KMITL Sci. Tech. J. Vol.16 No.2 Jul.-Dec. 2016

Bacteriocins from *Lactobacillus* strains and their anti-*Listeria monocytogenes* in Nham, Thai Fermented Pork

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Abstract

In this study, two bacteriocin-producing *Lactobacillus* strains (the isolates LS0602 and LT0904) isolated from Thai fermented pork products were identified by 16S rDNA sequence analysis. The isolate LS0602 was found to be *Lactobacillus plantarum*, whereas the isolate LT0904 was *Lactobacillus brevis*. Their bacteriocins were partially purified by ammonium sulfate precipitation, dialysis and trichloroacetic acid precipitation. Bacteriocin activity was determined by agar well diffusion assay and critical dilution method against *Listeria monocytogenes*. The partial purified bacteriocins from *L. plantarum* LS0602 and *L. brevis* LT0904 had strong inhibitory activity against the growth of *L. monocytogenes* at 819,200 AU/ml and 409,600 AU/ml, respectively. These bacteriocins were used to control the growth of *L. monocytogenes* in Nham during fermentation at 30°C. The partial purified bacteriocins (4 %) in combination with *L. plantarum* LS0602 starter culture could reduce the number of total presumptive *Listeria* counts in Nham by 1.5 log unit after 3-day fermentation.

Keywords: Lactic acid bacteria, bacteriocins, fermented meat, starter culture

1. Introduction

Bacteriocins, antimicrobial peptides are synthesized ribosomally by different bacterial strains. Some lactic acid bacteria (LAB) such as *Lactococcus lactis*, *Lactobacillus plantarum*, and others can produce bacteriocins with broad spectrum of antimicrobial activity against food-borne pathogenic bacteria. The bacteriocins are generally recognized as safe substances (non-toxic to the cells), and suitable for food preservation due to their desirable properties, e.g. heat and pH tolerance. They are inactivated by digestive protease, thus having little effect on gut microflora [1]. Food can be supplemented with partial purified bacteriocins or bacteriocin-producing organisms under optimum conditions that favour the bacteriocin production.

Listeria monocytogenes, a Gram-positive rod bacterium causes listeriosis. Meningitis and sepsis are most commonly recognized symptoms when the individuals contract the disease. Cooked and cured meat products, and fermented sausages have been contaminated with *L. monocytogenes* [2]. Nham, a Thai fermented pork product normally produces under non-sterile conditions. This product is exposed to the risk of contamination by undesirable microorganisms

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including *L. monocytogenes*. This pathogenic bacterium is frequently found in nham [3]. Therefore, this suggests the need to control the growth of *L. monocytogenes* in nham. In recent years, bacteriocins or bacteriocin-producing LAB were reported to be effective against *L. monocytogenes* in raw meat and meat products such as dry fermented sausage [4]. This research is aimed to study the effect of bacteriocins and bacteriocin-producing *Lactobacillus* on controlling the growth of *L. monocytogenes* in Nham.

2. Materials and Methods

2.1 Bacterial strains and culture condition

Two *Lactobacillus* strains used in this experiments were the isolates LS0602 and LT0904, isolated from Nham and Saigrork Esan, respectively. They have been presumptively identified by morphological characterization and biochemical tests as *Lactobacillus* [5]. These two LAB strains produced bacteriocins inhibiting the growth of *L. monocytogenes* [6]. The isolate LS0602 was cultivated at 37°C for 24 h in MRS broth (de Man Rogosa and Sharpe medium, Difco Laboratories, USA) supplemented with 30 g/l glucose, 10 g/l tryptone and 5 g/l K_2HPO_4, while the isolate LT0904 was cultivated at 37°C for 24 h in MRS broth supplemented with 40 g/l glucose, 10 g/l tryptone, 20 g/l yeast extract and 2 g/l KH_2PO_4 to obtain strongest bacteriocin activity [6]. *L. monocytogenes* DMST 11256 obtained from the culture collection of the Department of Medical Science, Ministry of Public Health, Thailand was used as an indicator strain. This bacterium was cultivated in Tryptic soy broth (TSB, Difco Laboratories, USA) at 37°C for 24 h.

2.2 Molecular identification by 16S rDNA sequencing

Genomic DNA of the isolates was extracted by using MasturePure™ Gram positive DNA purification kit (EPICENTRE®, USA.). The 16S rDNA gene was amplified by polymerase chain reaction using primers F16SrDNA-27bac (5´-AGAGTTTGATCCTGGCTCAG-3´) and R16SRDNA-1492bac (5´-GGTTACCTTGTTACGACTT-3´). PCR reaction and PCR condition were done as previously described [7]. PCR product with an approximate size of 1,500 bp was purified with QIAquick PCR purification kit (Qiagen, Germany), ligated to TA-cloning vector (QIAGEN PCR Cloning kit, Germany) and transformed into the competent cells *E. coli* DH5α. Plasmid DNA was isolated from the transformants by QIAprep® Spin Miniprep kit (Qiagen, Germany) and was then sequenced by Big-dye terminator reaction using ABI PRISM®3700 DNA analyzer (First Base Laboratories SdnBhd, Malaysia). Nucleotide sequence was analyzed using the BLAST server of National Center Biotechnology Information, National Institutes of Health, USA [8].

2.3 Partial purification of bacteriocins

The cell-free culture supernatant of each LAB strain was partially purified by ammonium sulfate precipitation, dialysis and trichloroacetic acid (TCA) precipitation. Briefly, the culture supernatant was precipitated with ammonium sulfate to 60 % saturation and stirred at 4 °C. Then, the mixture was centrifuged at 8,000 ×g for 30 min (4°C). The precipitate was resuspended in 0.05 M potassium phosphate buffer (pH 7.0, 50 ml). Then, the suspension was dialyzed in tubular cellulose membrane (Spectrapor, 1,000 dalton MWCO, Fisher Scientific, Pittsburgh, PA, USA) against 0.05 M potassium phosphate buffer (pH 7.0, 5 litres) for 24 h. After dialysis, the dialysate (30 ml) was precipitated with 5% TCA [9]. The mixture was centrifuged at 8,000 ×g for 30 min (4°C). The pellet was dissolved in potassium phosphate buffer (2 ml). These suspensions were analyzed for bacteriocin activity and protein content [10].

2.4 Bacteriocin activity assay

Bacteriocin activity was determined by agar well diffusion assay [11] and critical dilution method [12]. Briefly, the overnight culture of each *Lactobacillus* strain was centrifuged at 8,000 ×g, 4°C for 30 min. The pH of cell-free supernatant was adjusted to 6.5 and filtered through a 0.2 µm-pore-size membrane filter. Then, this supernatant was treated with catalase (5 mg/ml, C30 Sigma-Aldrich, Germany) and two-fold serially diluted using MRS broth. To perform this assay, TSA plus 0.6% yeast extract (TSAye) was overlaid with soft TSAye agar (7 ml) inoculated with *L. monocytogenes* cell suspension (10^8 CFU/ml, 0.1 ml). Agar wells (6 mm diameter) were cut. The supernatant (40 µl) at each dilution ($1:2^1$ to $1:2^{18}$) was placed into each well. The plates were incubated at 37 °C for 48 h, and examined for the inhibition zone. The bacteriocin activity was expressed as arbitary units (AU/ml). It is proportional to the reciprocal of the highest dilution factor, producing zone of inhibition (DFi). The bacteriocin activity was calculated as follows:

bacteriocin activity (AU/ml) = 1/DFi × 1000/volume spotted in µl

2.5 Effect of bacteriocin-producing starter cultures and their partial purified bacteriocins on controlling of *Listeria monocytogenes* in Nham

Nham was prepared from pork (70.3 %, lean), cooked pork rind (17.6 %), garlic (4.4 %), salt (2.0 %), sugar (0.4 %), monosodium glutamate (0.2 %) and cooked rice (5.1 %). The pork was minced and mixed with all ingredients using a decontaminated bowl mixer (KitchenAid modek 5K5SS, Large Appliances, Benton Harbor, MI, USA). Then, Nham batter was divided into six parts for six treatments: a negative control treatment (no bacteriocins or starter added) and the other five treatments added with either one of 1) 50 mg/kg nisin (a positive control treatment), 2) 10^5CFU/g *L. plantarum* LS0602 starter, 3) 4 % bacteriocins from *L. plantarum* LS0602 plus 10^5 CFU/g *L. plantarum* LS0602 starter, 4) 10^5 CFU/g *L. brevis* LT0904 starter, or 5) 4 % bacteriocins from *L. brevis* LT0904 plus 10^5 CFU/g *L. brevis* LT0904 starter. After mixing, all samples were inoculated with *L. monocytogenes* (10^5 CFU/g), tightly packed in sterile plastic bags, and incubated at 30 °C for 3 days. Total LAB counts, total presumptive *Listeria* counts (TPLC), and pH value in all samples were determined at 1-day interval. The remaining bacteriocin activity in all samples was analyzed at the end of fermentation using the method as described by Garriga *et al.* [13]. The experiments were done in triplicate.

2.6 Statistical analysis

The data of three replications were analyzed using analysis variance (ANOVA) and Duncan 's multiple range test to determine if significant difference ($P \leq 0.05$) existed between the treatment means using SPSS version 11.5 statistical package (SPSS Inc; Chicago IL, USA).

3. Results and Discussion

The isolate LS0602 was molecular identified by 16S rDNA sequence analysis as *Lactobacillus plantarum* (99% similarity to *L. plantarum*). The isolate LT0904 could be identified as *Lactobacillus brevis* (99% similarity to *L. brevis*). The partial purified bacteriocins produced by *L. plantarum* LS0602 and *L. brevis* LT0904 had the highest inhibitory activity against *L. monocytogenes* at 819,200 AU/ml and 409,600 AU/ml, respectively (Table 1). These results corresponded to the specific activity of bacteriocins from *L. plantarum* LS0602 which increased from 80.31 AU/µg in cell-free supernatant to 1,866.06 AU/µg in the partial purified bacteriocins. Similarly, the specific activity of bacteriocins of *L. brevis* LT0904 also increased from 42.67 AU/µg in cell-free supernatant to 1,170.29 AU/µg in the partial purified bacteriocins. The reason for testing the bacteriocin activity against only *L. monocytogenes* was due to their strongest

inhibitory activity against this bacterium which was found during our previous investigation. The increase of bacteriocin activity after the purification steps may be due to increasing bacteriocin concentration. Induction mechanism of protein precipitation with TCA relates to negative charged ions of TCA, resulting in stability of native protein conformation. Partial unfolding of proteins results in exposure of the non-polar surface with a solvent, thereby combining of protein molecules. This leads to the precipitation of the proteins [14].

Table 1. Characteristic of partial purified bacteriocins from *Lactobacillus plantarum* LS0602 and *Lactobacillus brevis* LT0904

Organism	Purification stage	Volume (ml)	Activity (AU/ml)	Total activity (AU)[a]	Protein content[b] (µg/ml)	Specific activity[c] (AU/µg)	Purification factor[d]	Recovery[e] (%)
***L. plantarum* LS0602**	Cell-free supernatant	200	102,400	20,480,000	1,275	80.31	1	100
	Partial purified bacteriocins	4	819,200	3,276,800	439	1,866.06	2.91	16
***L. brevis* LT0904**	Cell-free supernatant	200	51,200	10,240,000	1,200	42.67	1	100
	Partial purified bacteriocins	4	409,600	1,638,400	350	1,170.29	3.41	16

[a] Total activity was determined by multiplication of volume by activity.
[b] Protein concentration was determined by the Bradford method (µg/ml).
[c] Specific activity (AU/µg) is the activity unit divided by the protein concentration (µg/ml).
[d] Purification factor is the increase of initial specific activity.
[e] Recovery percentage is the remaining total activity as a percentage of the initial total activity.

The samples added with all types of bacteriocins or starter cultures had lower TPLC, compared to the control samples. The lowest TPLC were found in the samples added with *L. plantarum* LS0602 starter plus 4 % of their partial purified bacteriocins after 3-day fermentation (Figure 1a). This may be due to the antimicrobial action of their bacteriocins. The total LAB counts in the samples added with *L. plantarum* LS0602 either alone or plus 4 % of their bacteriocins increased from 6.0-6.05 log CFU/g at the beginning of fermentation to 8.25-8.35 log CFU/g at the end of fermentation. The samples added with 50 mg/kg nisin had lower LAB counts, compared to the others (Figure 1b). The initial pH of all samples was 5.41-5.52, while the final pH was 4.52-4.84. The sample added with starter plus their bacteriocins had significantly lower pH (4.52-4.53), compared to others (4.69-4.86). After 3-day fermentation, bacteriocin activity in almost all treatments of Nham was 12,800 AU/ml, except for the samples added with 50 mg/kg nisin. Nham added with 50 mg/kg nisin had maximum bacteriocin activity (25,600 AU/ml). The decrease in TPLC may be influenced by the inhibitory effect of the antimicrobial substances produced by these two LAB starters during fermentation. LAB produced metabolic products with inhibitory properties including organic acids, hydrogen peroxide, low molecular metabolites and bacteriocins during fermentation [15].

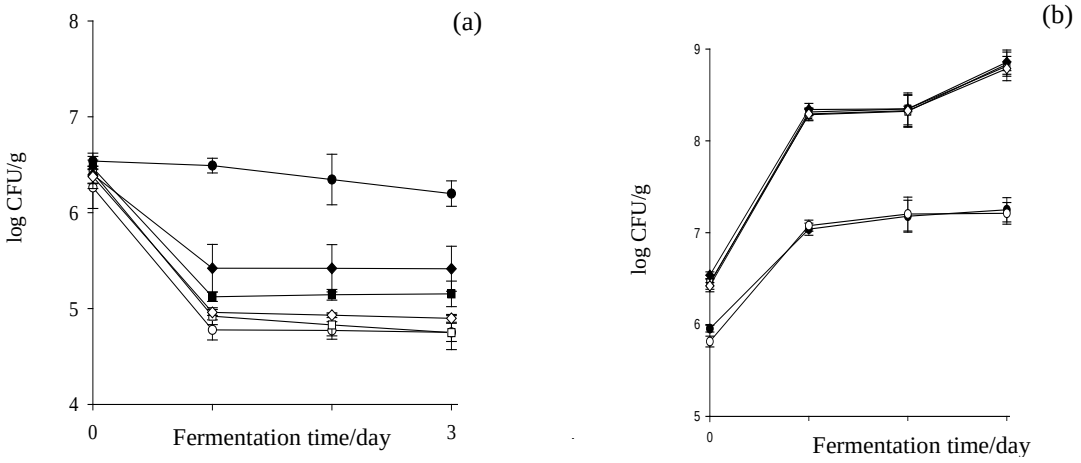

Figure 1 Change of total presumptive *Listeria* counts (a) and total lactic acid bacterial counts (b) in Nham during fermentation at 30 °C; (●) the control (no bacteriocins or starter culture added), (○) Nham added with 50 mg/kg nisin, (■) Nham added with *Lactobacillus plantarum* LS0602 starter cultures, (□) Nham added with *L. plantarum* LS0602 starter culture plus 4 % of their bacteriocins, (♦) Nham added with *Lactobacillus brevis* LT0904 starter cultures, and (◊) Nham added with *L. brevis* LT0904 starter cultures plus 4 % of their bacteriocins

4. Conclusions

According to the data obtained, it can be concluded that the bacteriocins from *L. plantarum* LS0602 and *L. brevis* LT0904 produced in appropriate condition effectively inhibited the growth of *L. monocytogenes* in Nham. Therefore, these two bacteriocin-producing *Lactobacillus* and their partial purified bacteriocins could potentially be used to control the growth of *L. monocytogenes* in fermented meat products.

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