

## Two Novel Strains of Bacteriocin-Producing *Lactobacillus plantarum* and their Application as Biopreservative in Chill-stored Fresh Cobia Meat

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The use of bacteriocins as a biopreservative has been shown to be effective in the control of food pathogenic and spoilage microorganisms. This research aims to screen novel strains of bacteriocin-producing lactic acid bacteria and investigate their application for the preservation of chilled fresh cobia meat. Among a total of 69 strains of lactic acid bacteria isolated from traditional Vietnamese fermented cabbage, two strains T8 and T13 were found to express the broadest, strongest and supplemented inhibitory spectra against 16 indicator strains of food-borne pathogenic and spoilage bacteria. Their bacteriocins may belong to Class I (Lantibiotic), which remain active at 121°C for 15 min, at pH 4-10 and with proteinase K but deactivated by  $\alpha$ -chymotrypsin treatment. These two strains were identified as *Lactobacillus plantarum* using a combined genotypic and phenotypic analysis. The application of culture extract from the strain T13 with cell concentration of  $10^{10}$  CFU/ml or crude bacteriocin extract from the strain T8 with bacteriocin activity of 800 AU/ml was shown to prolong the chilling preservation of fresh cobia meat compared to control within first 7 days. To our knowledge, this is the first study that has used bacteriocin or its producer as a biopreservative in fresh cobia meat.

**Key words:** Bacteriocin, Cobia, Food preservation, *Lactobacillus plantarum*, Lantibiotic.

Bacteriocins are ribosomally synthesized antibiotic peptides or proteins, which are the most abundant and diverse group of bacterial defenses. According to Riley<sup>1</sup>, this fascinating family of toxins offers potential solutions to human health and agriculture challenges. Traditionally bacteriocins were mainly isolated from lactic acid bacteria in food products for their applications in food preservation and nowadays have been looking for a positive health benefit to the host including human, livestock, and aquaculture animals. The use of antibiotics may induce an antibiotic-resistant mechanism of bacteria and an accumulation of unused residues of drugs in the environment which, in turn, results in long term

negative effects on human and animal health. Therefore, an alternative approach for the preservation of food and disease prevention in human and animals is the use of bacteriocin-producing bacteria with dual role of anti- and probiotic activity<sup>2</sup>.

Bacteriocins were first identified almost 100 years ago as a heat-labile product present in *Escherichia coli* V and toxic to *E. coli* S<sup>3</sup>. Then, Fredericq demonstrated that this product were proteins with a limited range of activity to specific receptors on sensitive cell surfaces<sup>4</sup>. By now bacteriocins have been found in all major lineages of Bacteria and some members of the Archaea. Two main features distinguish the majority of bacteriocins from classical antibiotics: bacteriocins are ribosomally synthesized and bacteriocins often have a narrow killing spectrum<sup>5</sup>. For example, bacteriocins from lactic acid bacteria directed

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against low-GC Gram-positive bacteria in general and sometimes against Gram-negative bacteria when the integrity of their outer membrane has been compromised.

Current progress in the research of bacteriocins and booming genomic data led to develop the genome mining softwares BAGEL, BAGEL2<sup>6</sup> and BAGEL3 (new). BAGEL2 identifies putative bacteriocins on the basis of conserved domains, physical properties and the presence of biosynthesis, transport and immunity genes in their genomic context<sup>6</sup>. To date, more than 200 bacteriocins have been characterized and classified mainly based on producer bacterial family, their molecular weight, their amino acid sequence homologies and/or gene cluster organization. Actually, bacteriocins have not been clearly classified yet. Up to 1993, the Klaenhammer's classification system is the most extensively cited<sup>7</sup>. Cotter proposed a revised classification scheme in which bacteriocins composed of two distinct categories: the lanthionine-containing (class I) and the non-lanthionine-containing bacteriocins (class II)<sup>8</sup>. Then, Desriac has proposed an update classification system, in which bacteriocins are divided into two main categories: (i) the protein-bacteriocins mainly produced by Gracilicutes, mostly enterobacteria; (ii) and the peptide-bacteriocins from Firmicutes, chiefly from lactic acid bacteria<sup>9</sup>.

Diverse applications of bacteriocins have been required for major challenges in food processing industry, in which consumers demand for food safety, minimally processed foods with a long shelf-life and without chemical preservatives. Bacteriocins are an excellent candidate as food additives because they are usually heat stable and they can inhibit a broad spectrum of pathogenic and spoilage organisms in minimally processed foodstuffs. However, at present, only nisin and pediocin PA1/AcH have found widespread use in food. The form of nisin used most widely in food is Nisaplin (Danisco®)<sup>5</sup>, and that of pediocin PA1 is ALTA2431 (Quest®)<sup>10</sup>.

Bacteriocins can be introduced into food in at least three different ways: produced *in situ* by bacterial hosts as starter cultures in fermented food; purified or semi-purified then directly added to food; or an ingredient based on a fermentation of a bacteriocin-producing bacterium can be used<sup>5</sup>.

Bacteriocins have been shown to have potential in the biopreservation of meat, dairy products, canned food, fish, alcoholic beverages, salads, egg products, high-moisture bakery products, and fermented vegetables<sup>11</sup>. Their effects are either alone, in combination with other methods of preservation, or through their incorporation into packaging film/food surfaces<sup>12, 13</sup>.

This research aims to screen novel strains of bacteriocin-producing lactic acid bacteria isolated from traditional Vietnamese fermented cabbage and investigate their application for the preservation of chilled fresh cobia meat based on two of three ways above: the use of cell culture extract as starter and crude bacteriocin extract directly immersed by food. To our knowledge, this is the first study that a bacteriocin or its producer has been used as a biopreservative in fresh cobia meat.

## MATERIALS AND METHODS

### Isolation of lactic acid bacteria

Lactic acid bacteria were isolated from the traditional Vietnamese fermented cabbage collected in Nha Trang city using methods described by Gao and coworkers<sup>14</sup>. The cabbage samples (0.1 ml, 10<sup>-4</sup>–10<sup>-6</sup> dilution) were spread directly on the surface of *Lactobacillus* MRS agar (HiMedia, India) and then were incubated at 30°C for 1–2 days. The representative colonies developed on the plates were picked up and purified.

### Identification and phylogenetic analysis of lactic acid bacteria

Isolates were primarily identified according to their physiological and biochemical characteristics<sup>15</sup> and to genus-level by 16S rDNA gene amplification and sequencing as described below. Further identification was based on carbohydrate fermentation reactions using the kit API 50CHL (Biomérieux, France) and ApiWeb software according to manufacturer's instructions.

The total DNA of bacterial strains was extracted by the solid-phase purification method using kit Wizard®SV Genomic DNA Purification System (Promega, USA). Purified DNA samples were used as templates for amplification of 16S rDNA gene segments using eubacterial universal primers (Integrated DNA Technologies, USA), namely forward primer 16S-27F (5'-AGA GTT TGA

TCC TGG CTC AG-3') and reverse primer 16S-1492R (5'-ACG GCT ACC TTG TTA CGA CT-3')<sup>16</sup>. The PCRs were performed in 50 µl reactions containing 2 µl of template DNA, 0.5 µM each primer, 1.5 mM of MgCl<sub>2</sub>, 250 µM each dNTP, and 1 U Taq DNA polymerase along with 1X Taq buffer as recommended (Thermo Scientific, USA). PCR conditions were an initial denaturation for 5 min at 95°C, followed by 35 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 1 min and a final extension of 72°C for 5 min. The amplified products were visualized on a 1% agarose gel stained with ethidium bromide. DNA sequencing of putative 16S rDNA segments was performed on an ABI PRISM® 3700 DNA Analyzer (Applied Biosystems). Raw sequence data was analysed using Geneious software (Biomatters, New Zealand).

The 16S rRNA gene sequences of bacterial isolates and reference sequences of type strains available in GenBank were used for sequence analysis at the National Center for Biotechnology Information (NCBI) using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>). All the 16S rRNA gene sequences were aligned using ClustalW<sup>17</sup>, and regions with gaps were removed using BioEdit<sup>18</sup>. Model selection was used to determine the best fit model with the lowest Bayesian Information Criterion score for Neighbor Joining<sup>19, 20</sup> and Maximum Likelihood analysis, which was then used to construct a phylogenetic tree using the MEGA5 program<sup>21</sup>. The robustness of the tree topology was tested by bootstrap analysis with 1,000 resamplings<sup>22</sup>.

#### Well diffusion assay for bacteriocin activity

Bacteriocin activity was determined by agar-well diffusion method. Isolates were grown on MRS agar media and incubated at 37°C for 24-48 hours. Cell-free supernatants were obtained by centrifugation (6000 rpm, for 30 min, 4°C) and adjusted to pH 7.0 with 1N NaOH. Plates were overlaid with 3 ml soft agar containing 1x10<sup>6</sup> cells of selected indicator bacteria. These indicator bacteria include 16 strains of food pathogenic and spoilage bacteria from our own culture collection at Nha Trang University (Table 1). Wells (diameter  $d = 5$  mm) were cut and 100 µL of supernatant fluid of the test organism was poured into each well. After 24 hours incubation, the diameter  $D$  of inhibition zone around the well was measured and

bacteriocin activity ( $D-d$ , mm) was calculated as described by Todorov and Dicks<sup>23</sup>. Each bacteriocin preparation was serially diluted and activity units (AU) per milliliter were calculated as previously reported<sup>24</sup>.

#### Bacteriocin production

Lactic acid bacteria were cultured on MRS medium at pH 6.2-6.8 and 37°C for 2 days, with agitation. Changes in optical density at 600 nm of the cultures were determined every three hours during the first 33 hours of cultivation. Bacteriocin activity was measured as described above with *Bacillus cereus* B1.1 (10<sup>6</sup> CFU/ml) cultured in Tryptone Soya Broth (TSB) (HiMedia) was used as an indicator strain.

#### Effect of enzymes, pH and temperature on bacteriocin activity

The neutralized supernatant fluid was first treated with catalase (Promega, USA) at a final concentration of 0.5 mg/ml for 30 min at 37°C to define the antimicrobial activity of hydrogen peroxide. To check the protein nature of bacteriocin, proteinase K or  $\alpha$ -chymotrypsin (Promega, USA) at a final concentration of 1 mg/ml were added. Afterward, the fluid was incubated for 3 hours at 50°C. To check the thermal stability, cell-free neutralized supernatants of bacteriocins were exposed to 60°C, 100°C for 60 min, 121°C for 15 min and bacteriocin activity was checked by agar-well diffusion method as described above. Similarly, the effect of pH on the bacteriocin was determined by adjusting the cell-free supernatant to pH 2.0–12.0 with 1N HCl or 1N NaOH. After 30 min of incubation at 30°C, the samples will be readjusted to pH 7.0 and the bacteriocin activity was determined.

#### Cobia meat preparation and sampling

Fresh cobia (*Rachycentron canadum*) with the body length of 60-70 cm and the weight of 5-7 kg were purchased from local farms in Luong Son, Nha Trang, Khanh Hoa, Vietnam. Fish were packaged in sterile plastic trays, stored in ice and transported to the lab within two hours.

Collected fish were washed and immersed in the culture extract of lactic acid bacteria (cell density  $\geq 10^{10}$  CFU/ml) for 2 min or in the crude bacteriocin extract (800 AU/ml) for 5 min, then packaged in sterile plastic bags, and stored in dark under refrigeration at 4°C until chilled fish sample deterioration. The fish without bacteriocin immersion were used as controls.

At days 1, 3, 5, and 7, fish were sampled for food quality evaluation through microbiological, physicochemical and sensory analyses. A total of 60 individual fish were used, in which each experiment used 5 individuals for bacteriocin-treated samples and 5 for controls. The experiment was done in triplicate.

#### Quality evaluation of fish samples

Sensory evaluation was applied according to Zhang<sup>24</sup> with some modifications. Chilled fish samples were assessed on the basis of color, texture, flavor, elasticity and taste. Bacteriocin-treated fish samples were compared with control which was taken as a reference value (five points for each parameter) according to Vietnamese hygienic standard TCVN 52-77-90 for fresh fish. Physicochemical change was recorded by the  $\text{NH}_3$  content, which was determined by Kjeldahl method as described by Meyer and Geissler<sup>25</sup> according to Vietnamese hygienic standard TCVN 3706-90.

Microbiological analyses: 25g chilled fish sample from the same position of each fish was cut using sterile knives and homogenized for 2 min with 225 ml of 0.1% (w/v) sterile peptone water containing 1% (v/v) Tween 80 and 0.85% (w/v) NaCl. Serial decimal dilutions were made and then plated in triplicate on total count and selective agar plates. Total mesophilic counts were determined on Plate Count Agar (PCA) (HiMedia), incubated at 30°C for 48 h.

Challenge test was carried out to determine the efficacy of bacteriocin or bacteriocinogenic lactic acid bacteria which can suppress growth of spoilage and pathogenic microorganism in chilled fish storage. Each of 25 g chilled fish sample was inoculated with either *Salmonella typhimurium* Sal1 or *Vibrio cholerae* V1.1 ( $10^5$  CFU/g), then immersed in preservative solution. Survival rate was monitored by enumeration on *Salmonella* selective agar (Xylose Lysine Deoxycholate agar – XLD) (HiMedia) and *Vibrio* selective agar (Thiosulphate Citrate Bile Salt Sucrose Agar – TCBS) (HiMedia), respectively.

#### Statistical analysis

All experiments were conducted in triplicate. Data sets were subjected to analysis of variance (ANOVA) and t-test on SPSS 8.0 for Windows.

## RESULTS

### Isolation and screening of bacteriocinogenic lactic acid bacteria

A total of 69 strains of lactic acid bacteria were isolated from traditional Vietnamese fermented cabbage. Among them, five strains expressed their antagonistic activity against 16 indicator strains of food borne pathogenic and spoilage bacteria (Table 1). Two strains T8 and T13 had the broadest and strongest inhibitory spectra, against almost all Gram positive (*Bacillus cereus*, *Staphylococcus aureus*, *Clostridium perfringens*) and Gram negative bacteria (*Salmonella typhimurium*, *Vibrio parahaemolyticus*, *V. cholerae* and *E.coli*). The result showed that the cell culture extract of the strain T13 was found to express a stronger antagonistic activity than that of the strain T8 while a reverse trend between these two strains was shown in their crude bacteriocin extracts. Therefore, the culture extract of the strain T13 and the crude bacteriocin extract of the strain T8 will be used as biopreservatives for further experiments.

### Identification of bacteriocinogenic lactic acid bacteria

The strains T8 and T13 were primarily identified as *Lactobacillus* spp. by physiological and biochemical characteristics, which was revealed by positive-Gram-stained cells, negative catalase activity and positive glucose fermentation. Further identification was carried out by 16S rDNA amplification and sequencing. Gene sequences were submitted to GenBank with the Gene Accession numbers KC213804 (712 bp) and KC213805 (1316 bp) for the strains T8 and T13, respectively.

The results from 16S rDNA gene sequencing of these two strains revealed 100% homology to *Lactobacillus plantarum* strain ST-III (GenBank Accession number CP002222), 99.9% homology to *L. plantarum* type strain NRRL B-14768<sup>T</sup> (NR042394) and *L. pentosus* type strain 124-2<sup>T</sup> (NR029133), 99.6-99.8% to *L. paraplantarum* type strain DSM 10667<sup>T</sup> (NR025447) and 98.6-99.2% to *L. fabifermentans* type strain LMG 24284<sup>T</sup> (NR042676). Phylogenetic analysis of the 16S rRNA gene sequences of T8 and T13 isolates showed significant differences with type strains of their closest species within the *Lactobacillus* genus (Fig. 1). However, these two strains along with type

strains of *L. plantarum* and *L. pentosus* show the same clusterings in the phylogenetic tree, which required further analysis for a species-level differentiation.

Further results from the kit API 50CHL analysis of 49 sugar fermentation reactions confirmed genotype-based identification. Only minor differences in fermentation yield of some reactions were shown between two strains T8 and

T13 (Table 2). Both strains were identified as *L. plantarum* type 1 (with %ID = 99.9 and T = 0.65-0.66), which was distinguished from *L. pentosus* by at least two characteristics: negative reaction to D-xylose fermentation and positive to D-melezitose fermentation.

#### Bacteriocin production and characterization

Maximum bacteriocin production activity (inhibitory zone diameter of 12 and 16 mm for strains

**Table 1.** Antimicrobial activity of five lactic acid bacteria strains

Indicators	Lactic acid bacteria	T4		T8		T12		T13		O16	
		(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Gram positives (G <sup>+</sup> )	<i>Bacillus cereus</i> B1.1	++ <sup>(c)</sup>	+	++++	++++	++	+	+++++	+++	++	+
	<i>Bacillus</i> sp. B2.3	++	+	++	+	++	+	+++	+	++	+
	<i>Staphylococcus aureus</i> SA	+	+	+	+	+	+	+++	++	+	+
	<i>Clostridium perfringens</i> CP	+	+	+	+	+	+	+++	++	+	+
Gram negatives (G <sup>-</sup> )	<i>Salmonella typhimurium</i> Sal1	++	+	++	+	++	+	+++	+	++	+
	<i>Escherichia coli</i> H10b	-	-	++	++	++	+	+++	++	++	+
	<i>E. coli</i> L2.1	-	-	++	++	++	++	++	+	++	+
	<i>E. coli</i> TN3	++	+	++	+	++	+	++	+	++	+
	<i>E. coli</i> TN4.1	-	-	++	+	++	+	++	+	-	-
	<i>E. coli</i> TN4.2	++	+	++	+	++	+	++++	++	+	+
	<i>E. coli</i> TN5.2	++++	+++	+++	+++	++	+	++	+	++	+
	<i>E. coli</i> GA	-	-	++	+	-	-	++	+	+	+
	<i>Vibrio cholerae</i> V1.1	-	-	++	+	-	-	++	+	-	-
	<i>V. parahaemolyticus</i> C1	+++	++	+++	++	+++	++	+++	+	++	+
	<i>Vibrio</i> sp. CR6	++	+	++	+	+++	++	+++	+	++	+
	<i>Vibrio</i> sp. CR7	-	-	++	+	-	-	-	-	-	-

(a) Culture extracts, (b) Crude bacteriocin extracts, (c) Antibacterial zone diameter (*D-d*), (-): < 5, (+): 5-7.5, (++) : 7.5-10, (+++): 10-12.5, (++++): 12.5-15, (+++++): > 15 (mm).

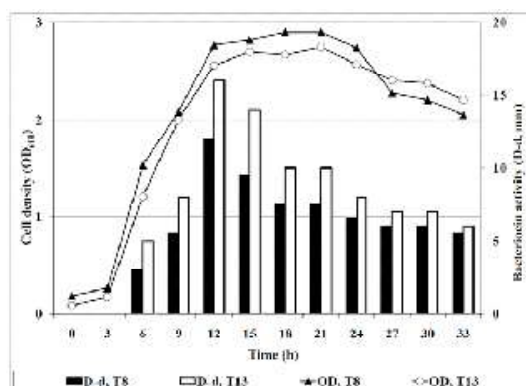
**Table 2.** Phenotypic differences among strains T8, T13, *L. plantarum* type 1 and *L. pentosus*

Carbon source utilization	T8	T13	<i>L. plantarum</i> type 1	<i>L. pentosus</i>
Glycerol	-	w	-	+
L-arabinose	+	+	+/-	+
D-xylose	-	-	-	+
D-sorbitol	w	-	+/-	+
Methyl-b-D-manopyranoside	w	w	+/-	-
Methyl-b-D-glucopyranoside	-	-	+/-	+/-
Inulin	w	-	-	-
D-melezitose	+	+	+	-
D-raffinose	w	-	+/-	+
Gentiobiose	w	+	+	+
D-turanose	-	-	+/-	+/-
D-arabitol	w	w	+/-	-
Potassium gluconate	w	+	+/-	+/-

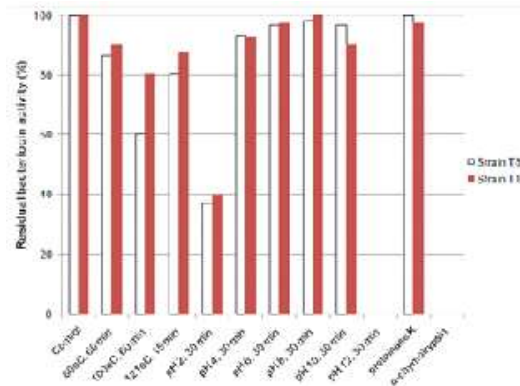
(+): >85% positive, (-): <15% positive, (+/-): 15-85% positive, w: weakly positive



**Fig. 1.** Neighbor-Joining phylogenetic tree based on the comparative analysis of 16S rRNA gene sequences showing the relationships between the strains T8 and T13 to closely related species. The percentage of replicate trees more than 50% in the bootstrap test (1000 replicates) is shown on the branches. The scale bar indicates the number of substitutions per nucleotide position. *Bacillus subtilis* GS1 (AB773829) was used as outgroup. (T) stands for type strain



**Fig. 2.** The relationship of growth and bacteriocin production of the strains T8 and T13



**Fig. 3.** Effect of enzymes, pH and temperature on bacteriocin activity. Bacteriocin activity of control samples of the strains T8 and T13 as 6000 AU/ml and 8000 AU/ml, respectively

T8 and T13, respectively) was recorded at the late exponential phase after 12 hours of growth on MRS medium when tested against *Bacillus cereus* B1.1 in TSB broth (Fig. 2). From a metabolic point of view, this trend is characteristic of primary metabolite production, as reported for bacteriocins produced by other lactic acid bacteria<sup>26</sup>. Then bacteriocin production decreased rapidly to a half of maximum level after 24-27 hours of the growth.

The reduction in activity recorded at the end of fermentation may be due to degradation of the bacteriocin by extracellular proteolytic enzymes.

To assess the effect of enzymes, pH and temperature on the activity of crude bacteriocin extracts from the strains T8 and T13, the pH value was adjusted to neutral and catalase was added to remove the antibacterial effect of organic acids and hydrogen peroxide. The results have indicated that

the crude bacteriocin extracts from both strains remained active after incubation in the autoclave conditions at 121°C for 15 min (80-88% residual activity) or at pH 4-10 for 30 min ( $\geq 90\%$  residual activity), or with Proteinase K (98-100% residual activity) (Fig. 3). However, complete inactivation in activity was recorded when bacteriocin extracts

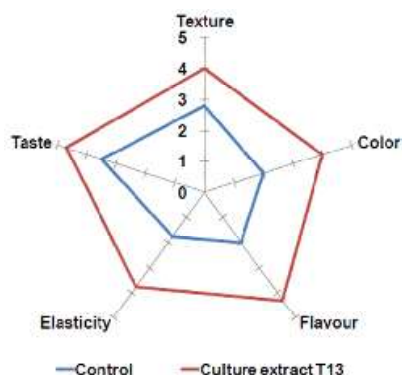


Fig 4. Sensory analysis of cobia meat treated with culture extract T13 compared to the control at day 7

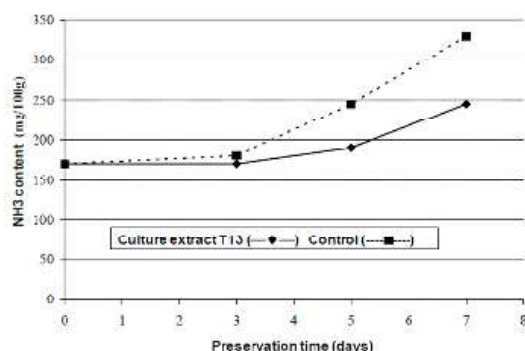


Fig 5. The change of  $\text{NH}_3$  content of cobia meat treated with culture extract T13 compared to the control

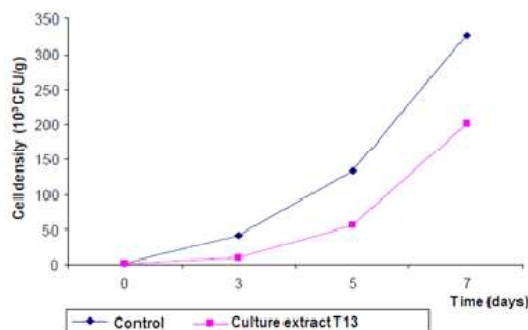


Fig 6. The change of total aerobic bacteria counts of cobia meat treated with culture extract T13 compared to the control

was treated with  $\alpha$ -chymotrypsin, indicating the proteinaceous nature of the bacteriocin extracts. Moreover, these bacteriocins from the strains T8 and T13 were not digested by Proteinase K, which showed that they belonged to the bacteriocin class I, or lantibiotics, a series of small heat-resistant peptides composing of post-translationally modified amino acids<sup>8</sup>.

#### Quality evaluation of chill-stored fresh cobia meat using the cell culture extract T13

##### Sensory analysis

The results of sensory evaluation of the fresh cobia sample treated with cell culture extract T13 (cell density  $10^{10}$  CFU/ml, bacteriocin activity 800 AU/ml) and control without bacteriocin immersion at day 7 was shown in the Fig. 4. It was described with higher scores on color, texture, flavor, elasticity, and taste of the bacteriocin-treated sample compared to the control.

##### Physicochemical change: $\text{NH}_3$ content

The  $\text{NH}_3$  content of both bacteriocin-treated sample and control increased gradually with the storage time (Fig. 5). By day 7, the  $\text{NH}_3$  content of control had increased from 170 to 330 mg/100 g of meat whereas that of the bacteriocin-treated sample could be controlled in a level of  $<250$  mg/100 g of meat with 74% lower concentration.

##### Microbiological analyses

As indicated in Fig. 6, the control started to deteriorate at day 7. Compared with the control, the sample treated with culture extract T13 could significantly inhibit the growth of total aerobic bacteria of tray-packaged chilled cobia meat, which reached the permitted Vietnamese hygienic standard for fresh fish ( $<10^6$  CFU/g of meat). Moreover, *Salmonella typhimurium* or *Vibrio cholerae* ( $10^7$  CFU/ml) was challenged to cobia meat at day 3, a four-fold and two-fold reduction in the cell count of these food pathogens, respectively. Similar results were also recorded in the bacteriocin-treated sample compared to the control at day 7 (data not shown).

Results from sensory, physicochemical and microbiological analyses on cobia meat sample treated with crude bacteriocin extract from the strain T8 (800 AU/ml) were also recorded in the same trend (data not shown). Therefore, the application of crude bacteriocin extract T8 or cell culture extract T13 could prolong the chilling preservation of fresh cobia meat compared to control until 7 days.

## DISCUSSION

Lactic acid bacteria have been used for food fermentation for centuries. They are generally regarded as safe (GRAS) by the US Food and Drug Administration (FDA). The regulation permits their use in fermented foods without additional regulatory approval. Production of bacteriocins has been found widespread among the lactic acid bacteria group<sup>1</sup>. Bacteriocins from lactic acid bacteria could be used as optimal food preservatives firstly because they have a wide inhibitory spectrum against sensitive spoilage and pathogenic bacteria. In addition, they are digested by intestinal proteases when supplemented into food leading to a harmless effect to consumers. Finally, they may be used as protective cultures to improve the microbial safety of foods, so no more costs necessary to be applied in fermented foods<sup>27</sup>.

The crude or purified bacteriocin forms or even bacteriocin producers may also be applied directly as food preservative. In this research, we isolated and screened two strains of *Lactobacillus plantarum* T8 and T13 from traditional Vietnamese fermented cabbage with strong and broad bacteriocin activity against 16 indicators of food spoilage and pathogenic bacteria. A similar result was reported from sakacin C2 of *Lactobacillus sakei* isolated from Chinese fermented cabbage, which expressed a broad antimicrobial spectrum against both Gram positives such as *Lactobacillus plantarum*, *L. delbrueckii*, *L. acidophilus*, *Staphylococcus aureus*, *Listeria innocua* and Gram negatives such as *E. coli*, *Salmonella typhimurium*, *Shigella flexneri*<sup>14</sup>. Sakacin C2 was found to inhibit the growth of *Bacillus cereus*, however, no effects were observed against for *Bacillus subtilis*, yeast (*Saccharomyces cerevisiae*) and mold (*Aspergillus niger*).

A combined phenotype- and genotype-based analysis has been revealed as an effective method to identify the lactic acid bacteria (T8, T13), which belonged to the commercially important species *L. plantarum*. The first bacteriocin which has been purified and characterized from *L. plantarum* named Plantaricin A<sup>28</sup>. Currently, this bacteriocin was shown as a cationic peptide pheromone that has not only an antimicrobial effect but also a membrane-permeabilizing effect on eukaryotic cells<sup>29</sup>. Up to now, a series of bacteriocins

from *L. plantarum* have been reported, for example, plantaricin B, plantaricin BN, plantaricin C, plantaricin S and T, plantaricin F, plantaricin C19, plantaricin SA6<sup>30</sup>, and plantaricin UG1<sup>31</sup>. Most of bacteriocin-producing *L. plantarum* strains were isolated from fermented food products.

Another bacteriocin, plantaricin 423, which was produced from *Lactobacillus plantarum* 423 isolated from sorghum beer, was found to inhibit food spoilage bacteria and food-borne pathogens, e.g., *Bacillus cereus*, *Clostridium sporogenes*, *Enterococcus faecalis*, *Listeria* and *Staphylococcus*<sup>32</sup>. Plantaricin 423 remained active after incubation at pH 1-10, after autoclaving at 121°C for 15 min (75% residual activity), after 60 min at 100°C (50% residual activity), but inactivated when treated with pepsin, papain,  $\alpha$ -chymotrypsin, trypsin and Proteinase K. Very similar results were shown for the crude bacteriocin extracts from both strains T8 and T13, which were resistant to autoclave conditions (80-88% residual activity), after 60 min at 100°C (60% residual activity) or at pH 4-10 for 30 min ( $\geq 90\%$  residual activity), and also inactivated with  $\alpha$ -chymotrypsin treatment. However, the bacteriocin extracts T8 and T13 remained nearly 100% activity after the treatment with Proteinase K. This is explained by the fact that bacteriocins from the strains T8 and T13 may belong to the class I, antibiotics, while almost all other plantaricins have been reported as bacteriocins class II<sup>8,11</sup>.

Bacteriocins have been grouped into three classes based on their structure<sup>7</sup>. Class I is composed of small heat-resistant peptides that undergo post-translational modifications. For example, reactions between dehydrated serine and threonine residues with the sulfhydryl group of cysteine can lead to the formation of rings in the peptide chain, which was not digested by Proteinase K. Class II is represented by heat-resistant unmodified peptides whereas heat-labile and high-molecular-weight proteins are grouped into class III. According to the revised classification scheme by Cotter<sup>8</sup>, bacteriocins from the strains T8 and T13 can belong to the lanthionine-containing (class I) while the other category includes the non-lanthionine-containing bacteriocins (class II).

Up to now, only plantaricin C, a bacteriocin produced by a *L. plantarum* strain of dairy origin,



has been considered as a lantibiotic from this species. It contains 27 amino acids, including one dehydroalanine, one lanthionine, and three beta-methyl-lanthionine residues that form the beta-methyl-lanthionine bridges between residues 12-15, 13-18 and 23-26<sup>27</sup>. Therefore, experiments on purification of bacteriocins from the strains T8 and T13 should be carried out for further characterization of their structure.

In this research, experiments on fresh cobia preservation using bacteriocins or their starter cultures were also carried out. Traditionally, a popularly-used and effective method of preserving the freshness of seafood in Vietnam and other countries is to chill with ice<sup>33, 34</sup>, which requires a large mass of ice, large ice container and transporter leading to a high preservation cost. Moreover, the recent development in cobia production in Vietnam made this nation become the 3rd largest producer of farmed cobia in the world, which requires the advanced technologies for cobia processing and preservation<sup>33</sup>. Despite the recent progress in food preservation technologies and safety concepts, the problem of seafood safety and security remains to be solved. We report here the application of culture extract from lactic acid bacteria strain T13 (cell density of 10<sup>10</sup> CFU/ml) or crude bacteriocin extract from the strain T8 (bacteriocin activity of 800 AU/ml) was shown to prolong the chilling preservation time of fresh cobia meat. This was revealed that the sensory quality, physicochemical change and microbiological counts of fresh cobia meat were well kept within first 7 days.

Basic study using bacteriocin-producing lactic acid bacteria or their crude bacteriocin extract for the preservation of aquatic food products has been just progress. To our knowledge, this is the first application of bacteriocin or its producer in preservation of fresh cobia meat. In a current report, lactic acid bacteria strains isolated from sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) have been characterized for their bacteriocin production activities against the most relevant seafood-spoilage and pathogenic bacteria. They are expected to have potential applications in the biopreservation of fish and shellfish products<sup>35</sup>.

## CONCLUSIONS

Among a total of 69 strains of lactic acid bacteria isolated from traditional Vietnamese fermented cabbage, five strains were found to express their antagonistic activity against 16 indicator strains of food borne pathogenic and spoilage bacteria. Two strains of lactic acid bacteria T8 and T13 have the broadest, strongest and supplemented inhibitory spectra. Their bacteriocins belong to Class I (Lantibiotic), which are stable at 121°C for 15 min, at pH 4-10 and with proteinase K but deactivated by  $\alpha$ -chymotrypsin treatment. Analyses of 16S rRNA gene sequence and API 50CHL tests identified these strains as *Lactobacillus plantarum*. The application of the culture extract from the strain T13 (cell concentration of 10<sup>10</sup> CFU/ml) or crude bacteriocin extract from the strain T8 (bacteriocin activity of 800 AU/ml) was shown to prolong the chilling preservation of fresh cobia meat compared to control. It was revealed that the sensory quality, physicochemical change and microbiological counts of fresh cobia meat were well kept within first 7 days, which reached the permitted Vietnamese hygienic standard for fresh fish.

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