, while *L. plantarum* ST III originally isolated from Kimchi (Wang *et al*., 2011) and *L. plantarum* JDM1 is Chinese commercial LAB with several probiotic functions (Zhang *et al*., 2009).

There were two types of plantaricin W. The first is plantaricin W which consists of two peptides plantaricin W alpha and plantaricin W beta, while the second is plantaricin W that has no plantaricin W alpha and beta but only consist of one peptides. Plantaricin W alpha and beta show significant sequence similarities with the corresponding peptides of two lantibiotic, namely lacticin 3147 (from *Lactococcus lactis*) and staphylococcin C55 (from *S. aureus*). Three plantaricins W belong to type A lantibiotic (class I bacteriocin) (Holo *et al*., 2001), while another plantaricins W belong to class II bacteriocin. The result showed that it might be different type of plantaricin W. Plantaricin W obtained from *L. plantarum* IIA-1A5, IIA-1B1 and IIA-2B2 might be included in class II bacteriocin that were in the difference cluster with plantaricin W beta (class I bacteriocin).

Dendogram of phylogenetic tree of plantaricin W-beta gene was shown in Figure 3. Plantaricin from Indonesian *L. plantarum* strains located in different cluster with plantaricin W- beta from *L. plantarum* LMG 2379 and plantaricin produced by *L. plantarum* isolated from Malaysian food. Not all strains of *L. plantarum* can reveale plantaricin W genes. The plantaricin locus is widespread among *L. plantarum* strains showed genetic diversity (Saenz *et al*., 2009). *L. plantarum* strains isolated from red wine from South Africa (Knoll *et al*., 2008) and *L. plantarum* strains isolated from fermented maize from Republic of Kongo (Ben Omar *et al*., 2008) did not reveal the presence of the plantaricin encoding plantaricin W alpha and plantaricin W beta genes.

Experiment 2. Purification and Characterization of Plantaricin

Purification of plantaricin. Plantaricin IIA-A5, IIA-1B1, and IIA-2B2 were succesfully purified from cell-free supernatant by ammonium sulfate precipitation and cation-exchange chromatography (Table 2, 3, and 4). The purification step could increase total protein, total activity of plantaricin, spesific activity except plantaricin IIA-1B1 and yield. Ammonium sulphate precipitation could not increase spesific activity of crude plantaricins IIA-1A5, IIA-1B1, and IIA-2B2. It might be because plantaricins were purified by ammonium sulphate precipitation were not in active site of peptides that had antimicrobial

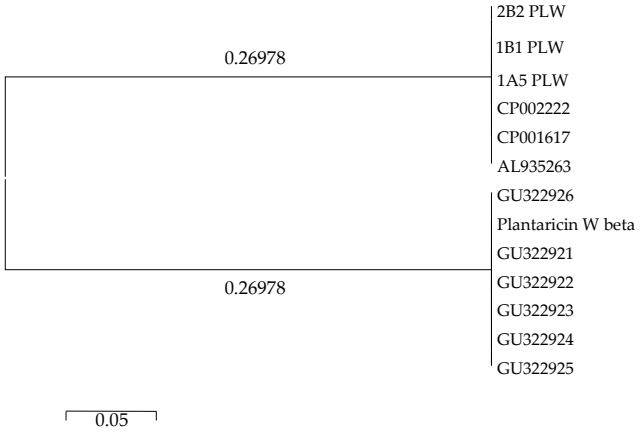

Figure 3. Phylogenetic three of plantaricin IIA-1A5, IIA-1B1, and IIA-2B2 genes compared with plantaricin from another strains deposited in GenBank

Table 1. Individual genetic distance of plantaricins

	AL935263	Plantaricin_W_beta	GU322921	GU322922	GU322923	GU322924	GU322925	GU322926	CP001617	CP002222	1A5_PLW	1B1_PLW	2B2_PLW
AL935263	*												
Plantaricin_W_beta	0.54	*											
GU322921	0.54	0.00	*										
GU322922	0.54	0.00	0.00	*									
GU322923	0.54	0.00	0.00	0.00	*								
GU322924	0.54	0.00	0.00	0.00	0.00	*							
GU322925	0.54	0.00	0.00	0.00	0.00	0.00	*						
GU322926	0.54	0.00	0.00	0.00	0.00	0.00	0.00	*					
CP001617	0.00	0.54	0.54	0.54	0.54	0.54	0.54	0.54	*				
CP002222	0.00	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.00	*			
1A5_PLW	0.00	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.00	0.00	*		
1B1_PLW	0.00	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.00	0.00	0.00	*	
1B2_PLW	0.00	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.00	0.00	0.00	0.00	*

Table 2. Purification of plantaricin IIA-1A5

Purification stage	Volume (mL)	Concentration (μg/mL)	Total protein (mg)	Total activity (AU)	Spesific activity (AU/mg)	Purification (%)
Cell free supernatant	500.00	138.00	69.00	582.51	8.44	100.00
Ammonium sulfate precipitation	10.00	143,853.00	1,438.53	808.92	0.56	138.87
Cation exchange chromatography	3.00	45,292.50	135.88	1,821.20	13.40	312.65

Table 3. Purification of plantaricin IIA-1B1

Purification stage	Volume (mL)	Concentration (μg/mL)	Total protein (mg)	Total activity (AU)	Spesific activity (AU/mg)	Purification (%)
Cell free supernatant	500.00	173.00	86.50	504.63	5.83	100.00
Ammonium sulfate precipitation	8.93	178,421.00	1593.30	684.11	0.43	135.57
Cation exchange chromatography	3.00	97,900.00	293.70	1502.38	5.12	297.72

Table 4. Purification of plantaricin IIA-2B2

Purification stage	Volume (mL)	Concentration (μg/mL)	Total protein (mg)	Total activity (AU)	Spesific activity (AU/mg)	Purification (%)
Cell free supernatant	500.00	238.00	119.00	673.95	5.66	100.00
Ammonium sulfate precipitation	7.00	117,118.00	819.83	814.97	0.99	120.92
Cation exchange chromatography	3.00	40,105.00	120.315	926.55	7.70	137.48

activities. Bacteriocin is peptides which can have antimicrobial activities in spesific active site. Positively charged C-terminus of bacteriocin is important for initial binding and antimicrobial activity (Bauer *et al*., 2005). Ammonium sulphate bind all protein from cell free supernatan, that still consist of protein from media (MRS broth), not only bacteriocin. Interaction protein-peptides of ammonium sulphate precipitation could reduce antimicrobial acitivites. For the purpose of getting pure plantaricins, purification of bacteriocin can not be achieved only by ammonium sulphate precipitation, but must be continued by cation exchange chromatography.

The experiments used SP Sepharose™ Fast Flow, a strong cation exchanger, as resin of column chromatography. The ion exchange group is a sulphopropyl group which remains charged and maintains consistently high capacities over the entire working range, pH 4–13. Purification step by cation exchange has been proven to purify many plantaricins succesfully, otherwise many research need another column chromatography technique such as gel filtration continued by HPLC (Gong *et al*., 2010). It was similar with plantaricin C19 (Atrih *et al*. 2001) and plantaricin ASM1 (Hata *et al*., 2010) that could be completely purified by chromatography technique.

In this research, cation exchange chromatoghraphy purification could increase spesific activity of the plantaricins except plantaricin IIA-1B1. Plantaricin IIA-1A5 had higher concentration of protein than plantaricin ASM1 and plantaricin LR14 (Table 2). Concentration of plantaricin IIA-1B1 and IIA-2B2 after cation exchange chromatography purification (Table 3 and 4) were lower than plantaricin ASM1 that had 17500 μg/mL (Hata *et al*., 2010), but higher than plantaricin LR14 that had 59.21 μg/mL (Tiwari & Srivastava, 2008). The type of plantaricins were different might be caused by differences of *L. plantarum* strains. Functional characteristics of antibacterial activity of plantaricin produced by *L. plantarum* depend on strains (Saenz *et al*., 2009).

Bacteriocin activity. Plantaricin IIA-1A5, IIA-1B1, and IIA-2B2 showed a broad antibacterial spectrum against pathogenic tested strains consisted of Gram positive and Gram negative bacteria (Table 5, 6). This result indicated that all plantaricins were effective against the most problematic food-borne pathogenic bacteria. Based on Gillor *et al* (2008), the general bacteriocins mode of action have been reported to be bactericidal due to its pore-forming action on the cell membrane.

Antimicrobial properties of all type plantaricin IIA produced by *L. plantarum* IIA-1A5, IIA-1B1, and IIA-2B2 differed significantly and affected by strains of pathogenic bacteria (Table 5). Plantaricin IIA-1A5 had

Table 5. Diameter of inhibition zone of plantaricins to pathogenic bacteria (mm)

Type of plantaricin	Indicator strains (pathogenic bacteria)			
	E. coli ATCC 25922	*B. cereus*	*S. typhimurium* ATCC 14028	*S. aureus* ATCC 25923
IIA/1A5	9.59±0.68^{a}	9.91±0.70^{a}	10.75±0.45^{c}	9.78±1.07^{ac}
IIA/1B1	8.51±1.08^{ab}	8.17±0.21^{b}	8.73±0.49^{ab}	8.35±0.37^{ab}
IIA/2B2	9.24±1.19^{ab}	9.17±1.60^{ab}	9.16±1.72^{abc}	9.09±1.03^{a}

Note: means in rows and column with different superscript differ significantly (P<0.05).

Table 6. Minimum inhibitory concentration of plantaricins against pathogenic bacteria (%)

Producer strains	Indicator strains (pathogenic bacteria)			
	E. coli ATCC 25922	*B. cereus*	*S. typhimurium* ATCC 14028	*S. aureus* ATCC 25923
L. plantarum IIA-1A5	93.33±2.89^{a}	88.33±7.64^{ab}	93.00±5.20^{a}	80.00±0.00^{c}
L. plantarum IIA-1B1	91.67±2.89^{a}	94.00±3.61^{a}	94.00±3.61^{a}	85.00±8.66^{abc}
L. plantarum IIA-2B2	92.33±4.04^{a}	94.67±4.04^{a}	94.67±4.04^{a}	86.67±5.77^{ab}

Note: means in rows and column with different superscript differ significantly (P<0.05).

higher antimicrobial activity againts *S. typhimurium* than *E. coli* and *B. cereus*. Generally, antimicrobial activities of plantaricin IIA-1A5 were higher than plantaricin IIA-1B1, but similar with plantaricin IIA-2B2. The result indicated that antimicrobial activities of plantaricins were spesific depend on strain of *L. plantarum* and strains of pathogenic bacteria. Gong *et al.* (2010) reported that plantaricin MG produced by *L. plantarum* KLDS.0391 isolated from "Jiaoke" a fermented cream from China had broad spectrum of antimicrobial activities againts a number of Gram positive and Gram negative bacteria. Ben-Omar *et al.* (2008) stated that plantaricins produced by *L. plantarum* isolated from poto-poto had narrow spectrum, only on Gram positive bacteria. Ben-Omar *et al.* (2006) reported that *L. plantarum* strains from ben saalga, a traditional fermented gruel from Burkino Faso had broad bateriocinogenic.

Minimun inhibitory concentration of plantaricins from Indonesian *L. plantarum* were affected by interaction beetwen strains of *L. plantarum* and pathogenic bacteria strains (Table 6). Gram positive pathogenic bacteria *S. aureus* were more sensitive than Gram negative pathogenic bacteria such as *E. coli* and *S. typhimurium* by the action of plantaricin IIA-1A5, IIA-1B1, and IIA-2B2. Plantaricin from *L. plantarum* IIA-1A5 had higher activity against *S. aureus*. It is related to Thomas *et al.* (2000) that most bacteriocins have limited spectrum of activity, generally effective only against bacteria related to the producer organism. Bacteriocins produced by Gram positive bacteria such as *Lactobacillus* species are normally more active against Gram positive than Gram negative bacteria, yeast and mold.

The general mode of action of bacteriocin has been reported to be bactericidal due to its pore-forming action on the cell membrane making it leaky (Tiwari & Srivastava, 2008). Most of the Gram positive bacteriocins are membrane active compounds that increase the permeability of the cytoplasmic membrane (Savadogo *et al.*, 2006). According to Asaduzzaman & Sonomoto (2009), the two most well-established mechanisms of pore formation by bacteriocin were the barrel-stave and wedge models. In the barrel-stave mechanism, the cationic bacteriocin monomer bind to the membrane surface through electrostatic interactions and are assembled into a pre-aggregrate, and the pores are formed at a certain membrane potential, where the bacteriocin is perpendicular to the membrane. In the case of wedge model, surface bound bacteriocin molecules bind parallel to the membrane surface and generate local strain, bending the membrane in such a way that the lipid moleculs, together with the bacteriocin, form a pore.

Stability against proteolytic enzyme and heat treatments. Since bacteriocins are defined as proteinaceous substances, they must be sensitive to at least one proteolytic enzymes (Moreno *et al.*, 2000). The activity unit of plantaricin IIA-1A5, IIA-1B1, and IIA-2B2 were significantly reduced by the trypsin (Table 7).

Partial inactivation acitivities were observed after treatment of plantaricins with trypsin. The reduction of plantaricins activity unit as the effect of trypsin ranged from 33.5 %-66.02% (Table 7). Trypsin could degrade plantaricins. These results indicated that plantaricin produced by *L. plantarum* IIA-1A5, IIA-1B1, and IIA-2B2 strains were proteinaceous in nature based on its sensitivity to tripsyn enzyme. Plantaricins could be abolised by trypsinization. It similar to most plantaricins produced by other *L. plantarum* strains such as bacteriocins produced by *L. plantarum* isolated from Tunisian traditional salted meat (Essid *et al.*, 2009); plantaricin ASM1 (Hata *et al.*, 2010); plantaricin W (Holo *et al.*, 2001) and bacteriocins from other strains such as bacteriocins from *Streptococcus bovis* J2 40-2 (Rashid *et al.*, 2009) and from *Fusobacterium nucleatum* (Riberio-Ribas *et al.*, 2009).

Trypsin is a natural proteloytic in gastrointestinal tract of human. Based on this data that plantaricin IIA-

Table 7. Sensitivity of bacteriocins produced by Indonesian lactic acid bacteria *L. plantarum* strains to treatment with proteolytic enzyme

Type of plantaricin	Enzyme treatment	Indicator pathogenic strains			
		E. coli ATCC 25922	*B. cereus*	*S. typhimurium* ATCC 14028	*S. aureus* ATCC 25923
IIA/1A5	Control	1,650.5±67.4[a]	1,251.5±46.5[c]	1,386.6±98.4[e]	943.9±57.9[g]
	Trypsin	740.1±72.6[b]	699.4±51.5[d]	342.5±40.5[f]	258.6±24.7[h]
IIA/1B1	Control	864.0±80.1[i]	975.8±40.7[k]	1,207.9±50.7[m]	821.0±33.4[o]
	Trypsin	343.6±10.3[j]	560.4±51.4[l]	520.2±58.6[m]	392.3±21.2[p]
IIA/2B2	Control	979.2±99.4[q]	1,004.6±43.7[s]	823.8±72.0[o]	783.9±33.7[x]
	Trypsin	496.5±83.1[r]	695.9±35.4[t]	510.7±63.7[p]	225.5±19.9[y]

Note: Statistic analysis every type of plantaricin and every pathogenic bacteria strains (compared between control and trypsin), means with different superscript differ significantly (P<0.05) for every type of plantaricin and pathogenic bacteria strain. Results were expressed in activity unit (mm^2/mL).

1A5, IIA-1B1, and IIA-2B2 could be degraded by trypsin, it also indicated that the plantaricins might be safe for human consumption as food biopreservative.

The stability of antimicrobial activities of plantaricins after heating treatments against *E. coli* ATCC 25922 is shown in Table 8. Stability of plantaricin activities were influenced significantly by interaction between type of plantaricin and heating treatments towards antimicrobial activities against *E. coli* ATCC 25922 (P<0.05). Plantaricin IIA/1B1 had the lowest antimicrobial activity expressed by lowest diamater of inhibitory zone against *E. coli* ATCC 25922. While, plantaricin IIA-1A5 had the best stability on heating treatments. Plantaricin is categorized in heat stable class II bacteriocins. Another type of plantaricin is categorized in class I bacteriocin, that is also heat stable. Plantaricin W from *L. plantarum* LMG 2379 was heat stable, categorized as lantibiotic, similar with Nisin as class I bacteriocin (Holo *et. al*., 2001). Sterilization could decrease plantaricin activity as antimicrobes. High temperature will denature protein, and conformation of protein will be canged and causing its inactivation.

The stability of inhibitory activity of plantaricins after obtaining different heating treatment against *B. cereus* can be seen in Table 9. Stability plantaricin activities after pasteurization and sterilization treatments against indicator bacteria *B. cereus* were affected by the type of plantaricin (P<0.05). Plantaricin IIA-1B1 had the lowest antimicrobial activity agains *B. cereus*. Plantaricin IIA-/1A5 and IIA-2B2 were heat stable as antimicrobial against *B. cereus.*

The observation results of plantaricin stability activity after a heating treatment against *Salmonella enteriditis* ser. Typhimurium ATCC 14028 can be seen in Table 9.

Table 8. Diameter of inhibition zone from plantaricin IIA-1A5, IIA-1B1, and IIA-2B2 by heating treatment against *E. coli* ATCC 25922 (mm)

Type of plantaricin	Heat treatments		
	Control	Pasteurization 80 °C, 30′	Sterilization 121 °C, 15′
IIA-1A5	9.59±0.68[a]	9.09±0.79[a]	9.30±1.23[a]
IIA-1B1	8.51±1.08[ab]	8.17±0.83[ab]	7.95±0.25[b]
IIA-2B2	9.24±1.19[ab]	8.49±1.85[ab]	8.65±1.42[ab]

Note: means in rows and column with different superscript differ significantly (P<0.05).

Table 9. Diameter of inhibition zone from plantaricin IIA-1A5, IIA-1B1, and IIA-2B2 by heating treatment against *B. cereus* (mm)

Type of plantaricin	Heat treatments		
	Control	Pasteurization 80 °C, 30′	Sterilization 121 °C, 15′
IIA-1A5	9.91±0.70[a]	9.29±1.57[ab]	8.65±1.40[ab]
IIA-1B1	8.17±0.21[ab]	9.03±0.87[ab]	7.93±0.22[b]
IIA-2B2	9.17±1.60[ab]	8.68±1.58[ab]	8.91±1.82[ab]

Note: means in rows and column with different superscript differ significantly (P<0.05).

Table 10. Diameter of inhibition zone from plantaricin IIA-1A5, IIA-1B1, and IIA-2B2 by heating treatment against *S. typhimurium* ATCC 14028 (mm)

Type of plantaricin	Heat treatments		
	Control	Pasteurization 80 °C, 30′	Sterilization 121 °C, 15′
IIA-1A5	10.75±0.45[a]	8.77±1.49[b]	9.21±1.50[ab]
IIA-1B1	8.73±0.49[b]	7.92±0.60[b]	8.36±0.64[b]
IIA-2B2	9.16±1.72[ab]	8.04±0.55[b]	9.06±1.35[ab]

Note: means in rows and column with different superscript differ significantly (P<0.05).

Table 11. Diameter of inhibition zone from plantaricin IIA-1A5, IIA-1B1, and IIA-2B2 by heating treatment against *S. aureus* ATCC 25923 (mm)

Type of plantaricin	Heat treatments			Average
	Control	Pasteurization 80 °C, 30′	Sterilization 121 °C, 15′	
IIA-1A5	9.78±1.07	9.69±0.73	9.91±1.18	9.79±0.88
IIA-1B1	8.35±0.37	8.07±0.34	8.74±2.23	8.39±1.18
IIA-2B2	9.09±1.03	8.93±1.88	9.28±1.88	9.10±1.44
Average	9.42±1.10	9.29±1.43	9.50±1.49	

Note: means in rows and column with different superscript differ significantly (P<0.05).

Antimicrobial activity of plantaricin IIA-1A5 and IIA-2B2 againts *S. typhimurium* ATCC 14028 were effected by heating treatments, but plantaricin IIA-1B1 was not effected. Pasteurization temperature decreased significantly antimicrobial activity of plantaricin IIA-1A5 and plantaricin IIA-2B2 againts *S. typhimurium* ATCC 14028 (Table 10). Plantaricin IIA-1B1 had lower activity than plantaricin IIA-1A5 and IIA-2B2.

Plantaricin IIA-1A5, IIA-1B1 and IIA-2B2 had stable properties on heating, by its ability to maintain inhibitory activity against *S. aureus* ATCC 25923 after heating at pasteurization or sterilization temperature (Table 11). Heat stability was the characteristic of almost all of the identified plantaricin, IIA-1A5, IIA-1B1, and IIA-2B2. The result had similarities with other plantaricins. Plantaricin ASM1 which was heat stable at 90 °C for 15 min (Hata *et al*., 2010), and plantaricin W as lantibiotic that was strongly heat stable (Holo *et al*., 2001).

CONCLUSION

L plantarum IIA-1A5, IIA-1B1, and IIA-2B2 reveal the presence of the plantaricin encoding plantaricin W gene. Plantaricin IIA-1A5, IIA-1B1, and IIA-2B2 have broad spectrum antimicrobial activities, againts Gram positive and Gram negative pathogenic bacteria, such as *E. coli,* S. *typhimurium*, *B cereus,* and *S. aureus*. All plantaricins are digested by trypsin and heat stable relatively. Generally, plantaricin IIA-1A5 is better as antimicrobial agent than plantaricin IIA-1B1 and plantaricin IIA-2B2.

ACKNOWLEDGEMENT

This study was financially supported by Indonesia Toray Science Foundation (ITSF) through Science and Technology Research Grant 2010/2011.

REFERENCES

Arief, I. I., B. S. L. Jenie, M. Astawan, & A. B. Witarto. 2010. Efektivitas probiotik *Lactobacillus plantarum* 2C12 dan *Lactobacillus acidophilus* 2B4 sebagai pencegah diare pada tikus percobaan. Med. Pet. 33:137-143. http://dx.doi.org/10.5398/medpet.2010.33.3.137

Arief, I. I. 2011. Characterization of Indigenous Lactic Acid Bacteria from Beef as Probiotic and Identification by 16S rRNA gene sequencing. Ph.D. Thesis. Bogor Agricultural University, Indonesia.

Atrih, A., N. Rekhif, A. J. G Moir, A. Lebrihi, & G. Lefebrve. 2001. Mode of action, pourification and amino acid sequence of plantaricin C19, an anti_listeria bacteriocin produced by Lactobacillus plantarum C19. Int. J. Food Microbiol. 68: 93-104. http://dx.doi.org/10.1016/S0168-1605(01)00482-2

Asaduzzaman, S. M. & K. Sonomoto. 2009. Lantibiotics: Diverse activities and unique modes of action. J. Biosci. & Bioeng. 107: 475-487. http://dx.doi.org/10.1016/j.jbiosc.2009.01.003

Bauer R, M. L. Chikindas, & L. M. T. Dicks. 2005. Purification, partial amino acid sequence and mode of action of pediocin PD-1, a bacteriocin produced by Pediococcus damnosus NCFB 1832. Int. J. Food Microbiol. 101: 17–27. http://dx.doi.org/10.1016/j.ijfoodmicro.2004.10.040

Ben-Omar, N., H. Abriouel, R. Lucas, M. Martinez-Canamero, J. P. Guyot, & A. Galvez. 2006. Isolation of bacteriocinogenic *Lactobacillus plantarum* strains from ben saalga, a traditional fermented gruel from Burkina Faso. Int. J. Food Microbiol. 112: 44-50. http://dx.doi.org/10.1016/j.ijfoodmicro.2006.06.014

Ben-Omar, N., H. Abriouel, S. Keleke, A. S.Valenzuela, M. Martínez-Ca-amero, R. L. López, E. Ortega, & A. Gálvez. 2008. Bacteriocin-producing Lactobacillus strains isolated from poto poto, a Congolese fermented maize product, and genetic fingerprinting of their plantaricin operons. Int. J. Food Microbiol. 127: 18-25. http://dx.doi.org/10.1016/j.ijfoodmicro.2008.05.037

Diep, D. B., D. Straume, M. Kjos, C. Torres, & I. F. Nes. 2009. An overview of the mosaic plantaricin pln loci

from Lactobacillus plantarum. Peptides 30: 1562-1574. http://dx.doi.org/10.1016/j.peptides.2009.05.014

Essid, I., M. Medini, & M. Hassouna. 2009. Technological and safety properties of *Lactobacillus plantarum* strains isolated from a Tunisian traditional salted meat. Meat Sci. 81: 203-208. http://dx.doi.org/10.1016/j.meatsci.2008.07.020

Gillor, O., A. Etzion, & M.A. Riley. 2008. The dual role of bacteriocins as anti-and probiotics. Appl. Microbiol. Biotechnol. 81: 591-606. http://dx.doi.org/10.1007/s00253-008-1726-5

Gong, H. S., X. C. Meng, & H. Wang. 2010. Plantaricin MG active against Gram-negative bacteria produced by Lactobacillus plantarum KLDS1.0391 isolated from ''Jiaoke", a traditional fermented cream from China. Food Control. 21: 89-96. http://dx.doi.org/10.1016/j.foodcont.2009.04.005

Hata, T., R. Tanaka, & S. Ohmomo. 2010. Isolation and characterization of plantaricin ASM 1: a new bacteriocin produced by Lactobacillus plantarum A-1. Int. J. Food Microbiol. 137: 94-99. http://dx.doi.org/10.1016/j.ijfoodmicro.2009.10.021

Holo, H., Z. Jeknic, M. Daeschel, S. Stevanovic, & I. F. Nes. 2001. Plantaricin W from *Lactobacillus plantarum* belongs to a new family of two–peptide lantibiotics. Microbiol. 147: 643-651

Knoll, C., B. Divol, & M. Toit. 2008. Genetic screening of lactic acid bacteria of oenological origin for bacteriocin-encoding genes. Food Microbiol. 25: 983-991. http://dx.doi.org/10.1016/j.fm.2008.06.010

Moreno, I., A. L. S. Lerayer, V. L. S. Baldini, & F. Mauro de F. Leitão. 2000. Characterization of bacteriocins produced by *Lactococcus lactis* strains. Brazillian J. Microbiol. 31:184-192.

Rashid, Md. H., K. Togo, M.Ueda, & T. Miyamoto. 2009. Characterization of bacteriocin produced by *Streptococcus bovis* J2 40-2 isolated from traditional fermented milk "Dahi". Anim. Sci. J. 80: 70-80. http://dx.doi.org/10.1111/j.1740-0929.2008.00596.x

Riberio-Ribas, R. N., M. A. R. de Carvalho, C. A. Vieira, A. C. M. Apolonia, P. P. Magalhaes, E. N. Mendes, J. S. Oliveira, M. M. Santoro, & L. de M. Fariaz. 2009. Purification and partial characterization of a bacteriocin produced by an oral Fusobacterium nucleatum isolate. J. Appl. Microbiol. 107: 699-705. http://dx.doi.org/10.1111/j.1365-2672.2009.04250.x

Sáenz, Y., B. Rojo-Bezares, L. Navarro, L. Díez, S Somalo, M Zarazaga, F. Ruiz-Larrea, & C. Torres. 2009. Genetic diversity of the *pln* locus among oenological *Lactobacillus plantarum* strains. Int. J. Food Microbiol. 134:176-183. http://dx.doi.org/10.1016/j.ijfoodmicro.2009.06.004

Sambrook, J., E. F. Fritsch, & T. Maniats. 1989. Molecular cloning: a laboratory manual. CSH Laboratory Press, USA.

Savadogo, A., C. A. T. Outtara, I. H. N. Bassole, & A. S. Traore. 2006. Bacteriocins and lactic acid bacteria – a minireview. Afr. J Biotechnol. 5: 678-683.

Steel, R. G. D. & J. H. Torrie. 1995. Principles and Procedures of Statistic a Biomedical Approach. 3rd Ed. McGraw Hill, Inc., Singapore.

Thomas, L.V, M. R. Clarkson, & J. Delves-Broughton. 2000. Nisin. In: Naidu (Ed). Natural Food Antimicrobial Systems. CRC Press LLC, USA.

Tiwari, S. K. & S. Srivastava. 2008. Purification and characterization of plantaricin LR14: a novel bacteriocin produced by *Lactobacillus plantarum* LR/14. Appl. Microbiol. Biotechnol. 79: 759-767. http://dx.doi.org/10.1007/s00253-008-1482-6

Todorov, S. D., P. Hob, M. Vaz-Velho, & L. M. T. Dicks. 2010. Characterization of bacteriocins produced by two strains of *Lactobacillus plantarum* isolated from Beloura and Chouriço, traditional pork products from Portugal. Meat Sci. 84 : 334-343. http://dx.doi.org/10.1016/j.meatsci.2009.08.053

Wang, Y., C. Chen, L. Ai, F. Zhou, Z. Zhou, L. Wang, H. Zhang, W. Chen, & B. Guo. 2011. Complete genome sequence of the probiotic *Lactobacillus plantarum* ST-III. J. Bacteriol. 193: 313. http://dx.doi.org/10.1128/JB.01159-10

Zhang, Z., C. Liu, Y. Zhu, Y. Zhong, Y. Zhu, H. Zheng, G. Zhao, S. Wang, & X. Guo. 2009. Complete genome sequence of *Lactobacillus plantarum* JDM1. J. Bacteriol. 191:5020. http://dx.doi.org/10.1128/JB.00587-09