ISOLATION AND PHYLOGENETIC ANALYSIS OF MARINE BACTERIA IN ASSOCIATION WITH OTTER CLAM (*LUTRARIA PHILIPPINARUM*) REVEALING BACTERIOCIN PRODUCTION BY *CRONOBACTER SAKAZAKII* AND *ENTEROBACTER CLOACAE*

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SUMMARY

Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins, which are produced by bacteria to inhibit the growth of similar or closely related bacterial strains. They have been looking for a positive health benefit to the host including human, livestock, aquaculture animals and some plants. The aims of this study were to isolate, assess the bacteriocinogenic activity and analyze phylogenetic relationship of marine bacteria in association with otter clam (*Lutraria philippinarum*) captured in Nha Trang Bay and Cam Ranh Bay. Among a total of 128 bacterial isolates, 19 strains (15%) were found to produce bacteriocins against at least one of selected indicator bacteria. The highest bacteriocin production activity was shown by 6 strains, named H9, H18, H51, H61, H77 and H108, which were then selected for further study. The antimicrobial activity of crude bacteriocin extracts from these strains was completely inactivated after the treatment with proteinase K and trypsin while they still remained the activity after the exposure to lipase and α-amylase. All six strains are rod - shape bacteria, facultatively anaerobic and Gram-negative. The result of sequencing of the 16S rRNA and *rpoB* genes presented that the strain H77 was identified as *Enterobacter cloacae* and the other strains as *Cronobacter sakazakii*. This is the first study on bacteriocins produced by a member of the *Cronobacter* genus. The research contributes to the collection of the marine bacteriocinogenic bacteria and further using them as potential probiotics or drugs in aquaculture.

Keywords: 16S rRNA, bacteriocin, *Cronobacter*, *Enterobacter*, marine bacteria, *rpoB*, probiotic

INTRODUCTION

Vietnam has excellent natural conditions for the development of aquaculture, especially various species of marine animals. Aquaculture is one of the major sources of income for the poor farmers in this field. However, farmers have faced to serious diseases of opportunistic bacterial pathogens, in which *Vibrio* species is one of the most important pathogens recognized in larval culture that break out dramatically causing heavy losses (Toranzo *et al.*, 2005). In addition, the fear of aquaculture farming increases with climate change, because the virulence and transmission of pathogens perhaps increases at higher temperatures (Gillor *et al.*, 2010).

Antibiotics have been utilized in animal research almost from the time of their discovery and quickly found widespread use in the farm environment as therapeutic agents. Perhaps, due to overuse in aquaculture, it presents disease resistance by bacteria, damaged normal microflora and caused microdysbiosis. It also made residues accumulated in aquatic products to be harmful for human. So, scientific communities have proposed friendly alternatives such as vaccines, antibiotic substitutes or probiotics (Corripio-Myar *et al.*, 2007). However, vaccine use is often laborious, costly and stressful to the animals. Therefore, bacteriocinogenic bacteria strains appear to be an excellent candidate with dual role because bacteriocin would be an antibiotic substitute, whereas bacteria would be a potential probiotic (Gillor *et al.*, 2008).

Evidence is abundant that bacteriocins are important mediator of intra- and interspecies interactions and, consequently, a significant factor in maintaining microbial biodiversity (Riley and Wertz, 2002).Bacteriocin is considered that is not harmful to human heath. For using bacteriocin as potential probiotics and drugs in aquaculture, bacteriocin-producing bacteria must be isolated from marine animals and marine-environment to adapt with the change of temperature and salinity in the conditional culture (Desnac *et al.*, 2010). Thus, local marine animals might be suitable for being isolated and screened potential probiotics or drugs. In this research, otter clam (*Lutraria philippinarum*) is chosen to study. In our knowledge, it is the first time that a bacteriocin-producing bacterium was found in *L. philippinarum* and also the first bacteriocin produced by a member of the *Cronobacter* genus.

MATERIALS AND METHODS

Indicator bacteria

Selected target bacteria strains including Enterococcus *faecalis* B1.1, *Vibrio parahaemolyticus* C1 and *Vibrio alginolyticus* V3.3 are seafood-borne pathogens obtained from the culture collection of local microorganisms at Nha Trang University.

Otter clam sampling

Otter clams (*Lutraria philippinarum*) (n=9) were collected from Nha Trang Bay and Cam Ranh Bay from February to October 2012. At the sampling sites, the intestinal content and mucus of clams were separated using sterile sharp knife and forceps, and dispensed in pre-weighed sterile 1% peptone water taken in a screw cap test tube. Samples were then brought to the laboratory in insulated containers for further analysis.

Bacterial isolation

Marine bacteria were isolated by the spread plate method. Clam samples were homogenized using tissue grinders and vortex in sterile saline solution (8.5 g.l^{-1} NaCl). Tenfold serial dilutions of samples were prepared and plated on trypticase

soy agar (TSA, HiMedia) supplemented with 1% NaCl and 1.5 % agar. All plates were incubacted for 24 hours at 37° C. Colonies with different morphological characteristics from each sample were picked up and purified, subcultured in suitable media and stored in sterile glycerol (20% v/v) at -80° C (Nithyanand and Pandia, 2009).

Assay for bacteriocin activity

Antibacterial activity was determined by agar-well diffusion method. Isolates were grown in Trypticase Soy Broth (TSB, HiMedia) and incubated at 37° C for 12 - 24 hours. Then cell-free supernatants were harvested by centrifugation (6000 rpm, for 30 min, 4° C). The cells were discarded and pH of the supernatant fluid was adjusted to 7.0 with 1 N NaOH or 1N HCl to remove the effect of organic acid and then treated with catalase (Promega, USA) at the final concentration of 0.5 mg/ml at 37° C for 30 mins to remove the effect of hydrogen peroxide. Plates were overlaid with 3 ml soft agar containing 10^6 cells of selected target bacteria. Wells (7 mm diameter) were cut and 100 µl of the supernatant fluid of the test organism was poured into each well. Next day the diameter of inhibitory zone around the well was measured (Todorov and Dicks, 2009) To check the protein nature of bacteriocin, trypsin (Promega, USA) at the final concentration of 1 mg/ml were treated with supernatant fluid at 50°C for 3 hours.

Effect of physicochemical factors on the activity of crude bacteriocin

The supernatant fluid was first adjusted pH-to 7 and treated with catalase as described above. To check the chemical nature of bacteriocin, proteinase K, trypsin, lipase or α-amylase (Promega, USA) at a final concentration of 1 mg/ml were added. Afterward, the fluids were incubated at their optimal temperatures as recommended by the manufacture. In particular, trypsin and proteinase K were incubated at 50° C for 3 hours, and lipase and α-amylase at 37° C and 20° C for 2 hours, respectively. The residual activity after enzyme treatment was determined as described previously.

To check the thermal stability, cell-free neutralized supernatants of bacteriocins were exposed to 30° C, 60° C, 100° C for 30 min, 121° C for 15 min (autoclave condition) 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 1 N HCl or 1 N NaOH. After 30 min of incubation at 30° C, the samples will be readjusted to pH 7.0 and the bacteriocin activity was determined

Genomic DNA extraction and Polymerase Chain Reaction of 16S rRNA and *rpoB* genes

The DNA of bacterial strains was extracted by alkaline lysis method using the kit Wizard®SV Genomic DNA Purification System (Promega, USA). Purified DNA samples were used as templates for amplification of 16S rRNA and *rpoB* gene segments using respective primers (Integrated DNA Technologies, USA) as follows: 16S-27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 16S-1492R (5'-ACG GCT ACC TTG TTA CGA CT-3') (Luan *et al.*, 2007); CM7 (5'-AAC CAG TTC CGC GTT GGC CTG G-3') and CM31b (5'-CCT GAA CAA CAC GCT CGG A-3') (Mollet *et al.*, (1997). The PCRs were performed in 50 µl reactions containing 2 µl (10 ng) of template DNA, 0.5 µM each primer, 1.5 mM of $MgCl_2$, 50 µM each dNTP, and 1 U Taq DNA polymerase along with 1 X Taq buffer as recommended (Promega, USA). Amplification was performed in a DNA thermal cycler (Biorad) as follows: 40 cycles of 1 min per cycle at 95° C, and 1 min at 55° C, followed by an increase to 72° C over 2 min. Extension of the amplified product was at 72° C for 5 min. The amplified products were be visualized in a 1% agarose gel stained with ethidium bromide.

Gene sequencing and phylogenetic analyses

The PCR product of the 16S rRNA and *rpoB* genes of bacterial isolates was purified using the PCR Clean Up System Kit (Promega, USA) and used as template for sequencing using dye-labelled dideoxy terminator (Big Dye Terminator v. 3.1, Applied Biosystems) on an ABI Prism 3700 DNA Analyser (Applied Biosystems). The 16S rRNA and *rpoB* gene sequences of bacterial isolates and reference sequences available in GenBank were used for sequence analysis at the NCBI using BLAST tool. Phylogenetic trees were constructed using the MEGA5 program (Tamura *et al.*, 2011). The robustness of the tree topology was tested by bootstrap analysis with 1,000 resamplings (Felsenstein, 1985).

RERULTS AND DISCUSSION

Isolation and screening of marine bacteriocin-producing bacteria from otter clam

Total 128 strains were isolated from otter clam (*Lutraria philippinarum*). The result from Table 1 showed that 26/128 strains expressed the inhibitory activity against at least one of three indicator bacteria. In particular, 14 strains could inhibit the growth of *Enterococcus faecalis* B1.1, 19 strains against *Vibrio alginolyticus* V3.3, and 2 strains against *V. parahaemolyticus* C1. Among of them, 8 strains showed their activity against both B1.1 and V3.3, and the only strain H50 against both C1 and V3.3. Interestingly, out of 26 strains with antimicrobial activity, 19 bacteriocin-producing strains were screened as revealed by the complete inactivation of their supernatant fluids after trypsin treatment compared to controls (Table 1). Based on the strong and stable inhibitory activity against at least two indicator bacteria, 6 bacteriocinogenic strains H9, H16, H51, H61, H77 and H108 were selected for further study.

Table 1. The antimicrobial activity of isolates from otter clam against selected target strains

No.	Strains	Inhibitory zone diameter (mm)				No.	Strains	Inhibitory zone diameter (mm)			
		V3.3	C1	B1.1	Trypsin treatment			V3.3	C1	B1.1	Trypsin treatment
1	X1.4	9.5 ± 0.5			0	14	H21	6.5 ± 0.5			6.5 ± 0.5
2	X1.5	10.5 ± 0.5			0	15	H50	9 ± 2	6.5 ± 0.5		0
3	X1.9	7.5 ± 0.5			7.5 ± 0.5	16	H51	8 ± 1		10.5 ± 0.5	0
4	X1.10		10.5 ± 0.5		0	17	H53	11 ± 1			0
5	X1.11	7.5 ± 0.5			7.5 ± 0.5	18	H58			13.5 ± 0.5	0
6	H1			7.5 ± 0.5	0	19	H61	11.5 ± 0.5		11.5 ± 0.5	0

7	H2	7 ± 1			7 ± 1	20	H64			8	0
8	H5	5± 0.5			5± 0.5	21	H73	6 ± 1			6 ± 1
9	H7			12 ± 1	0	22	H74			6 ± 1	0
10	H8	6.5 ± 0.5		16 ± 1	0	23	H76			15 ± 1	0
11	H9	8 ± 1		15.5 ± 0.5	0	24	H77	9.5 ± 0.5		17.5 ± 0.5	0
12	H15	6.5 ± 0.5			6.5 ± 0.5	25	H78			19	0
13	H18	10		16 ± 1	0	26	H108	9 ± 1		18 ± 1	0

The effect of physiochemical factors on the activity of the crude bacteriocin

The effect of physiochemical factors on the activity of crude bacteriocin is presented in table 2 and fig 2. In short, the crude bacteriocin extracts were completely activited by proteolytic enzymes, proteinase K and trypsin, indicating a proteinaceous nature of the inhibitory compounds, called bacteriocins or bacteriocin-like substances (BLIS). However, the treatment with lipase or α-amylase was found to have no effects on the bacteriocin activity resulting from normal size of zone of inhibition, which suggested the absence of a lipid and carbohydrate moiety in these bacteriocins (Table 2a & Fig. 2). Baladzar *et al.* (2010) obtained similar results in the case the cell-free culture supernatants of 13 isolates from seahhorses (*Hippocampus guttulatus*) were also treated with proteinase K and trypsin. The results showed that the cell – free culture supernants freom 3 strains (HG-14F, HG-12F and HG-3F) were inactivated by proteinase K, whereas only 2 strains were inactivated by trypsin. Bacteriocin are ribosomally synthesized peptides or proteins that are generally effective against closely related species. Bacteriocins are differentially cleavaged by distinguished-enzymes such as trypsin, proteinase K and α- chymotrypsin according to the protein structure (Riley and Wertz, 2002).

Table 2. The effect of enzymes, temperature and pH on the activity of crude bacteriocin extracts

a. Enzymes

Strains	Inhibitory zone diameter in mm (residual activity, %)						
	Control 1 (55°C, 3 h)	Proteinase K (55°C, 3 h)	Trypsin (55°C, 3 h)	Control 2 (37°C, 2 h)	Lipase (37°C, 2 h)	Control 3 (20°C, 2 h)	α- amylase (20°C, 2 h)
H9	11 (100)	0	0	10 (100)	8.5 (85)	10.5 (100)	9.5 (90.5)
H18	14 (100)	0	0	15 (100)	13 (86.6)	12.5 (100)	13 (104)
H51	16 (100)	0	0	17.5 (100)	13 (74.3)	16 (100)	12 (75)
H61	10 (100)	0	0	9 (100)	9 (100)	10 (100)	7.5 (75)
H77	13 (100)	0	0	14 (100)	12.5 (89.3)	17 (100)	12.5 (73.5)
H108	16 (100)	0	0	16 (100)	15 (93.4)	10.5 (100)	8 (76.2)

b. Temperature

Strains	Inhibitory zone diameter in mm (residual activity, %)			
	Control (30° C, 30 min)	60° C, 30 min	100° C, 30 min	121° C, 15 min
H9	12 (100)	11 (91.7)	0	0
H18	13 (100)	10 (76.9)	0	0
H51	13 (100)	13 (100)	0	0
H61	15 (100)	11 (73.3)	0	0
H77	10 (100)	0	0	0
H108	17 (100)	12 (70.6)	5 (29.4)	0

c. pH

Strain s	Inhibitory zone diameter in mm (residual activity, %)						
	pH=2 (30 min)	pH=4 (30 min)	pH=5 (30 min)	pH=7 (30 min)	pH=8 (30 min)	pH=10 (30 min)	pH=12 (30 min)
H9	7(70)	8(80)	9(90)	10(100)	11(110)	7(70)	0
H18	0	7(87.5)	10(125)	8(100)	8(100)	6(75)	4(50)
H51	0	8(80)	11(110)	10(100)	9(90)	4(40)	4(40)
H61	4(36.4)	6(54.5)	8(72.7)	11(100)	8(72.7)	7(63.6)	0
H77	6(46.1)	10(76.9)	12(92.3)	13(100)	11(84.6)	9(69.2)	0
H108	4(28.5)	7(50)	16(114.3)	14(100)	13(92.9)	10(71.4)	6(42.9)

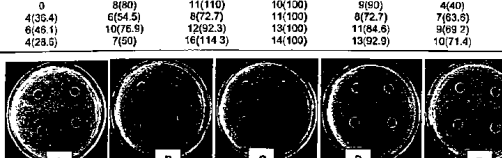

Fig. 2. The effect of enzymes (proteinase K, trypsin, lipase, α-amylase) (A-B), temperatures (60° C for 30 min, 100° C for 30 min, 121° C for 15 min) (C) and pH 2-12 (D-E) on the activity of crude bacteriocin extracts from the strain H108

The results from assessing the effect of temperature on the activity of the crude bacteriocin have shown that bacteriocin activity remained until 60° C for 30 min (expect the strain H77).(Table 2b & Fig 2). Especially, the bacteriocin extracted from the strain H108 remained nearly 30% activity at 100° C for 30 min but completely inactivated at autoclave condition. However, the bacteriocin activity of 6 strains was relatively stable between pH 4 - 10 (Table 2c & Fig. 2). Even the crude bacteriocins from the strains H18, H51 and H108 remained more than 40% activity at pH 12.

Overall, these results are similar to data recorded by bacteriocin class III, including large, heat-labile bacteriocins (Klaenhammer, 1993), suggesting that the bacteriocins from the isolates from otter clam in our research could belong to

Class III. The physico-chemical characteristics of bacteriocins from 6 marine bactena support important data for further studying on bacteriocin diversity in marine bacteria of Vietnam ongin and applying these bacteria as potential probiotics in aquaculture and food technology.

Identification and phylogenetic analysis of marine bacteriocin-producing bacteria

Morphological and cultural studies have shown that all six strains are facultatively anaerobic Gram-negative bacteria with rod - shaped cells, 4 - 8 mm in diameter of colonies. Further identification was carried out by 16S rDNA amplification and sequencing. Gene sequences were submitted to GenBank with the Gene Accession numbers KC894665, KC894666, KC894667, KC894668, KC894669 and KC894670 for the strains H9, H18, H51, H61, H77 and H108, respectively. The results from 16S rDNA gene sequencing of five strains H9, H18, H51, H61 and H108 revealed 100% homology to *Cronobacter sakazakii* strain Lc10g (GenBank Accession number JQ963902), 99.6% homology to *C. malonaticus* PHLTA-12 (FN401344), while the strain H77 had 100% homology to *Enterobacter cloacae* AB2 (JX188069), 99.2% to *E. kobei* BM0593 (JQ680938) and 98.1% to *E. cowanii* 6L (DQ919062).

Phylogenetic analysis of the 16S rRNA gene sequences of these six isolates showed significant differences with type strains of their closest species within the *Cronobacter* and *Enterobacter* genera (Fig. 3 & 4). The strain H77 along with the type strain of *E. cloacae* was well distinguished with type strains of *E. kobei* and *E. cowanii*, suggesting the strain H77 could belong to *E. cloacae* (Fig. 3). However, five strains H9, H18, H51, H61 and H108 along with type strains of *C. sakazakii* and *C. malonaticus* show the same clusterings in the phylogenetic tree, which required further analysis for a species-level differentiation (Fig. 4).

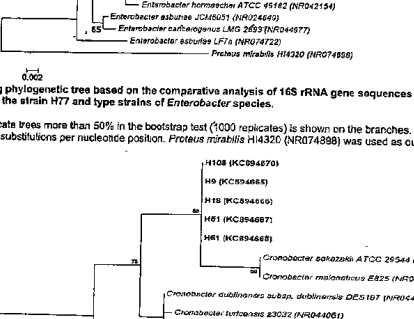

Fig 3. Neighbor joining phylogenetic tree based on the comparative analysis of 16S rRNA gene sequences showing the relationships between the strain H77 and type strains of *Enterobacter* species.

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. *Proteus mirabilis* HI4320 (NR074898) was used as outgroup.

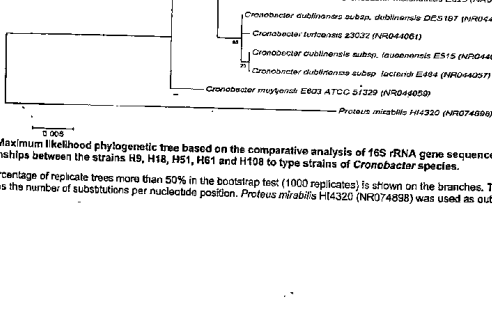

Fig 4. Maximum likelihood phylogenetic tree based on the comparative analysis of 16S rRNA gene sequences showing the relationships between the strains H9, H18, H51, H61 and H108 to type strains of *Cronobacter* species.

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. *Proteus mirabilis* HI4320 (NR074898) was used as outgroup.

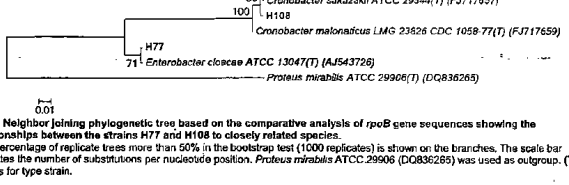

Fig 5. Neighbor joining phylogenetic tree based on the comparative analysis of *rpoB* gene sequences showing the relationships between the strains H77 and H108 to closely related species.
The percentage of replicate trees more than 50% in the bootstrap test (1000 replicates) is shown on the branches. The scale bar indicates the number of substitutions per nucleotide position. *Proteus mirabilis* ATCC 29906 (DQ836265) was used as outgroup. (T) stands for type strain.

Therefore, the *rpoB* genes of the strains H77 and H108 was further amplified and sequenced with the Gene Accession numbers KF028398 and KF028399, respectively. Phylogenetic analysis of the *rpoB* gene sequences of these two strains with closely related species confirmed H77 belonging to *E. cloacae* and revealed H108 as *C. sakazakii*, which was well separated with *C. malonaticus* in the evolutionary tree (Fig. 5). These results are agreement with the previous studies (Mollet *et al.*, 1997; Glammanco *et al.*, 2011), in which *rpoB* sequence analysis with better resolution than 16S rDNA sequencing could be used as a marker for bacterial identification in the Enterobacteriaceae family.

CONCLUSIONS

Total 128 marine bacteria strains were isolated from otter clam (*Lutraria philippinarum*) captured in Nha Trang Bay and Cam Ranh Bay. Among them, 6 strains H9, H18, H51, H61, H77 and H108 were found to express the highest and stable bacteriocin activity. They perhaps produced bacteriocins class III, including large, heat-labile bacteriocins but these substances were relatively stable at pH 4 - 10. Finally, sequence and phylogeny analysis of the 16S rRNA and *rpoB* genes indicated that the strain H77 was identified as *Enterobacter cloacae* and the other strains as *Cronobacter sakazakii*.

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PHÂN LẬP VÀ PHÂN TÍCH PHÁT SINH LOÀI CỦA VI KHUẨN BIỂN SỐNG TRÊN TU HÀI (*LUTRARIA PHILIPPINARUM*) CHO THẤY KHẢ NĂNG SINH BACTERIOCIN BỞI *CRONOBACTER SAKAZAKII* VÀ *ENTEROBACTER CLOACAE*

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TÓM TẮT

Bacteriocin là các peptide kháng khuẩn được sản sinh bởi một vi khuẩn này để ức chế sinh trưởng của các vi khuẩn khác có quan hệ tiến hóa gần gũi. Chúng đang được tìm kiếm nhằm bảo vệ sức khỏe cho người, vật nuôi và một số thực vật. Mục tiêu của nghiên cứu này là phân lập, xác định hoạt tính sinh bacteriocin và phân tích quan hệ phát sinh loài của các chủng vi khuẩn biển sống trên ốc hải (*Lutraria philippinarum*) thu từ Vịnh Nha Trang và Vịnh Cam Ranh. Trong số 128 chủng vi khuẩn phân lập được, có 19 chủng (15%) có khả năng sinh bacteriocin kháng lại ít nhất một trong các chủng chỉ thị. Trong số này, 6 chủng H9, H18, H51, H61, H77 và H108 đã thể hiện hoạt tính sinh bacteriocin mạnh nhất. Hoạt tính kháng khuẩn của dịch bacteriocin thô từ các chủng này đã bị hất hoạt hoàn toàn khi xử lý với proteinase K và trypsin trong khi không bị ảnh hưởng bởi lipase và α-amylase. Tất cả 6 chủng đều là vi khuẩn Gram âm, kị khí tùy tiện, và có tế bào hình que. Kết quả giải trình tự đoạn gen 16S rDNA và *rpoB* chỉ ra rằng chủng H77 thuộc về loài *Enterobacter cloacae* và các chủng còn lại thuộc về loài *Cronobacter sakazakii*. Đây là thông báo đầu tiên về bacteriocin được sản sinh bởi chi *Cronobacter*. Nghiên cứu này làm giàu bộ sưu tập chủng vi khuẩn biển sinh bacteriocin và mở ra tiềm năng ứng dụng của chúng làm probiotic hoặc thuốc chữa bệnh trong nuôi trồng thủy sản.

Từ khóa: 16S rDNA, bacteriocin, *Cronobacter*, *Enterobacter*, vi khuẩn biển, *rpoB*, probiotic .

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