ISOLATION AND SELECTION OF LACTIC ACID BACTERIA FROM VIETNAMESE FERMENTED PORK MEAT PRODUCT WITH ANTIMICROBIAL ACTIVITY AND CHARACTERIZATION OF BACTERIOCIN

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ABSTRACT

The objective of this study was to isolate lactic acid bacteria (LAB) from Vietnamese fermented pork meat (nem chua) and to determine their antimicrobial activity. A total of 101 LAB isolates were screened for their inhibitory effect on the indicator organism *Lactobacillus plantarum* JCM 1149 in the first selection by the agar spot test. Results showed that 58 isolates had activity against *Lb. plantarum* JCM 1149. From the 58 LAB, the second selection by agar well diffusion method used *Lb. plantarum* JCM 1149, *Listeria monocytogenes*, *Bacillus cereus*, *E. coli*, and *Salmonella typhimurium* as indicator organisms, and were inhibited by 18, 17, 6, 16, and 0 isolates, respectively. There were 5 strains (NH3.6, NT1.3, NT1.6, NT2.9, and NT3.20) with wide spectrum inhibition antimicrobial activity of both pathogenic gram-positive *B. cereus* and *L. monocytogenes*, and gram-negative *E. coli*. Furthermore, protease K enzyme was applied to test the antimicrobial activity of the 5 LAB isolates by bacteriocin, acid, or H_2O_2. Results indicated the NH3.6 strain had antimicrobial activity by bacteriocin. In addition, this research characterized the bacteriocin of the NH3.6 isolate and found it was stable in temperatures at 100°C and 120°C for 10 minutes, a pH = 3, and tolerant of NaCl concentration less than 8%.

Keywords: Antimicrobial activity, Bacteriocin, indicator bacteria, lactic acid bacteria.

Phân lập, tuyển chọn vi khuẩn lactic từ nem chua với khả năng kháng vi sinh vật và đặc điểm của bacteriocin

TÓM TẮT

Mục đích của nghiên cứu này là phân lập vi khuẩn lactic từ nem chua và xác định khả năng kháng vi sinh vật của chúng. 101 chủng vi khuẩn lactic đã được tuyển chọn tiến hành nghiên cứu tác động hạn chế vi sinh vật kiểm định *Lactobacillus plantarum* JCM 1149 trong tuyển chọn lần đầu bằng phương pháp cấy chấm điểm. Kết quả đã chỉ ra 58 chủng có khả năng kháng *Lb. plantarum* JCM 1149. Từ 58 chủng này sau tuyển chọn lần 2 bằng phương pháp khuếch tán đĩa thạch với vi sinh vật kiểm định là *Lb. plantarum* JCM 1149, *Listeria monocytogenes*, *Bacillus cereus*, *E. coli*, *Salmonella typhimurium* đã chỉ ra có 18, 17, 6, 16, 0 chủng kháng *Lb. plantarum* JCM 1149, *B. cereus*, *L. monocytogenes*, *E. coli, Sal. typhimurium*, tương ứng. Đặc biệt có 5 chủng (NH3.6, NT1.3, NT1.6, NT2.9, NT3.20) có hoạt tính kháng khuẩn rộng hạn chế cả vi khuẩn gram dương và gram âm. Ngoài ra, protease K enzyme cũng được ứng dụng để nghiên cứu khả năng kháng vi sinh vật của 5 chủng vi khuẩn lactic này do sinh bactericocin hay do sinh acid, H_2O_2 …. Kết quả đã chỉ ra có 1 chủng NH3.6 có khả năng sinh chất kháng khuẩn là bacteriocin. Thêm vào đó, nghiên cứu này cũng nghiên cứu một số đặc điểm của bacteriocin do chủng NH3.6 sinh ra có thể chịu nhiệt độ lên tới 100°C và 120°C trong 10 phút, hoạt động tốt nhất ở pH = 3, và có khả năng chịu được nồng độ muối nhỏ hơn 8%.

Từ khóa: Bacteriocin, hoạt tính kháng khuẩn, vi khuẩn kiểm định, vi khuẩn lactic.

1. NTRODUCTION

Fermented pork meat (nem chua) is a traditional product of Vietnam. The fermentation takes place without the addition of a starter culture or any further cooking or heating (Nguyen *et al.*, 2011; Nguyen *et al.*, 2013). Nem chua is a good source of lactic acid bacteria (LAB). LAB strains isolated from nem chua include *Lactobacillus pentosus, Lb. plantarum*, *Lb. brevis*, *Lb paracasei*, *Lb. fermentum*, *Lb. acidipiscis*, *Lb. farciminis*, *Lb. rossiae*, *Lb. fuchuensis*, *Lb. namurensis*, *Lc. lactis*, *Leuconostoc citreum*, *Leuconostoc fallax*, *Pediococcus acidipiscis*, *Pediococcus pentosaceus*, *Pediococcus stilesii, Weissella cibaria,* and *Weissella paramesenteroides* (Nguyen *et al.*, 2013).

Bacteriocins may be produced by both gram-positive and gram-negative species (Savadogo *et al.*, 2006). In recent years, bacteriocin produced by LAB have attracted significant attention because of their generally recognized as safe (GRAS) status and potential use as safe additives for food preservation (Diop *et al.*, 2007; Leah *et al.*, 2011). According to the definition of Klaenhammer (Klaenhammer, 1988), bacteriocins produced by LAB are active against closely related bacteria. The structure and composition of the outer membrane of gram-negative bacteria does not allow bacteriocins access to the cytoplasmic membrane. However, a few exceptions have been described of bacteriocins that possess activities against gram-negative bacteria, including bacteriocin ST34BR produced by *Lc.* subsp. *lactis* (Todorov and Dicks, 2004), and bacteriocins ST26MS and ST28MS produced by *Lb. plantarum* (Todorov and Dicks, 2005).

Although a large number of LAB bacteriocins have been identified and characterized, only nisin, produced by *Lc. lactis*, the most thoroughly studied bacteriocin to date, has been applied as an additive to certain foods worldwide (Delves *et al.*, 1996). Substantial work has been done on the effectiveness of nisin on various spoilage and pathogenic microorganisms, such as *Listeria monocytogenes*, and its application in different food products (Freitas *et al.*, 2008; Staszewski and Jagus 2008). Other bacteriocins, such as pediocin, may also have potential applications in foods, though they are not currently approved as antimicrobial food additives (Naghmouchi *et al.*, 2007).

Isolation and screening of LAB from natural sources has always been the most powerful means for obtaining useful and genetically stable strains (Ibourahema *et al.*, 2008). In the present study, we isolated and selected LAB from nem chua, evaluated their antimicrobial effects *in vitro*, and characterized the peptides, with the hope of finding new bacteriocin to be used in applications as safe additives for food preservation.

2. MATERIALS AND METHODS

2.1. Sample Collection

Six samples of nem chua were collected from various households in Hanoi and Thanh Hoa. In Hanoi, samples were obtained from three households, i.e. NH1, NH2, and NH3. In addition, three samples were collected from 3 households in Thanh Hoa, designated as NT1, NT2, and NT3. Nem chua samples were collected at the same stage of fermentation, i.e after approximately 48 to 72 hours of fermentation at ambient temperature. Ambient temperatures varied between 32°C in Hanoi and 35°C in Thanh Hoa. All nem chua samples were naturally fermented.

2.2. Indicator strains

Lb. plantarum JCM 1149*, L. monocytogenes, Bacillus cereus, Escherichia coli,* and *Salmolnella typhimurium* were supplied by the Institute of Biotechnology, Vietnam Academy of Science and Technology, Hanoi, Vietnam for antimicrobial activity tests.

2.3. Isolation of lactic acid bacteria

Isolation of LAB was described by Nguyen *et al.* (2013). For microbial analysis, each nem chua sample (25 grams) was homogenized in 225 ml sterilized water after which 10-fold serial dilutions were prepared. MRS (De Man

Rogosa Sharpe) agar was used for the isolation of LAB. One-hundred µl of diluted solution was spread directly onto the surface of MRS agar plates. Then, these plates were incubated for 48 h at 30°C. Identification of colonies as LAB was performed by morphological tests as described by Barnali and Subhankar (2010). All isolates were initially screened for the production of catalase. Only catalase negative isolates (n = 101) were considered as LAB and were stored in MRS media supplemented with glycerol 40% at -80°C until further analysis.

2.4. Screening of LAB isolates for antibacterial activity using the agar spot method (the first method for screening)

The inhibitory activity of the selected LAB isolates against the indicator strain (*Lb. plantarum* JCM 1149) was assayed by the agar spot test described by Fleming *et al.* (1975). The LAB isolates were spotted onto the surface of MRS agar plates and incubated at 30°C for 18 h to allow the colonies to develop. The indicator strain (*Lb. plantarum* JCM 1149) was inoculated into 10 ml of soft MRS agar (0.9% agar) and poured over the plate on which the LAB isolates were grown. These plates were kept in the fridge at 4°C for 4 h, and then incubated at 30°C for 18 h. After that, the plates were examined for the presence of inhibition zones. Inhibition was considered positive when the width of the clear zone around the colonies of the LAB isolates was 0.5 mm or larger. The selected antimicrobial isolates were used for further study.

2.5. Screening of LAB isolates for antibacterial activity using the well agar diffusion method (the second method for screening)

The well agar diffusion bioassay was the second method used to study the antibacterial effect of the LAB isolates that were selected in section 2.4. In this study, the agar diffusion method was performed following the method of Herreros *et al.* (2005). *Lb. plantarum* JCM 1149, *L. monocytogenes*, *B. cereus*, *E. coli*, and *Sal. typhimurium* were used as indicator organisms.

LAB isolates were cultured in MRS broth, and incubated at 30°C for 18-20 h. Cells were removed by centrifuging at 6000 g for 20 minutes. Cell-free supernatant (CFS) from each LAB isolate, which was adjusted to pH 6.8-7 with NaOH 1 N in order to eliminate possible inhibition effects due to organic acids, was added to each well.

For the indicator organisms, *Lb. plantarum* JCM 1149 was cultured in MRS broth, and *L. monocytogenes*, *B. cereus*, *E. coli*, and *Sal. typhimurium* were cultured in LB broth, and incubated at 30°C for 24 h. Then 30 µl of each solution was spread on the MRS agar plate (*Lb. plantarum* JCM 1149) and LB agar plates (*L. monocytogenes*, *B. cereus*, *E. coli*, and *Sal. typhimurium*). Four to six wells in each plate (8 mm diameter) were cut and 80 µl of cell-free supernatant (CFS) from each LAB isolate, with an appropriate adjustment to pH 6.8-7, was added to each well. These plates were kept in the fridge at 4°C for 4 h, and then incubated at 30°C for 24 h. If inhibition zones were found in the well, the isolates were considered to be able to produce bacteriocin-like substances (BLS).

2.6. Enzymatic test for presence of bacteriocins in lactic acid bacteria cell free supernatant

The possible presence of proteinaceous compounds in the CFS from the LAB isolates showed antibacterial activity after acid neutralization. They were further analyzed in our studies by incubating the treated CFS with 1 mg/ml proteolytic enzyme (proteinase K) at 37°C for 2 h following the method of Bonade *et al.* (2001). Both the control and the samples were assayed for antimicrobial activity by using the well diffusion method as described above (section 2.5). In this experiment, *Lb. plantarum* JCM 1149 and *E. coli* were used as indicator organisms.

2.7. Determining the activity units (AU/ml)

The method for determining the activity units was the continuous dilution method according to Tran Thi Thuy (1999). Studied LAB isolates were cultured in MRS solution at

30°C for 24 h. Then, this solution was centrifuged at 6000 g for 20 minutes to remove the cell pellet. The supernatant was diluted by MRS and put in tubes. An indicator organism was added with a 103 CFU/ml concentration and incubated at 30°C for 24 h. Next, all the tubes were checked for having cloudiness or not. Antibacterial activity units of LAB isolates were determined by using AU/ml: the largest concentration in a range of diluted continuous solutions at which the indicator organism (Lb. plantarum JCM 1149) is still inhibited. AU/ml was calculated using the formula:

$AU / ml = 1 : (V \times 2^n \times 1000)$

V: the volume of each dilution into each well to determine activity (µl).

2^n: the n dilution which still expresses bacteriocin activity.

The method for determining the activity unit (AU%) was by the following formula:

$AU(\%) = AU_{(treated)} : AU_{(control)} \times 100\%$

$AU_{(treated)}$: the activity unit of the cell-free supernatant (crude bacteriocin) that were treated in the different environmental conditions such as heat, pH, and NaCl.

$AU_{(control)}$:the activity unit of the cell-free supernatant (crude bacteriocin) before adjusting to different environmental conditions such as heat, pH, and NaCl.

2.8. Characterization of bacteriocin

In this study, the methods for characterization of bacteriocin were performed following the methods of Tran Thi Thuy (1999).

All control samples were not treated.

Heat stability: A volume of 5 ml of cell cell-free supernatant (crude bacteriocin) in different test tubes was overlaid with paraffin oil to prevent evaporation and then heated to 40, 60, 80, 100, and 121°C for 10, 20, 40, and 60 minutes. The heat-treated samples were then assayed for AU/ml as described previously.

pH sensitivity: A volume of 5 ml of cell-free supernatant (crude bacteriocin) was pipetted into test tubes and each tube adjusted to a different pH (2-10) using either sterile NaOH 5N or HCl 1N. Treated samples were incubated for 1 hour at room temperature before determination of AU/ml as described previously.

NaCl sensitivity: Different NaCl concentrations were added to the centrifugal services to achieve concentrations of 2, 4, 6, 8, and 10% NaCl for 60 minutes and then AU/ml was determined.

2.9. Statistical analysis

Data was statistically analyzed using Irristat program.

3. RESULTS AND DISCUSSION

3.1. Enumeration of LAB isolates from fermented pork meat

In the present study, 6 samples of nem chua from different households in two cities (Hanoi and Thanh Hoa) in Northern Vietnam were used. LAB counts on MRS agar ranged from 10^6 to 10^7 CFU/g (Table 1). At the moment of sampling, nem chua samples differed in fermentation time (48 h or 72 h), in pH (ranging from 4.3 to 5.0), and ambient temperature of fermentation (32°C in Hanoi or 35°C in Thanh Hoa).

The highest counts on the MRS agar were observed for NH2 with 34 x 10^7 CFU/g, whereas the lowest number, 33 x 10^6 CFU/g, was associated with NH1. Nem chua samples collected from Thanh Hoa were characterized by having lower pH values than samples collected in Hanoi city.

Starting from conventional culturing using MRS agar, a total of 150 isolates were picked based on morphological tests. All isolates were initially screened for the production of catalase. Of these, 101 isolates were negative for catalase activity, were considered to be LAB, and were used to test antimicrobial activity. The number of presumptive LAB isolates retrieved from each sample is depicted in Table 1.

Table 1. Main charateristics of 6 nem chua samples included in this study

Samples	City	Fermentation			CFU/g	Number of LAB isolates recovered
	Designation	time (h)	temperature (°C)	pH		
NH1	Hanoi	72	32	5.0	33×10^6	13
NH2		72	32	4.9	37×10^7	22
NH3		72	32	5.0	28×10^7	18
NT1	Thanh Hoa	48	35	4.6	53×10^6	13
NT2		48	35	4.3	64×10^6	15
NT3		48	35	4.4	40×10^6	20

Table 2. Zone of inhibition (mm) of the isolated LAB against *Lb.platarum* JCM1149

Orders	Isolates	Zone of inhibition (D-d) (mm)	Orders	Isolates	Zone of inhibition (D-d) (mm)	Orders	Isolates	Zone of inhibition (D-d) (mm)	Orders	Isolates	Zone of inhibition (D-d) (mm)
1	NH1.1	4	16	NH2.17	12	31	NT1.4	5	46	NT2.14	6
2	NH1.2	7	17	NH2.18	9	32	NT1.6	11	47	NT3.1	5
3	NH1.3	4	18	NH2.20	8	33	NT1.9	9	48	NT3.2	6
4	NH1.4	5	19	NH3.2	6	34	NT1.10	5	49	NT3.3	9
5	NH1.5	5	20	NH3.3	10	35	NT1.11	6	50	NT3.5	6
6	NH1.6	3	21	NH3.5	8	36	NT1.13	8	51	NT3.8	4
7	NH1.7	4	22	NH3.6	11	37	NT2.1	9	52	NT3.9	4
8	NH1.8	6	23	NH3.7	6	38	NT2.3	4	53	NT3.14	5
9	NH1.12	4	24	NH3.11	4	39	NT2.5	6	54	NT3.15	8
10	NH1.13	8	25	NH3.12	4	40	NT2.6	5	55	NT3.17	6
11	NH2.1	5	26	NH3.13	6	41	NT2.7	6	56	NT3.18	9
12	NH2.6	4	27	NH3.14	5	42	NT2.9	10	57	NT3.20	10
13	NH2.9	5	28	NH3.15	8	43	NT2.11	4	58	NT3.21	5
14	NH2.11	9	29	NT1.2	10	44	NT2.12	5			
15	NH2.13	6	30	NT1.3	7	45	NT2.13	4			

3.2. Screening of LAB isolates for antibacterial activity using the agar spot method (the first method for screening)

Bacteriocins are ribosomally synthesized antimicrobial peptides and often inhibit similar or closely related bacteria (Bowdish *et al.*, 2005; Cotter *et al.*, 2005). Therefore, in the first screening method for antimicrobial activity, we used *Lb. plantarum* JCM 1149 as the indicator microorganism.

From 101 LAB strains, there were 58 strains with different profiles of antibacterial activity against *Lb. plantarum* JCM 1149 (as shown in Table 2). Similar results were reported by Tran Thi Thuy (1999) who showed that LAB isolates from chicken intestine also had antibacterial activity against *Lb. plantarum* JCM 1149. Of the 58 strains, seven isolates (accounting for 12.1%) (NH2.17, NH3.3, NH3.6, NT1.2, NT1.6, NT2.9, and NT3.20, highlighted in gray in Table 2) were characterized by having high spectrum inhibition with a zone of inhibition (D-d) ≥ 10 mm. In addition, 25.9% of the isolates had a (D - d) between 7-9 mm, and 62% of the isolates had a (D - d) ≤ 6 mm.

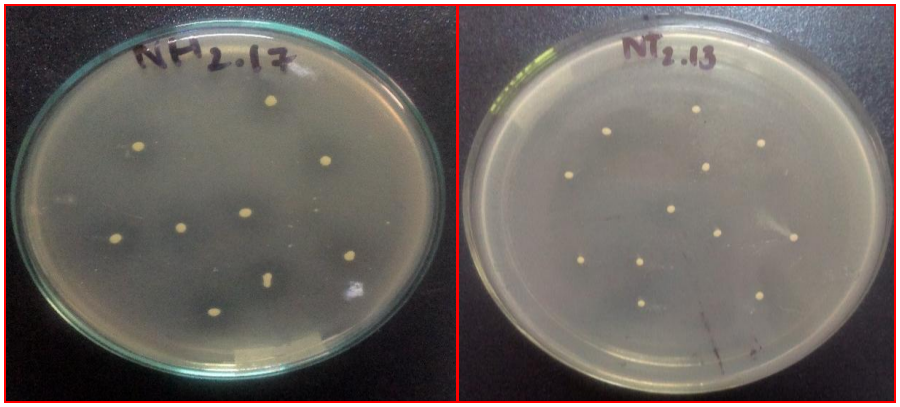

Figure 1. Against *Lb. plantarum* JCM 1149 activity of some LAB isolates

Figure 1 shows that the NH2.17 isolate has antibacterial inhibition that is clearer, rounder, and larger than NT2.13. Fifty-eight strains had activity against *Lb. plantarum* JCM 1149 by bacteriocin or acid. The first selection results, however, cannot determine whether these strains produced bacteriocin or not. We continued to proceed with a second selection for the 58 strains by the well agar diffusion method.

3.3. Screening of LAB isolates for antibacterial activity using the well agar diffusion method (the second method for screening)

The well agar diffusion method was performed as described in section 2.5 with *Lb. plantarum* JCM 1149, *L. monocytogenes*, *B. cereus*, *E. coli*, and *Sal. typhimurium* used as indicator organisms.

In the second selection, cell-free supernatant (CFS) from each LAB isolate was adjusted to pH 6.8-7 with NaOH 1 M in order to eliminate possible inhibition effects due to organic acids before being added each well. The study results showed that all 58 strains of lactic acid bacteria had antibacterial activity against *Lb. plantarum* JCM 1149 in the first test, but there were only 18 isolates of LAB with activity against *Lb. plantarum* JCM 1149 with different profiles of inhibition against indicator organisms in the second test as shown in Table 3. In these 18 strains, there were 5 strains (NH3.6, NT1.3, NT1.6, NT2.9, and NT3.20, highlighted in gray in Table 3) that inhibited both gram-positive and gram-negative bacteria and had a large zone of inhibition diameter. Similar results were reported by Todorv and Dicks (2004) who showed that bacteriocin ST34BR produced by *Lc. lactis* subsp. *lactis* inhibits the growth of *Enterococcus faecalis, Lb. plantarum, Lb. casei, Pseudomonas aeruginosa,* and *Staphylococcus aureus*. Two additional bacteriocins, ST28MS and ST26MS, produced by *Lb. plantarum* isolated from molasses, inhibited the growth of *Lb. casei, Lb. sakei,* and *Staphylococcus aureus* (Todorov and Dicks, 2005).

No strain had activity against *Sal. typhimurium*. Similar results were reported by previous research showing bacteriocins from gram-positive bacteria had difficulty affecting gram-negative bacteria (Bowdish *et al.*, 2005; Cotter *et al.*, 2005). The structure and composition of the outer membrane of gram-negative bacteria does not allow access of bacteriocins to the cytoplasmic membrane (Gong *et al.*, 2010).

However, a few exceptions have been described of bacteriocins possessing activities against gram-negative bacteria, e.g. bacteriocin ST34BR produced by *Lc. lactis* subsp. *lactis* inhibits the growth of *E. coli*, (Todorov and Dicks, 2004), and two bacteriocins, ST28MS and ST26MS, produced by *Lb. plantarum* isolated from molasses, inhibited the growth of *E. coli* and *Acinetobacter baumanii* (Todorov and Dicks, 2005).

Table 3. Zone of inhibition (mm) of the isolated LAB against some indicator microorganism

Ký hiệu	Zone of inhibition diameter (D-d) (mm)				
	Lb. plantarum JCM1149	*E. coli*	*Listeria monocytogenes*	*Bacillus cereus*	*Salmonella typhimurium*
NH1.2	5	4	-	5	-
NH1.14	5	-	-	5	-
NH2.11	4	4	-	6	-
NH2.17	4	5	-	3	-
NH2.18	4	4	-	4	-
NH3.5	4	-	-	5	-
NH3.6	5	6	5	5	-
NH3.15	4	3	-	4	-
NT1.2	6	4	-	5	-
NT1.3	5	6	-	6	-
NT1.6	6	5	-	5	-
NT1.9	4	4	-	6	-
NT1.13	4	4	1.5	2	-
NT2.1	4	3	1.5	2	-
NT2.9	6	5	5	6	-
NT3.3	4	4	4	4	-
NT3.18	3	4	2	-	-
NT3.20	6	6	-	5	-

Note: (-) : no antimicrobial with indicator organisms

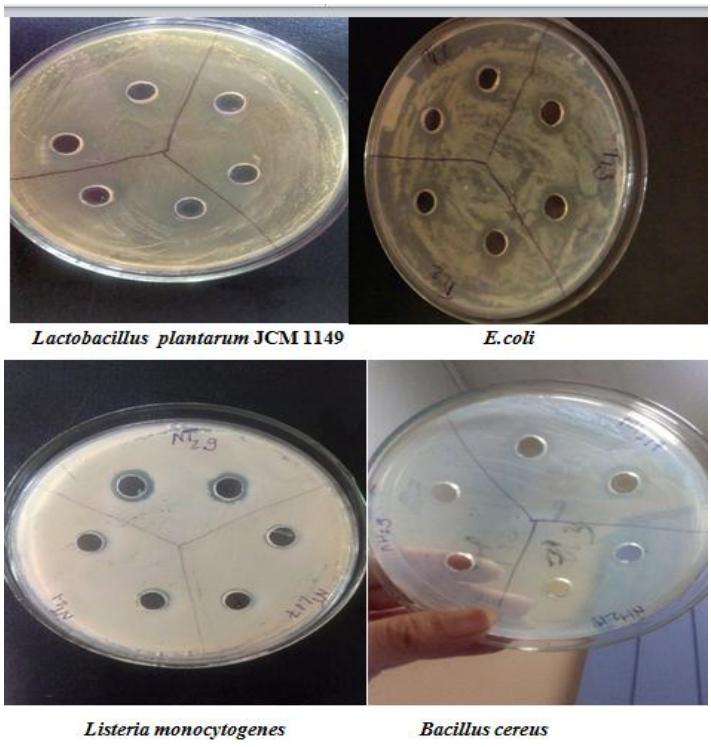

Figure 2. Antibacterial activity of a selection of LAB isolates for indicator microorganism

a. *Lb. plantarum* JCM1149 b. *E. coli*

Figure 3. Recognize bacteriocin by proteinase K for NH3.6 isolate

Note: a. 12, 13, Control, the other well: proteinase K

The results of the present study showed that the cell free supernatant of lactic acid bacteria has antimicrobial abilities from bacteriocin, CO_2, and H_2O_2. Therefore, we could not confirm whether these strains produce bacteriocin or not, and they continue to be studied in the next experiment.

3.4. Antimicrobial lactic acid bacteria isolates capable of yielding bacteriocin (the third selection)

Bacteriocins are antimicrobial peptides. To confirm the production of antimicrobial compounds by bacteriocin, proteinase K was used. Cell free supernatant of 5 strains (NH3.6, NT1.3, NT1.6, NT2.9, and NT3.20) with high antimicrobial activity against the indicator organisms were neutralized and treated with proteinase K as described in section 2.6. There was only one cell free supernatant of the NH3.6 strain that did not have antibacterial activity against both gram-positive and gram-negative bacteria after treatment with proteinase K (Figure 3). This result confirms that the NH3.6 strain produces bacteriocin. The present study has similar results with several previous studies. Tran Thi Thuy (1999) also chose proteinase K for identification of bacteriocin produced by lactic acid bacteria isolates. Besides, another study also indicated that the treatment of plantaricin MG produced by Lb plantarum KLDS1.0391 with proteinase K lead to loss of activity (Gong *et al.,* 2010).

3.4. Effect of temperature and time, pH, and NaCl on antibacterial ability of selected isolate

In the storage process, the factors of temperature, time, and pH are all very important. Bacteriocins are selected not only for preserving foods but also in processing. Therefore, the factors of temperature, time, and pH were used to study their effects on the antibacterial ability of the selected isolate.

Effect of temperature and time on the activity of NH3.6 bacteriocin

We chose a set of temperatures (4, 40, 60, 80, 100, and 121°C) and a set of times (10, 20, 40, and 60 minutes) to conduct this research. The experiment results are presented in Table 4.

According to the results presented in Table 4, at cold temperatures (4°C) the activity of lactic acid bacteria NH3.6 bacteriocin did not change compared to the control (AU > 90%), and from 40-60°C the activity of the bacteriocin decreased from 30% to 35% but depended very little on the time. From 80-121°C, the activity of the bacteriocin decreased slightly and depended clearly on time, i.e. treatment time increases led to activity decreases.

Moreover, the results of the study also showed that bacteriocin born from the NH3.6 strain has a heat tolerant ability as it retains over 50% activity units when exposed to 100°C to 121°C temperatures for 10 minutes and retains 18% activity units when exposed for 60

minutes. This feature is necessary for food preservatives because there are some products that need to be pasteurized before preserving. There are very few lactic acid bacteria able to produce bacteriocin with high thermal stability. Some researchers reported that plantaricin 423, a bacteriocin produced by *Lb. plantarum*, is resistant to treatment at 80°C, but loses 50% of its activity after 60 min at 100°C and 75% of its activity after autoclaving (121°C, 15 min) (Van *et al.*, 1998) Acidocin B, a bacteriocin produced by *Lb. acidophilus*, is active at 70°C for 10 minutes but at 100°C is no longer active (Zaheer *et al.*, 2010).

Table 4. Effect of temperature and time on the activity of NH3.6 bacteriocin

Temperature (°C)	Time (Minute)	Activity unit		Temperature (°C)	Time (Minute)	Activity unit	
		AU/ml	AU (%)			AU/ml	AU (%)
4	10	191	95.5	100	10	111	55.5
	20	186.3	93.15		20	84	42
	40	186.7	93.35		40	62	31
	60	185	92.5		60	46	23
40	10	133	66.5	121	10	102	51
	20	132	66		20	76	38
	40	132	66		40	55	27.5
	60	130	65		60	36	18
60	10	132	66	Control (no treating temperature)		200	100
	20	132	66				
	40	131	65.5				
	60	129	64.5				
80	10	115	57.5				
	20	106	53				
	40	96	48				
	60	85	42.5				

Effect of pH on the activity of NH3.6 bacteriocin

Cell-free supernatant of the NH3.6 strain was pipetted into test tubes and adjusted to different pH values (2-10), and kept for 1 hour at room temperature before determining the AU. The results are indicated in Table 5.

The results showed that the optimum pH for activity of the lactic acid bacteria NH3.6 bacteriocin was in a range from 3 to 4 (highlighted in gray in Table 5), outside this pH range the AU decreased. However, the obtained AU was still over 50% at pH 7, and over 22% at the pH range of 8-10. Therefore, it can be said that the lactic acid bacteria NH3.6 bacteriocin is a tolerant acid bacteriocin (suitable for food preservation fermentation), and is also relatively stable at a neutral pH (can be used for preservation for non-fermented foods). Similar results were obtained in previous studies, such as plantaricin 423, a bacteriocin produced by *Lb. plantarum*, remained active after incubation at a pH range of 1-10 (Van *et al.*, 1998), and acidocin B, a bacteriocin produced by *Lb. acidophilus*, remained active at a pH range of 2-10, and was only inactivated under high alkaline conditions (Zaheer *et al.*, 2010).

Effect of NaCl on the activity of lactic acid bacteria NH3.6 bacteriocin

Preserved fish and seafood products are usually high in salt content. Therefore, salt concentration is one of the important factors during preservation. To select the appropriate preservative compounds, we evaluated the

effects of NaCl concentration on the activity of lactic acid bacteria NH3.6 bacteriocin as follows: Cell-free supernatant of the NH3.6 strain was pipetted into test tubes, NaCl was added to reach different concentrations (2-10%), and tubes were kept for 1 hour at roomtemperature before determining the AU. The results are indicated in Table 6.

Table 6 shows that the strain NH3.6 bacteriocin has unchanged activity at the NaCl concentrations from 2% to 8%, but at the 10% concentration, the bacteriocin activity decreased markedly. This result suggests that lactic acid bacteria NH3.6 bacteriocin is suitable for food preservation with high NaCl. This result is similar the results of Tran Thi Thuy (1999) who reported that Enterocin Tn143, a bacteriocin from *Enterococcus* Tn143, has a tolerance ability in NaCl concentrations from 2 to 8%.

Table 5. Effect of pH on the activity of NH3.6 bacteriocin

pH	Activity Unit	
	AU/ml	AU (%)
2	136.3	68.15
3	164.67	82.3
4	160	80
5	143.33	71.7
6	120.33	60.2
7	104.33	52.2
8	85.7	42.85
9	75.3	36.75
10	45	22.5
Control	200	100

Table 6. Effect of NaCl on the activity of lactic acid bacteria NH3.6 bacteriocin

NaCl (%)	Activity Unit	
	AU/ml	AU(%)
2	184.3	92.15
4	182.7	91.35
6	176.67	88.3
8	171.3	85.65
10	122	61
Control	200	100

4. CONCLUSIONS

Our results revealed that 58 strains of the 101 isolates obtained from nem chua had antimicrobial abilities against the indicator organism. There were 5 strains (NH3.6, NT1.3, NT1.6, NT2.9, and NT3.20) with wide spectrum activity that inhibited both pathogenic gram-positive *B. cereus* and *L. monocytogenes*, and gram-negative *E. coli*. Of the 5 strains, only the NH3.6 strain was determined to have antimicrobial activity by bacteriocin. Moreover, bacteriocin NH3.6 strain was also characterized as being stable in temperatures of 100°C and 121°C for 10 minutes, having the most efficient activity at pH 3, and being salt tolerant at NaCl concentrations < 8%. With all the characterizations this bacteriocin may possess, it has the potential for many applications, especially for food preservation at low pH values, high salt concentrations such as fermented food, or foods that need high temperatures for processing.

REFENENCES

Barnali, A. and P. Subhankar (2010). Isolation and characterization off lactic acid bacteria from dairy effluents. Journal of Environmental Research And Development, 4: 983-992.

Bonade, A., F. Murelli, M. Vescovo, and G. Scolari (2001). Partial characterization of a bacteriocin produced by *Lactobacillus helveticus*. Letters in Applied Microbiology, 33: 153-158.

Bowdish, D.M., D.J. Davidson, and R.E. Hancock (2005). A re-evaluation of the role of host defence peptides in mammalian immunity. Current Protein and Peptide Science, 6(1): 35-51.

Cotter, P.D., C. Hill, and R.P. Ross (2005). Bacteriocins: developing innate immunity for food. Food Microbiology, 3(10): 777-788.

Delves, B.J., P. Blackburn, R.J. Evans, and J. Hugenholtz (1996). Applications of the bacteriocin, nisin. Antonie Van Leeuwenhoek International Journal of General, 69: 193-202.

Diop, M.B., D.R. Dibois, E. Tine, A.N. Jacqueline, and P. Thonart (2007). Bacteriocin producers from traditional food products. Biotechnology Agronomie Socciety Environnement, 11: 275-281.

Fleming, H.P., J.L. Etchells, and R.N. Costilow (1975) Microbial inhibition by an isolate of *Pediococcus*

from cucumber brines. Applied Microbiology, 30: 1040-1042.

Freitas, W.C., E.L. Souza, C.P. Sousa, and A.E.R. Travassos (2008). Anti- staphylococcal effectiveness of nisaplin in refrigerated pizza doughs. Brazilian Archives of Biology and Technology, 51: 95-599.

Gong H.S., X.C. Meng, and 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(1): 89-96.

Herreros, M.A., H. Sandoval, L. Gonzalez, J.M. Castro, J.M. Fresno, and M.E. Tornadijo (2005). Antimicrobial activity and antibiotic resistance of lactic acid bacteria isolated from Armada cheese (a Spanish goats’ milk cheese). Food Microbiology, 22: 455-459.

Ibourahema, C., R.D. Dauphin, D. Jacqueline, and P. Thonart (2008). Characterization of lactic acid bacteria isolated from poultry farms in Senegal. African Journal of Biotechnology, 7: 2006-2012.

Klaenhammer, T. R. (1988). Bacteriocins of lactic acid bacteria. Biochimie, 70: 337- 349.

Leah, A.M.V., Y. Sabesan, S.S. Clarissa, T.L. Christopher, and C.V. John (2011). The activity of bacteriocins from *Carnobacterium maltaromaticum* UAL307 against gram-negative bacteria in combination with EDTA treatment. FEMS Microbiology Letters, 317: 152-159.

Naghmouchi, K., E. Kheadr, C. Lacroix, and I. Fliss (2007). Class I /Class IIa bacteriocin crossresistance phenomenon in *Listeria monocytogenes*. Food Microbiology, 24: 718-727.

Nguyen, H., F. Elegado, N. Librojo-Basilio, R. Mabesa, and E. Dizon (2011). Isolation and characterisation of selected lactic acid bacteria for improved processing of nem chua, a traditional fermented meat from Vietnam. Beneficial Microbes, 1: 67-74.

Nguyen, T.L.D., H.K. Van, M. Cnockaert, B.E. De, B.K. De, T.B. Le, and P. Vandamme (2013). A culture-dependent and independent approach for the identification of lactic acid bacteria associated with the production of nem chua, a Vietnamese fermented meat product. Journal of Food Research International, 50: 232-240

Savadogo, A., C.A.T. Ouattara, I.H.N. Basssole, and S.A. Traoer (2006). Bacteriocins and lactic acid bacteria - a minireview. Africa Journal of Biotechnology, 5: 678-683.

Staszewski, M. and R.J. Jagus (2008). Natural antimicrobials: Effect of Microgard and nisin against *Listeria innocua* in liquid cheese whey. International Dairy Journal, 18: 255-259.

Todorov, S.D. and L.M.T. Dicks (2004). Influence of growth conditions on the production of a bacteriocin by *Lactococcus lactis* subsp. *lactis* ST 34BR, a strain isolated from barley beer. Journal of Basic Microbiology, 44: 305-316.

Todorov, S.D. and L.M.T. Dicks (2005). *Lactobacillus plantarum* isolated from molasses produces bacteriocins active against Gram negative bacteria. Enzyme and Microbial Technology Journal, 36: 318-326.

Tran Thi Thuy (1999). Phân lập và tuyển chọn và nghiên cứu vi khuẩn sinh Bacteriocin cao từ thực phẩm giàu protein lên men lactic. Luận án thạc sĩ khoa sinh học, Đại học quốc gia, Trường đại học Sư phạm.

Van, R.C.A., L.M. Dicks, and M.L. Chikindas (1998). Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. Journal of Applied Microbiology, 84: 1131-1137.

Zaheer, A., W. Yaing, C. Qiaoling, and M. Imran (2010). *Lactobacillus acidophilus* bacteriocin, from production to their application: an overview. African Journal of Biotechnology, 9: 2843-2850.