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# LAB ISOLATED, SCREENED AND SELECTED FROM LOCAL INDIGENOUS FERMENTED BEEF SAUSAGE (NAEM-NEUA) PRODUCTS FOR PROBIOTIC STARTER CULTURES IMPLEMENTATIONS

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**Abstract –** The high potential lactic acid bacteria (LAB) were isolated from local indigenous fermented beef sausage (Naem-Neua) products collected from three different small-scale food fermentation industries which are famous for their good quality beef in Sakon Nakhon province located in the Northeastern region of Thailand. Naem-Neua products were collected after they had been produced for 48-72 h and subsequently maintained under refrigerated condition (below 4 °C). The sample location codes were SN1, SN2 and SN3. Naem-Neua products were investigated for their physicochemical characteristics including pH, Aw, % titratable acidity, surface color and texture profile analysis (TPA). It was found that pH of SN3 was the highest value (pH 5.05) whereas Aw values of SN2 and SN3 were the highest ones (0.97) ($p \leq 0.05$). Also, % titratable acidity of SN1 was the highest value (0.45) ($p \leq 0.05$). However, only the pH of SN1 was complied with Thai Community Product Standard; Fermented Beef Sausage, Naem-Neua; TCP 470-2555 (2012). In addition, L* of SN1 was the highest value (41.32) while a* and b* of SN2 and SN3 were the highest ones (a* = 4.63 and 5.33, respectively, b* = 20.80 and 20.23, respectively) ($p \leq 0.05$). Moreover, hardness, gumminess and chewiness values of SN2 were the highest ones as they were 22.05 N, 12.45N and 7.35N.mm, respectively whereas springiness values of SN2 and SN3 were the highest ones as they were 0.59 mm and 0.63 mm, respectively ($p \leq 0.05$). Besides, cohesiveness value of SN1 was the highest one (0.69) ($p \leq 0.05$). For microbiological analysis, total aerobic plate count, lactic acid bacteria, yeast and mold were reported for all samples. In addition, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella* were not found in any samples which were complied with Thai Community Product Standard; Fermented Beef Sausage, Naem-Neua; TCP 470-2555 (2012) regardless of *Clostridium perfringens* in SN2 (3.43 Log CFU/g). For enumeration, isolation and screening of LAB as starter cultures from Naem-Neua products, colonies were randomly selected from MRS agar plates of Naem-Neua samples (SN1, SN2, SN3) and streaked again on MRS agar for further purification of the isolates. Primary screening of 45 LAB isolates were conducted using agar well diffusion assay and clear zones were recorded. The results showed that only 16 out of 45 LAB isolates could inhibit the growth of the tested pathogens. After that, secondary screening of 16 LAB isolates was then explored for their tolerances to high temperatures, acidic pHs, bile, high concentrations of lactic acid and sodium chloride. It was revealed that LAB isolates could not grow at 4 °C, 15 °C and 50 °C whereas most LAB isolates could grow at 30 °C, 37 °C and 45 °C. However, some LAB isolates including ITP01, ITP07, ITP12, ITP14 and ITP15 could not grow at 45 °C. In addition, for tolerances of LAB isolates to acidic pHs and bile salt, it was discovered that ITS14 could survive the most in MRS broth at pH 3.0 (adjusted with 1N HCl), however, all of LAB isolates did not appear in MRS broth at pH 2.0. Also, all of LAB isolates could survive in MRS broth supplemented with bile salt and it was found that ITP01 and ITP09 could survive the most in MRS broth added with 1% bile salt ($p \leq 0.05$). Furthermore, all of LAB isolates were not tolerant to lactic acid whereas most of them could tolerate to NaCl from 1.5% up to 5% as well as ITY11 and ITS14. Finally, two high potential LAB isolates including ITY11 and ITS14 were selected for probiotic starter cultures implementations and they were subsequently identified by 16s rDNA sequencing as *Lactobacillus plantarum* for ITY11 and *Lactobacillus pentosus* for ITS14 with 99.86 and 100% similarity, respectively.

## INTRODUCTION

Probiotics have been presented to supply a wide assortment of goods in health effects and the effects have shown to be strain dependent. Most of microorganisms used for probiotic intents belong to the genus *Lactobacillus* and *Bifidobacterium*. For the probiotic in the gastrointestinal tract (GIT), bacteria must be able to survive under the acidic conditions of the stomach and live in bile status at the beginning of small intestine (Rubio *et al.*, 2014). Lactic acid bacteria (LAB) have therapeutic properties such as balancing microorganisms in gastrointestinal system, protecting or decreasing violence disease in gastrointestinal system, stimulating immunity, lowering cancer risk, decreasing sugar in blood, and enhancing nutrients absorption, particularly on calcium and iron (Tongluang, 2008).

Naem-Neua is a native fermented beef sausage of Thailand prepared from ground beef. Generally, most Naem (fermented pork) production is in the North of Thailand. The ingredients for Naem are different depending on locations. Naem-Neua is made from ground beef, garlic, cooked rice, salt and sodium nitrate, then mixed and wrapped up with banana leaf or plastic bag to create anaerobic condition. Finally, it was incubated for 3-4 days so that Naem-Neua will under golactic acid fermentation and have a sour taste. Most microorganisms which were found include *Pediococcus* sp., *Lactobacillus plantarum*, and *Lactobacillus brevis*. Today, Naem-Neua has various recipes with different taste, odor, and characteristics depending on production sites and associated flora particularly lactic acid bacteria. Generally, *defatted chopped beef* is used to produce Naem-Neua since the manufacturer gives a reason that it contributes a good characteristic product. In 2018, *defatted chopped beef*was sold at 295 baht/kg but if one wants tosave cost, there was an alternative called "Scraps"sold at 190 baht/kg (Pon-Yang-Kham livestock breeding cooperative NSC. LTD., 2016). Scraps were collected from small cuttings trimmed from chuck, brisket, rib, short loin and round parts which were partially defatted. Previously, the producer had used scraps to produce Naem-Neua products under indigenous fermentation condition and it was found that their qualities were not consistency with shorter life time. Thus, this research was aimed to enumerate, isolate, screen, select and also identify LAB isolates from indigenous fermented beef sausage products to be used as potential starter cultures in its future production with great consistency in high quality and food safety aspects.

## MATERIALS AND METHODS

### Sampling, physicochemical and microbiological characteristics of Naem-Neua products

#### Sampling procedure

Raw Naem-Neua products were sampled from three locations in Mueang district, Sakon Nakhon province. Triplicates of samples were collected after they were produced for 48-72 h and immediately transported to the laboratory at the Department of Food Technology, Faculty of Technology, Khon Kaen University, Khon Kaen, Thailand. The samples were kept under refrigerated conditions (below 4 °C) for further characteristics analysis.

#### Physicochemical analysis

The pH of Naem-Neua samples were determined using a pH meter equipped with an electrode for solid samples (Five Easy, Mettler Toledo, China)

The Aw of Naem-Neua samples were determined using Aqua Lab 3TE water activity meter (Aqua Lab, WA, USA).

The titratable acidity (%) of Naem-Neua samples were analysed according the AOAC method (2005). Ten grams of sample and 90 mL of boiling distilled water were mixed and then homogenized for 2 min using stomacher. Twenty-five milliliters of the mixtures were transferred to a 125 mL Erlenmeyer flask and titrated with standardized 0.1 N NaOH to pH 8.0 using phenolphthalein as an indicator. The acidity expressed as lactic acid was calculated as % titratable acidity (w/w) using a factor of 0.09.

Surface color of Naem-Neua samples were measured immediately when opened according to the method described by Suman *et al.* (2011). L*, a*, and b* at ten random areas were determined for each sample using a HunterLab which provides L*; a*; and b* values.

Texture profile analysis of Naem-Neua samples were determined following the method reported by Devatkal *et al.* (2004). A texture analyzer was used. Five slices from each part (1.5 cm height and 2.5 cm diameter) were compressed twice to 50% of their original height. The parameters determined were hardness (N), springiness (cm), cohesiveness, gumminess (N) and chewiness (N cm). Five measurements were taken from each sample and

averaged for statistical analysis.

Analysis of those investigations was considered in triplicate for each sample. The values of mean and standard deviation of each sample were estimated.

**Microbiological analysis**

Microbiological analyses were carried out according to Thai Community Product Standard; Fermented Ground Beefs, Naem-Neua; TCP 470-2555 (2012). All samples were evaluated for (i) Aerobic plate count, (ii) Lactic acid bacteria count, (iii) *Salmonella*, (iv) *Staphylococcus aureus*, (v) *Bacillus cereus*, (vi) *Clostridium perfringens*, (vii) *Escherichia coli*, and (vii) Yeast and mold.

Twenty-five grams of Naem-Neua samples were aseptically weighed into the sterile stomacher bag. Two hundred and twenty-five milliliters of saline peptone water (0.85% (w/v) NaCl, 0.1% (w/v) bacteriological peptone) were added. The samples were mixed for 3 min in a stomacher. From the resulting dilution, the serial dilutions were prepared and plated in triplicate to enumerate the following microorganisms:

(i) Aerobic plate count (APC). APC was enumerated in Plate count agar (Himedia, India) and incubated at 37 °C for 48 h (Tournas *et al*., 2001).

(ii) Lactic acid bacteria count. LAB count was performed in de Man, Rogosa and Sharpe (MRS) agar (Difco, USA) modified by addition of 0.5% (w/v) calcium carbonate (0.5% $CaCO_3$, MR Sagar) and incubated at 37 °C for 48 h.

(iii) *Salmonella* spp. The samples were tested for the presence of *Salmonella* spp. (Andrews *et al*., 2014) using selective enrichment in Rappaport-Vassiliades (RV) Enrichment broth (Himedia, India), and incubated at 42 °C for 24 h. Then, they were plated on Xylose lysine deoxycholate (XLD) agar (Himedia, India) and incubated at 37 °C for 24 h. The biochemical tests were subsequently conducted in Triple sugar iron (TSI) agar (Himedia, India) and Lysine indole motility (LIM) medium (Himedia, India) incubated at 37 °C for 24 h.

(iv) *Staphylococcus aureus*. Coagulase positive *Staphylococcus aureus* count was determined using Baird Parker agar (Himedia, India), containing egg yolk tellurite emulsion and incubated at 37 °C for 48 h. Then, coagulase test from Brain Heart Infusion (BHI) broth (Himedia, India) was investigated with coagulase plasma (Himedia, India) for coagulase activity (Bennett and Lancette, 2001) and incubated at 37 °C for 24 h.

(v) *Bacillus cereus*. The sample dilutions were enumerated in Mannitol-egg yolk-polymyxin (MYP) agar (Merck, Germany) containing egg yolk solution (Himedia, India) and plates were incubated for 18-24 h at 30°C. For confirmation, phenol red glucose broth was used and anaerobically incubated for 24 h at 35 °C in Bactron anaerobic chamber (Bactron I, Shel Lab, Sheldom Manufacturing Inc., Cornelius, OR, USA). Tubes were shaken vigorously and observed for growth as indicated by increased turbidity and color change from red to yellow, which indicates that acid has been produced anaerobically from glucose (Tallent *et al*., 2012).

(vi) *Clostridium perfringens*. The sample dilutions were enumerated in Tryptose Sulfite Cycloserine (TSC) agar (Merck, Germany) containing egg yolk solution (Himedia, India) and incubated at 37 °C for 24 h under anaerobic conditions in a Bactron anaerobic chamber (Bactron I, Shel Lab, Sheldom Manufacturing Inc., Cornelius, OR, USA) (Rhodehamel and Harmon, 2001)

(vii) Coliform and *Escherichia coli*. They were detected using the Most Probable Number (MPN) method. Coliform was determined using Lauryl Sulfate Tryptose (LST) broth (Himedia, India) incubated at 37 °C for 24 to 48 h. Then, samples were confirmed for coliforms as gassing LST or lactose broth tubes by transferring a loopful of suspension to a tube of Brilliant Green Bile Broth (BGBB) (Himedia, India) and incubated at 37 °C for 24 to 48 h. Also, the fecal coliforms and *E. coli* were confirmed from each gassing LST or Lactose broth tube from the Presumptive test as a loopful of each suspension were transferred to a tube of EC broth (Himedia, India) and incubated at 44 °C for 24 to 48 h. Finally, *E. coli* was tested with Eosin Methylene Blue (EMB) agar (Himedia, India) incubated at 37 °C for 24 h (Feng *et al*., 2002).

(viii) Yeast and Mold Count. The sample dilutions were counted in Potato Dextrose Agar (PDA; Himedia, India) incubated at 25 °C for 3 to 5 days (Tournas *et al*., 2001).

Analyses of those above were conducted in duplicate for each sample. After counting the colonies, the values of mean and standard deviation

of each sample were estimated.

**Enumeration, isolation, screening and selection of LAB as the starter cultures from Naem-Neua products**

**LAB enumeration, isolation and their growth conditions**

Lactic acid bacteria were isolated from Naem-Neua samples obtained from three different locations of Mueang district, Sakon Nakhon province, Thailand. After 48-72 h of Naem-Neua production, the samples were enumerated for LAB. The colonies of LAB isolates were selected from the MRS agar plates of each Naem-Neua sample and streaked again on MRS agar incubated at 37 °C for 48 h for further purification of the isolates. The pure cultures were then examined for their cells morphologies using the Gram stain and also some other characteristics. Gram-positive and catalase-negative isolates were stored at -18 °C in MRS broth containing 30% glycerol (Merck, Darmstadt, Germany) for future use and further identification by molecular methods.

**Screening and selection of LAB isolates for probiotic starter cultures implementations**

**Primary screening of LAB isolates**

LAB isolates were screened for using as probiotic starter cultures through the procedures listed below:

**Detection of LAB antimicrobial activity**

The detection was conducted following the method described by Gonçalves de Almeida Júnior *et al.* (2015). The inhibitory effects of different LAB isolates over pathogens were investigated by using the agar disc diffusion assay. *Salmonella* spp., *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens* and *Escherichia coli* O157:H7 were grown on Tryptone Soya Agar (TSA, Himedia) supplemented with 0.6% yeast extract for 24 h at 37 °C. Each pathogen was suspended in 4 mL of peptone water and standardized to approximately $10^8$cfu/mL, comparable to the standard turbidity no. 0.5 of McFarland. Sterile swabs soaked in those pathogens suspensions were applied on the surfaces of TSA agar plates and allowed to absorb. After that, three of 6 mm diameter sterile filter paper discs (Whatman no.1) moistened with 20 mL of cell free supernatant obtained by centrifugation (2,500 x g/10 min) of each LAB isolate growing in exponential growth phase was put on distant area in plates. Also, the sterile filter paper discs without LAB were used as controls. Afterwards, the susceptibilities of pathogens to the discs were measured for the inhibition zones of bacterial growth around the discs (radius as mm) after incubation for 24 h at 37 °C. A clear zone of inhibition of at least 2 mm radius was recorded as positive. The experiment was performed in duplicateand the mean values were estimated.

**Secondary screening of LAB isolates**

Tolerances of LAB isolates to high temperatures, acidic pHs, bile, high concentrations of lactic acid and sodium chloride.

This study was conducted following method reported by Samappito (2010). The basal MRS medium enhanced with bromocresol purple as the pH indicator of 7.0 at the concentration of 0.17 g/L was used in this study. When the medium had a lower pH, the color of medium was changed from purple to yellow, and this change indicatedthe LAB growth because lactic acid production was growth-related. If the medium color did not change, it indicated no acid and no cells. Test tubes with screw caps were each filled with 20 mL of the basal MRS medium and sterilized in autoclave. Then, twenty-four hour cultures of each LAB isolate were used as the inocula and incubated for 24 h at 37 ºC. After that, the cells were spun down, suspended in 0.85% NaCl and 50 µL of the suspension was then inoculated into each test tube.

For temperatures tolerances, the test tubes of LAB cultures were incubated in the water bath equipped with a temperature controlling system and set at the specific test temperatures at 4, 15, 30, 37, 45 and 50 °C. At the end of 48 h, the changes of color and turbidity of the medium in each test tube were recorded as the simple indication of growth or no growth of LAB. The experiments were conducted in triplicate.

For acid tolerance (Wang *et al.*, 2014), the pH of MRS broth supplemented with 0.30% oxgall was adjusted to 3.0 and 2.0 with HCl compared to control (MRS broth at pH 6.2) then sterilized by heating at 121 °C for 15 min. Each of LAB isolate was subcultured at least 3 times before use, followed by centrifugation after the final subculture. The cultures were then inoculated at 10% (v/v) into the broth and growth was monitored at 37 °C for 3 h using the plate count method. The experiments were done induplicate.

For bile tolerance (Wang *et al.*, 2014), the MRS

broths at concentrations of 0%, 0.5% and 1.0% (w/v) of oxgall were prepared and dispensed in 10 mL volumes and subsequently sterilized by heating at 121 °C for 15 min. Each of the LAB isolates was subcultured at least 3 times before use, followed by centrifugation after the final subculture. Then, the cultures were inoculated at 10% (v/v) into the broth, and growth was monitored at 37 ºC for 24 h using the plate count method. The experiments were done in duplicate.

For lactic acid and NaCl tolerances, they were determined at the concentrations of 2.5%, 5%, 7.5%, 10%, 15% (w/v) and the concentrations of 1.5%, 2.5%, 5%, 7.5%, 10% (w/v), respectively. At the end of 48 h incubation at 37 °C, the changes of color and turbidity of the medium in each test tube wererecorded as the simple indication of growth or no growth of LAB. The experiments were conducted in triplicate.

**Selection of LAB isolates**

LAB isolates with high potential in antimicrobial activities and tolerances to temperatures, acidic pHs, bile, high concentrations of lactic acid and NaCl were selected for further identification.

**Molecular characterization and identification of LAB isolates using RAPD-PCR**

LAB isolates from previous step were identified for the genus and species using RAPD-PCR with molecular characterization method as described below.

LAB isolates were streaked across MRS agar plates and incubated overnight at 32°C. Single bacterial colonies were then inoculated into broth (TSB with 0.5% Yeast Extract) and incubated at 32 °C for overnight. A volume of 1.5 mL of the broth cultures was pipetted into 2 mL microtube and centrifuged. Pelleted cells were washed with 1.5 mL of saline (0.85% NaCl), and then centrifuged at 10,000xg for 5 min. Washed cells were subsequently suspended in 20 µL of saline solution (Samappito, 2010).

The RAPD-PCR assay was performed as described previously (Samappito, 2010) using the Gene Amp PCR System 9600 thermocycler (Applied Biosystems, USA). Each RAPD-PCR reaction was performed in a total volume of 50 µL containing 1 x PCR buffer, 25 mM $MgCl_2$, 100 µM dNTP of each, 0.3 µM of each primer, i.e., LMPBI 5′-GGAACTGCTA-3′ and LMPB4 5′-AAGGATCAGC-3′ (Boerlin *et al.*, 1995), 1.25 units Taq DNA polymerase, 5 µL of suspension cells, then $dH_2O$ is used to adjust to a final volume of 50 µL. All reagents were added along (except for the bacterial cells) into the reaction vial and mixed thoroughly. In the initialization step, the samples were initially incubated for 6.5 min at 94 °C and lysed cells hot stand PCR subsequently. Sample was amplified for 35 cycles. Each cycle consists of a denaturation step of 1 min at 94 °C, an annealing step of 2 min at 35 °C, an extension step of 1 min at 72 °C, and finally an elongation step of 10 min at 72 °C to ensure that any remaining single-stranded DNA was fully extended. When the PCR completed, the RAPD-PCR products were electrophoresed at 100 V on 2.5% agarose gels and stained with ethidium bromide as described previously (Fontana *et al.*, 2005). The RAPD-PCR profiles were analyzed via the NTSYS-PC software package and a dendrogram was generated by using the UPGMA program.

**Characterization and identification of LAB using 16S rDNA gene sequencing**

Chromosomal DNA of LAB was extracted with DNA extraction kit according to the manufacturer's instruction. A 345-bp fragment of the 16S rDNA gene of the bacterial isolates was amplified by LABF (5′-ACGGGAGGCAGCAGTAGGGA-3′) and LABR (5′-CGCTACACATGGAGTTCCAC-3′). Each RAPD-PCR reaction was performed in a total volume of 50 µL containing 1x PCR buffer, 25 mM MgCl2, 100 µM dNTP of each, 0.3 µM of each primer, 1.25 units Taq DNA polymerase, approximately 10 ng of genomic DNA and adjusted $dH_2O$ to a final volume 50 µL. Initial heating at 97 °C for 4 min, was followed by 40 cycles of the following sequence : 97 °C for 2 min, 65 °C for 4 min, 80 °C for 2 min. DNA sequencing was continually performed (Samappito, 2010; Fontana *et al*., 2005; Mellmann *et al*., 2003; Woo *et al*., 2003; Muyzer *et al*., 1993; Klijn *et al*., 1991).

**Statistical Analysis**

The data was carried out by analysis of the variance (ANOVA) and mean differences were evaluated by Duncan's new multiple range tests ($p \leq 0.05$). All data were expressed as mean ± S.D.

## RESULTS

**Sampling, physicochemical and microbiological characteristics of Naem-Neua products**

Samples of raw Naem-Neua products were

collected from three locations in Mueang district, Sakon Nakhon province. The samples were evaluated for their physicochemical and microbiological analyses.

The values of pH, aw and % titratable acidity of Naem-Neua were shown in Table 1. The SN3 showed the higher pH value than the one of SN1 ($p \leq 0.05$) but it was not different from the one of SN2 ($p > 0.05$). Aw values of SN2 and SN3 were the same and they were higher than the one of SN1 ($p \leq 0.05$). For titratable acidity (%), it was found that SN2 had the highest value while SN1 had the lowest one and they were significantly different ($p \leq 0.05$).

For surface color measurement of all three samples (Table 2), it was found that L* values were different significantly. SN1 had the brightest tone (L*=41.32) which was higher than the L* values of SN2 and SN3 with the values of 39.38 and 31.67 respectively ($p \leq 0.05$). When considering the values of a* and b*, it was found that a* values of SN2 and SN3 were not significantly different ($p > 0.05$) with the values of 4.63 and 5.33 respectively but they were significantly higher than the one of SN1 with the value of 2.95 ($p \leq 0.05$). Similarly, b* values of SN2 and SN3 were not significantly different ($p > 0.05$) with the values of 20.80 and 20.23 respectively but they were significantly higher than the one of SN1 with the value of 12.58 ($p \leq 0.05$).

For texture profile analysis, it was often called the "two bite test" because the texture analyzer mimics the mouth's biting action and it was a popular double compression test for determining the textural properties of foods. The results of texture profile analysis of Naem-Neua were shown in Table 3. It was found that hardness (N), gumminess (N) and chewiness (N.mm) values were 10.39, 7.06 and 3.33 for SN1 and 13.43, 6.27, 3.92 for SN3 respectively which were not significantly different ($p > 0.05$) but they were significantly lower than the ones of SN2 with the values of 22.05, 12.45 and 7.35 respectively ($p \leq 0.05$). Also, springiness (mm) values of SN2 and SN3 were 0.59 and 0.63 respectively which were not significantly different ($p > 0.05$) but they were significantly higher than the one of SN1 with a value of 0.47($p \leq 0.05$). Obviously, cohesiveness values of SN1 SN2 and SN3 were 0.69, 0.56 and 0.46 respectively which were significantly different ($p \leq 0.05$).

The indigenous microorganisms in Naem-Neua were analyzed according to Thai Community

**Table 1.** pH, Aw and % Titratable acidity of Naem-Neua samples.

| Location | pH | Aw | % Titratable acidity |
|---|---|---|---|
| SN1 | $4.56 \pm 0.04^{b}$ | $0.96 \pm 0.00^{b}$ | $0.45 \pm 0.05^{a}$ |
| SN2 | $4.85 \pm 0.61^{ab}$ | $0.97 \pm 0.01^{a}$ | $0.31 \pm 0.05^{b}$ |
| SN3 | $5.05 \pm 0.13^{a}$ | $0.97 \pm 0.01^{a}$ | $0.21 \pm 0.02^{c}$ |

Different superscripts in the same column indicate significant differences ($p \leq 0.05$) analyzed by Duncan's new multiple range tests.

**Table 2.** Surface color of Naem-Neua samples.

| Location | L* | a* | b* |
|---|---|---|---|
| SN1 | $41.32 \pm 2.40^{a}$ | $2.95 \pm 0.97^{b}$ | $12.58 \pm 2.88^{b}$ |
| SN2 | $39.38 \pm 4.37^{b}$ | $4.63 \pm 1.82^{a}$ | $20.80 \pm 4.70^{a}$ |
| SN3 | $31.67 \pm 4.03^{c}$ | $5.33 \pm 1.79^{a}$ | $20.23 \pm 4.58^{a}$ |

Different superscripts in the same column indicate significant differences ($p \leq 0.05$) analyzed by Duncan's new multiple range tests.

**Table 3.** Texture profile analysis of Naem-Neua samples.

| Location | Hardness (N) | Springiness (mm) | Cohesiveness | Gumminess (N) | Chewiness (N.mm) |
|---|---|---|---|---|---|
| SN1 | $10.39 \pm 0.45^{a}$ | $0.47 \pm 0.05^{a}$ | $0.69 \pm 0.07^{c}$ | $7.06 \pm 0.30^{a}$ | $3.33 \pm 0.15^{a}$ |
| SN2 | $22.05 \pm 0.70^{b}$ | $0.59 \pm 0.08^{b}$ | $0.56 \pm 0.06^{b}$ | $12.45 \pm 0.46^{b}$ | $7.35 \pm 0.29^{b}$ |
| SN3 | $13.43 \pm 0.21^{a}$ | $0.63 \pm 0.06^{b}$ | $0.46 \pm 0.05^{a}$ | $6.27 \pm 0.11^{a}$ | $3.92 \pm 0.08^{a}$ |

Different superscripts in the same column indicate significant differences ($p \leq 0.05$) analyzed by Duncan's new multiple range tests.

Product Standard; Fermented Ground Beefs, Naem-Neua; TCP 470-2555 (2012). The results were shown in Table 4. Microbiological analyses included aerobic plate count, lactic acid bacteria count, yeast and mold count, and also some pathogenic bacteria concerning *Clostridium perfringens*, *Staphylococcus aureus*, *Bacillus cereus* and *Salmonella* spp. It was shown that the aerobic plate count of SN3 was 9.54 Log CFU/g which was significantly higher than the ones of SN2 and SN1 with the values of 9.08 and 8.68 Log CFU/g respectively ($p \leq 0.05$). In addition, lactic acid bacteria counts of SN1 and SN2 were not significantly different with the values of 9.15 and 9.07 Log CFU/g respectively ($p > 0.05$) but they were significantly lower than the one of SN3 with the value of 9.50 Log CFU/g ($p \leq 0.05$). Yeast and mold count of SN1 was 8.89 Log CFU/g which was significantly higher than the ones of SN3 and SN2 with the values of 7.92 and 6.64 Log CFU/g respectively ($p \leq 0.05$). For pathogens, it was revealed that *Staphylococcus aureus*, *Bacillus cereus* and *Salmonella* spp in all three samples of SN1, SN2 and SN3 were less than 1 Log CFU/g. However, *Clostridium perfringens* count of SN2 was 3.43 Log CFU/g whereas the counts of SN1 and SN3 were less than 1 Log CFU/g.

For coliform and fecal coliform counts using MPN method as shown in Table 5, it was found that coliform counts of SN1, SN2 and SN3 were not significantly different with the values of 1200, 1113 and 1200 MPN/g respectively and they were also appeared as metallic sheen on EMB agar. Also, fecal coliform counts of SN1 and SN3 were the same with a value of 893 MPN/g but they were significantly lower than the one of SN2 with a value of 1666 MPN/g ($p \leq 0.05$).

**Enumeration, isolation and screening of LAB as the starter cultures from Naem-Neua products**

LAB isolates were obtained from Naem-Neua products sampled from three locations (SN1, SN2 and SN3) of Mueang district, Sakon Nakhon province, Thailand. After 48-72 h of Naem-Neua production, the samples were enumerated for LAB using a method described in a previous step. Then, fifteen LAB isolates from each location as shown in Table 6 were screened and selected for the appropriate ones as followings.

**Detection of LAB antimicrobial activity**

The inhibitory effects of different LAB isolates over pathogens were investigated by using the agar disc diffusion assay. Bacterial pathogens including *Salmonella* spp., *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli* O157:H7 were grown in Tryptone Soya Agar. A clear zone of inhibition of at least 2 mm radius was recorded as positive. It was found that sixteen out of forty-five LAB isolates showed the positive effects as they were illustrated in Table 7. For gram positive pathogens, it was revealed that

**Table 4.** Microbiological analysis of Naem-Neua samples.

| Location | Aerobic plate count (log cfu/g) | Lactic acid bacteria (log cfu/g) | Yeast and Mold (log cfu/g) | *C. perfringens* (log cfu/g) | *S. aureus* (log cfu/g) | *B. cereus* (log cfu/g) | *Salmonella* (log cfu/g) |
|---|---|---|---|---|---|---|---|
| SN1 | $8.68 \pm 0.25^{a}$ | $9.15 \pm 0.16^{a}$ | $8.89 \pm 0.10^{c}$ | <1 | <1 | <1 | <1 |
| SN2 | $9.08 \pm 0.62^{b}$ | $9.07 \pm 0.48^{a}$ | $6.64 \pm 0.12^{a}$ | 3.43±0.89 | <1 | <1 | <1 |
| SN3 | $9.54 \pm 0.42^{c}$ | $9.50 \pm 0.45^{b}$ | $7.92 \pm 0.15^{b}$ | <1 | <1 | <1 | <1 |

Different superscripts in the same column indicate significant differences ($p<0.05$) analyzed by Duncan's new multiple range tests.

**Table 5.** Coliform and Fecal coliform by MPN method.

| Locations | Coliform (MPN/g) | Fecal coliform (MPN/g) | EMB Agar | |
|---|---|---|---|---|
| SN1 | $1200 \pm 173^{a}$ | $893 \pm 179^{a}$ | metallic Sheen |  |
| SN2 | $1113 \pm 630^{a}$ | $1666 \pm 551^{b}$ | metallic Sheen |  |
| SN3 | $1200 \pm 173^{a}$ | $893 \pm 179^{a}$ | metallic Sheen |  |

Different superscripts in the same row indicate significant differences (p d" 0.05) analyzed by Duncan's new multiple range tests.

**Table 6.** Names of LAB isolatesfrom Naem-Neua samplesfor each location (SN1, SN2 and SN3).

| SN1 | SN2 | SN3 |
|---|---|---|
| ITY01 | ITS01 | ITP01 |
| ITY02 | ITS02 | ITP02 |
| ITY03 | ITS03 | ITP03 |
| ITY04 | ITS04 | ITP04 |
| ITY05 | ITS05 | ITP05 |
| ITY06 | ITS06 | ITP06 |
| ITY07 | ITS07 | ITP07 |
| ITY08 | ITS08 | ITP08 |
| ITY09 | ITS09 | ITP09 |
| ITY10 | ITS10 | ITP10 |
| ITY11 | ITS11 | ITP11 |
| ITY12 | ITS12 | ITP12 |
| ITY13 | ITS13 | ITP13 |
| ITY14 | ITS14 | ITP14 |
| ITY15 | ITS15 | ITP15 |

ITS06 LAB isolate could significantly inhibit both *Staphylococcus aureus* and *Bacillus cereus* the most compared to other LAB isolates with the clear zones of 1.12 and 1.30 mm, respectively. For gram negative pathogens, it was shown that ITY11 and ITS14 LAB isolates could significantly inhibit both *Salmonella* spp and *Escherichia coli* O157:H7 the most compared to other LAB isolates with the clear zones of 1.92 and 1.66 mm for ITY11, 1.82 and 1.74 mm for ITS14, respectively ($p \leq 0.05$). Surprisingly, some LAB isolates including ITY11, ITS06 and ITS14 could inhibit both gram positive and negative pathogens the most compared to other isolates ($p \leq 0.05$). For instance, ITY11 could inhibit *Staphylococcus aureus*, *Salmonella* spp and *Escherichia coli* O157:H7 with the clear zones of 1.08, 1.92 and 1.66 mm; ITS06 could inhibit *Staphylococcus aureus*, *Salmonella* spp and *Bacillus cereus* with the clear zones of 1.12, 1.82 and 1.30 mm and ITS14 could inhibit *Salmonella* spp, *Escherichia coli* O157:H7 and *Bacillus cereus* with the clear zones of 1.82, 1.74 and 1.46 mm respectively.

### Tolerances of LAB isolates to high temperatures, acidic pHs, bile, and different concentrations of lactic acid and sodium chloride

Tolerances of LAB isolates to high temperatures, acidic pHs, bile and different concentrations of lactic acid and sodium chloride were shown in Table 8, 9, 10, 11 and 12 respectively.

Tolerances of LAB isolates to different levels of temperatures including 4°C, 15°C, 30°C, 37C, 45°C and 50°C were determined (Table 8) and it was shown that all sixteen of LAB isolates could grow at both 30 °C and 37 °C while eleven out of sixteen LAB isolates including ITY01, ITY04, ITY011, ITS04, ITS05ITS06, ITS08, ITS11, ITS14, ITP09 and ITP10 could grow at 45 °C. However, no LAB isolates could grow at 4, 15 and 50 °C.

Tolerances of LAB isolates to different levels of pH including 2.0, 3.0 and 6.2 (control) were studied and it was shown that at pH 3.0, LAB isolate ITS14 could survive the most with a number of 8.89 Log

**Table 7.** Clear zones (millimeters) of LAB isolates against tested pathogenic microorganisms.

| No. | Names of LAB Isolates | *S. aureus* | *Salmonella* spp. | *E. coli* O157: H7 | *B. cereus* |
|---|---|---|---|---|---|
| 1 | ITY01 | $1.08\pm1.30^{efgh}$ | $1.32\pm0.20^{cd}$ | $1.00\pm0.34^{bc}$ | $1.20\pm0.14^{e}$ |
| 2 | ITY04 | $0.96\pm0.05^{cdef}$ | $0.84\pm0.09^{b}$ | $1.38\pm0.31^{def}$ | $1.09\pm0.09^{cde}$ |
| 3 | ITY11 | $1.08\pm0.20^{efgh}$ | $1.92\pm0.13^{gh}$ | $1.66\pm0.32^{fg}$ | $1.16\pm0.23^{de}$ |
| 4 | ITS04 | $1.22\pm0.11^{h}$ | $1.12\pm0.11^{c}$ | $1.62\pm0.18^{fg}$ | $1.14\pm0.21^{de}$ |
| 5 | ITS05 | $1.02\pm0.04^{defg}$ | $1.62\pm0.37^{ef}$ | $1.58\pm0.40^{efg}$ | $1.26\pm0.23^{ef}$ |
| 6 | ITS06 | $1.12\pm0.22^{fgh}$ | $1.82\pm0.18^{fgh}$ | $1.44\pm0.11^{def}$ | $1.30\pm0.41^{ef}$ |
| 7 | ITS08 | $0.90\pm0.10^{bcd}$ | $1.32\pm0.11^{cd}$ | $0.96\pm0.09^{bc}$ | $1.30\pm0.25^{ef}$ |
| 8 | ITS11 | $0.76\pm0.15^{b}$ | $1.50\pm0.21^{de}$ | $1.38\pm0.18^{def}$ | $0.88\pm0.04^{bc}$ |
| 9 | ITS14 | $1.00\pm0.00^{defg}$ | $1.82\pm0.20^{fgh}$ | $1.74\pm0.09^{g}$ | $1.46\pm0.18^{f}$ |
| 10 | ITP01 | $0.00\pm0.00^{a}$ | $1.98\pm0.18^{h}$ | $0.98\pm0.08^{bc}$ | $0.72\pm0.04^{b}$ |
| 11 | ITP07 | $0.90\pm0.14^{bcd}$ | $1.08\pm0.24^{bc}$ | $0.82\pm0.16^{b}$ | $0.94\pm0.11^{bcd}$ |
| 12 | ITP09 | $0.96\pm0.15^{cdef}$ | $1.54\pm0.23^{de}$ | $1.16\pm0.19^{cd}$ | $0.74\pm0.15^{b}$ |
| 13 | ITP10 | $0.92\pm0.11^{bcde}$ | $1.56\pm0.22^{def}$ | $0.82\pm0.13^{b}$ | $0.86\pm0.17^{bc}$ |
| 14 | ITP12 | $0.82\pm0.08^{bc}$ | $1.90\pm0.14^{gh}$ | $1.18\pm0.11^{cd}$ | $0.80\pm0.10^{b}$ |
| 15 | ITP14 | $0.96\pm0.11^{cdef}$ | $1.68\pm0.22^{efg}$ | $1.44\pm0.09^{def}$ | $0.80\pm0.14^{b}$ |
| 16 | ITP15 | $1.14\pm0.05^{gh}$ | $1.30\pm0.16^{cd}$ | $1.30\pm0.12^{de}$ | $0.78\pm0.04^{b}$ |
| 17 | Control | $0.00\pm0.00^{a}$ | $0.00\pm0.00^{a}$ | $0.00\pm0.00^{a}$ | $0.00\pm0.00^{a}$ |

Different superscripts in the same column indicate significant differences ($p \leq 0.05$) analyzed by Duncan's new multiple range tests.

cfu/g which was significantly higher than other LAB isolates ($p \leq 0.05$). However, all LAB isolates could not survive in MRS broth at pH 2.0 with a number less than 1 Log CFU/g. For control in MRS broth at pH 6.2, LAB isolates could grow in the range of 8.11-9.89 Log cfu/g.

For bile tolerance, all LAB isolates could survive in both 0.5 and 1% bile and it was shown that three out of sixteen LAB isolates including ITS08, ITP01 and ITP07 could survive the most in 0.5% bile with the numbers of 9.08, 9.00 and 9.00 Log cfu/g respectively which were significantly higher than the ones of other LAB isolates ($p \leq 0.05$). Furthermore, two out of sixteen LAB isolates including ITP01 and ITP09 could survive the most in 1% bile with the numbers of 9.45 and 9.47 Log cfu/g respectively which were significantly higher than the ones of other LAB isolates ($p \leq 0.05$).

Moreover, it was revealed that all LAB isolates could not survive in the medium at all levels of lactic

**Table 8.** High temperature tolerances of LAB isolates.

| No. | LAB Isolates | 4°C | 15°C | 30°C | 37°C(control) | 45°C | 50°C |
|---|---|---|---|---|---|---|---|
| 1 | ITY01 | — | — | ++ | ++ | ++ | — |
| 2 | ITY04 | — | — | ++ | ++ | ++ | — |
| 3 | ITY11 | — | — | ++ | ++ | ++ | — |
| 4 | ITS04 | — | — | ++ | ++ | ++ | — |
| 5 | ITS05 | — | — | ++ | ++ | ++ | — |
| 6 | ITS06 | — | — | ++ | ++ | ++ | — |
| 7 | ITS08 | — | — | ++ | ++ | ++ | — |
| 8 | ITS11 | — | — | ++ | ++ | ++ | — |
| 9 | ITS14 | — | — | ++ | ++ | ++ | — |
| 10 | ITP01 | — | — | ++ | ++ | — | — |
| 11 | ITP07 | — | — | ++ | ++ | — | — |
| 12 | ITP09 | — | — | ++ | ++ | ++ | — |
| 13 | ITP10 | — | — | ++ | ++ | ++ | — |
| 14 | ITP12 | — | — | ++ | ++ | — | — |
| 15 | ITP14 | — | — | ++ | ++ | — | — |
| 16 | ITP15 | — | — | ++ | ++ | — | — |

\+ in first position means had color change
\+ in second position means had turbidity change
\- means no change

**Table 9.** Tolerances of LAB isolates (log CFU/g) to acidic pHs acidified by HCl.

| No. | LAB Isolates | pH 6.2 (control) | pH 2.0 | pH 3.0 |
|---|---|---|---|---|
| 1 | ITY01 | $9.33 \pm 0.82^{cd}$ | <1 | $4.16 \pm 0.02^{a}$ |
| 2 | ITY04 | $9.89 \pm 0.16^{d}$ | < 1 | $3.93 \pm 0.05^{a}$ |
| 3 | ITY11 | $9.07 \pm 0.14^{bc}$ | < 1 | $7.14 \pm 0.01^{cde}$ |
| 4 | ITS04 | $9.26 \pm 0.69^{cd}$ | < 1 | $8.08 \pm 0.01^{g}$ |
| 5 | ITS05 | $9.40 \pm 0.06^{cd}$ | < 1 | $7.90 \pm 0.06^{fg}$ |
| 6 | ITS06 | $9.46 \pm 0.39^{cd}$ | < 1 | $7.90 \pm 0.06^{fg}$ |
| 7 | ITS08 | $9.23 \pm 0.08^{cd}$ | < 1 | $7.97 \pm 0.04^{g}$ |
| 8 | ITS11 | $8.71 \pm 0.20^{abc}$ | < 1 | $5.67 \pm 0.07^{b}$ |
| 9 | ITS14 | $8.77 \pm 0.00^{abc}$ | < 1 | $8.89 \pm 0.05^{h}$ |
| 10 | ITP01 | $8.44 \pm 0.36^{ab}$ | < 1 | $6.00 \pm 0.04^{b}$ |
| 11 | ITP07 | $8.77 \pm 0.16^{abc}$ | < 1 | $7.02 \pm 0.04^{cd}$ |
| 12 | ITP09 | $8.21 \pm 0.01^{a}$ | < 1 | $7.52 \pm 0.12^{ef}$ |
| 13 | ITP10 | $8.20 \pm 0.04^{a}$ | < 1 | $7.82 \pm 0.05^{fg}$ |
| 14 | ITP12 | $8.11 \pm 0.14^{a}$ | < 1 | $6.89 \pm 0.06^{c}$ |
| 15 | ITP14 | $8.71 \pm 0.02^{abc}$ | < 1 | $6.89 \pm 0.06^{de}$ |
| 16 | ITP15 | $8.35 \pm 0.03^{ab}$ | < 1 | $7.94 \pm 0.01^{fg}$ |

Different superscripts in the same column indicate significant differences ($p < 0.05$) analyzed by Duncan's new multiple range tests.

acid concentrations including 2.5, 5, 7.5, 10 and 15% compared to control (0%). However, all LAB isolates except LAB isolate ITP01, could survive in the medium at both levels of 1.5 and 2.5 % NaCl. However, nine out of sixteen LAB isolates including ITY01, ITY04, ITY11, ITS04, ITS05, ITS06, ITS08, ITS14 and ITP09 could survive in the medium at 5 % NaCl and only LAB isolate ITS06 could survive in the medium up to 7.5%NaCl. Obviously, all LAB isolates could not survive in the medium at 10% NaCl.

### Characterization and identification of LAB isolates using 16S rDNA gene sequencing

Two LAB isolates including ITS14 and ITY11 were identified based on 16s rDNA sequencing which were shown in Table 13. It was revealed that ITS14 was identified as *Lactobacillus pentosus* with 100%

**Table 10.** Tolerances of LAB isolates to different concentrations of bile.

| No. | LAB Isolates | 0% (control) (Log CFU/g) | 0.5% (Log CFU/g) | 1% (Log CFU/g) |
|---|---|---|---|---|
| 1 | ITY01 | $9.70 \pm 0.02^{g}$ | $8.62 \pm 0.04^{a}$ | $8.77 \pm 0.03^{ab}$ |
| 2 | ITY04 | $10.08 \pm 0.05^{h}$ | $8.98 \pm 0.03^{e}$ | $8.81 \pm 0.03^{b}$ |
| 3 | ITY11 | $10.03 \pm 0.01^{h}$ | $8.75 \pm 0.06^{b}$ | $8.80 \pm 0.07^{b}$ |
| 4 | ITS04 | $10.22 \pm 0.02^{i}$ | $8.96 \pm 0.02^{de}$ | $9.12 \pm 0.02^{de}$ |
| 5 | ITS05 | $10.90 \pm 0.03^{j}$ | $8.95 \pm 0.06^{cde}$ | $8.70 \pm 0.12^{ab}$ |
| 6 | ITS06 | $9.36 \pm 0.02^{f}$ | $8.95 \pm 0.05^{cde}$ | $9.15 \pm 0.04^{e}$ |
| 7 | ITS08 | $9.00 \pm 0.04^{c}$ | $9.08 \pm 0.01^{f}$ | $8.95 \pm 0.04^{c}$ |
| 8 | ITS11 | $8.77 \pm 0.10^{b}$ | $8.63 \pm 0.08^{a}$ | $8.65 \pm 0.10^{a}$ |
| 9 | ITS14 | $9.30 \pm 0.02^{ef}$ | $8.86 \pm 0.04^{c}$ | $8.76 \pm 0.05^{ab}$ |
| 10 | ITP01 | $8.15 \pm 0.04^{a}$ | $9.00 \pm 0.01^{ef}$ | $9.45 \pm 0.01^{f}$ |
| 11 | ITP07 | $9.18 \pm 0.01^{d}$ | $9.00 \pm 0.02^{ef}$ | $9.11 \pm 0.02^{de}$ |
| 12 | ITP09 | $9.22 \pm 0.02^{de}$ | $9.32 \pm 0.02^{g}$ | $9.47 \pm 0.01^{f}$ |
| 13 | ITP10 | $9.20 \pm 0.04^{d}$ | $9.28 \pm 0.04^{g}$ | $9.03 \pm 0.05^{cde}$ |
| 14 | ITP12 | $9.05 \pm 0.05^{c}$ | $8.77 \pm 0.03^{b}$ | $8.69 \pm 0.10^{ab}$ |
| 15 | ITP14 | $9.03 \pm 0.01^{c}$ | $8.71 \pm 0.04^{ab}$ | $9.01 \pm 0.03^{cd}$ |
| 16 | ITP15 | $9.32 \pm 0.02^{f}$ | $8.87 \pm 0.03^{cd}$ | $9.05 \pm 0.01^{cde}$ |

Different superscripts in the same column indicate significant differences ($p \leq 0.05$) analyzed by Duncan's new multiple range tests.

**Table 11.** Tolerances of LAB isolates to different concentrations of lactic acid.

| No. | Isolated | 0% (control) | 2.5% | 5% | 7.5% | 10% | 15% |
|---|---|---|---|---|---|---|---|
| 1 | ITY01 | ++ | — | — | — | — | — |
| 2 | ITY04 | ++ | — | — | — | — | — |
| 3 | ITY11 | ++ | — | — | — | — | — |
| 4 | ITS04 | ++ | — | — | — | — | — |
| 5 | ITS05 | ++ | — | — | — | — | — |
| 6 | ITS06 | ++ | — | — | — | — | — |
| 7 | ITS08 | ++ | — | — | — | — | — |
| 8 | ITS11 | ++ | — | — | — | — | — |
| 9 | ITS14 | ++ | — | — | — | — | — |
| 10 | ITP01 | ++ | — | — | — | — | — |
| 11 | ITP07 | ++ | — | — | — | — | — |
| 12 | ITP09 | ++ | — | — | — | — | — |
| 13 | ITP10 | ++ | — | — | — | — | — |
| 14 | ITP12 | ++ | — | — | — | — | — |
| 15 | ITP14 | ++ | — | — | — | — | — |
| 16 | ITP15 | ++ | — | — | — | — | — |

\+ in first position means had color change
\+ in second position means had turbidity change
\- means no change

**Table 12**. Tolerances of LAB isolates to different concentrations of sodium chloride.

| No. | LAB Isolates | 0% (control) | 1.5% | 2.5% | 5% | 7.5% | 10% |
|---|---|---|---|---|---|---|---|
| 1 | ITY01 | ++ | ++ | ++ | ++ | — | — |
| 2 | ITY04 | ++ | ++ | ++ | ++ | — | — |
| 3 | ITY11 | ++ | ++ | ++ | ++ | — | — |
| 4 | ITS04 | ++ | ++ | ++ | ++ | — | — |
| 5 | ITS05 | ++ | ++ | ++ | ++ | — | — |
| 6 | ITS06 | ++ | ++ | ++ | ++ | ++ | — |
| 7 | ITS08 | ++ | ++ | ++ | ++ | — | — |
| 8 | ITS11 | ++ | ++ | ++ | — | — | — |
| 9 | ITS14 | ++ | ++ | ++ | ++ | — | — |
| 10 | ITP01 | ++ | — | — | — | — | — |
| 11 | ITP07 | ++ | ++ | ++ | — | — | — |
| 12 | ITP09 | ++ | ++ | ++ | ++ | — | — |
| 13 | ITP10 | ++ | ++ | ++ | — | — | — |
| 14 | ITP12 | ++ | ++ | ++ | — | — | — |
| 15 | ITP14 | ++ | ++ | ++ | — | — | — |
| 16 | ITP15 | ++ | ++ | ++ | — | — | — |

\+ in first position means had color change
\+ in second position means had turbidity change
\- means no change

**Table 13.** Two identified bacterial strains based on 16s rDNA sequencing.

| No. | Sample code | Identified as | % Similarity |
|---|---|---|---|
| 1 | ITY11 | *Lactobacillus plantarum* | 99.86 |
| 2 | ITS14 | *Lactobacillus pentosus* | 100 |

similarity whereas ITY11 was identified as *Lactobacillus plantarum* with 99.86 % similarity. The bands of PCR products analyzed by agarose gel electrophoresis were shown in Fig. 1.

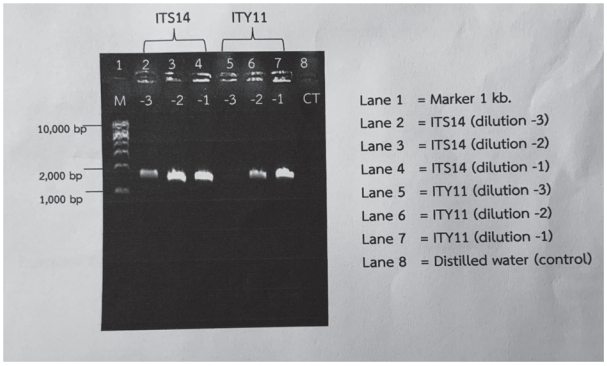


**Fig. 1.** PCR products analyzed by agarose gel electrophoresis.

## DISCUSSION

After Naem-Neua products were fermented for 72 hours, their pHs was decreased to the range of 4.56-5.05 mainly due to lactic acid producing bacteria during fermentation. De Maere *et al*. (2018) reported that the pH value of fermented sausages was reduced from 5.3 to 4.1-4.9 after three days of fermentation. Titratable acidity (%) was a value that indicated the existing acid content associated with the pH; thus, SN1 Naem-Neua sample with the lowest pH (4.56), also had the highest acid content or % titratable acidity (0.45%). In addition, Aw values of Naem-Neua samples from three sampling locations (SN1, SN2 and SN3) were in the range of 0.96-0.97 which also resembled the ones revealed by De Maere *et al*. (2018) based on the characteristic of the fermented pickled meat products, thereby making it a high Aw value.

The color values of Naem-Neua products after fermented for 48-72 hours from three sampling locations (SN1, SN2 and SN3) were similar in both lightness and color tones. They were in red and yellow tones, which were characterized by the nature of beef in accordance with the research reported by Sucu and Turp (2018) who had studied the use of the beet root powder in fermented beef sausage products instead of using nitrite. They were in red and yellow tones, whether they were a controlled formula or a formula that used beetroot powder, on day 0, 56 and 84. Also, differences in color values depended on meat from different sources and components of the formula.

The textural attributes of Naem-Neua samples from three sampling locations (SN1, SN2 and SN3)

when tested with Texture profile analysis (TPA), were different. Obviously, the hardness, gumminess and chewiness of SN2 Naem-Neua samples were the highest at values of 22.05 N, 12.45 N, and 7.35 N.mm, respectively. However, Savadkoohi *et al.* (2014) had reported that the Hardness and Chewiness of beef frank furter and beef ham were higher than the ones of fermented meat at values of 25.42 N and 31.21 N for hardness and 51.18 N.mm and 37.79 N.mm for chewiness, respectively.

It is evident that aerobic plate count and lactic acid bacteria count were similar in numbers ranging from 8.68 to 9.54 Log cfu/ml and 9.07 to 9.50 Log cfu/g, respectively since lactic acid bacteria were generally included in aerobic plate count for fermented meat products. Furthermore, yeast and mold count was considerably high in numbers ranging from 6.64 to 8.89 Log cfu/g. Ahmad and Srivastava (2007) had determined the effect of heart incorporation (0%, 15% and 20%) and increasing levels of fat (20% and 25%) on microbiological (total plate count and yeast and mold count) quality and shelf life of fermented sausages of buffalo meat during refrigerated storage (4°C) and it was found that total plate counts and yeast and mold counts were found to increase significantly ($p \leq 0.05$) during refrigerated storage.

It was revealed that aerobic plate count, lactic acid bacteria count, yeast and mold count, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella* spp. were complied with Thai Community Product standard; Fermented Beef Sausage, Naem-Neua; TCP 470-2555 (2012) except *Clostridium perfringens* which was enumerated as 3.43 Log CFU/g (exceeding the standard limit of 3 Log CFU/g).

*C. perfringens* is a gram positive bacterium, anaerobic and it forms heat resistant endospores (Brynestad and Granum, 2002). Miki *et al.* (2008) had reported that *Clostridium perfringens* is a major cause of foodborne diseases, usually associated with consumption of insalubrious meat goods and some strains are able to produce and release the enterotoxin in the gastrointestinal tract, causing nausea, abdominal pain and diarrhea (McClane *et al.*, 2006; McClane and Robertson, 2013). Ed-Dra *et al*. (2017) had reported that 77.56% of sausages samples marketed in Meknes city are contaminated with *C. perfringens* which reflect the neglect of hygiene practices throughout the manufacturing chain, storage, transport, and distribution of these products. Besides, the critical points of sausages contamination are concise in the origin of raw material, storage temperature, hygiene of preparation places, and the seasonal variations.

Although historically considered as safe, the characteristics of dry or semidry fermented sausages (DFSs) can provide survival and even growth of certain pathogens in these products. Surveys have shown the presence of pathogenic *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Listeria monocytogenes* in dry fermented sausages. *Clostridium botulinum* and *Toxoplasma gondii* have also been reported as potential microbial risks for consumers of DFSs. Pathogenic microorganisms can be introduced through contaminated raw materials or through cross-contamination from equipment or personnel during processing or at retail. Conditions during sausage processing and pathogen characteristics determine the ability for pathogen growth and survival and also determine possible strategies for pathogen elimination to ensure product safety (Holck *et al*., 2017).

Coliform and fecal coliform were analyzed and obviously MPN value of *Escherichia coli* of all Naem-Neua samples exceeded the food standard limit (3 MPN/g) along with metallic sheen on EMB Agar.

The presence of coliforms in meat and meat products indicates a potable fecal source of contamination which begins from slaughter houses as a result of skinning of animals by knives and workers, also during evisceration. Contamination may come from animal intestine, air and water used for washing and rinsing of carcasses. Also, the plant itself may be due to difference in manufacture practice, handling from producers to consumers and the effectiveness of hygienic measures applied during production (Gaafer, 2009).

The single bacterial colonies of SN1, SN2, and SN3 LAB isolates were separately picked for 15 isolates each, and finally the best two LAB isolates were selected. Beginning with antimicrobial activities of LAB isolates against tested pathogens including *Salmonella* spp., *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens* and *Escherichia coli* O157:H7 using agar disc diffusion assayon Tryptone Soya Agar were determined and then the clear zone areas were recorded. It was shown that there were 16 LAB isolates which could inhibit all kinds of pathogens with certain clear zone areas. Obviously, the results demonstrated that the antimicrobial activities of LAB isolates against pathogens were species-and strain-dependent which was in accordance with Abushelaibi *et al.*(2017). The antimicrobial activities of these LAB

isolates were mainly due to the production of one or more active metabolites during growth such as organic acids, hydrogen peroxide and bacteriocins.

According to the tolerances of LAB isolates to high temperature, acidic pHs, bile, high concentrations of lactic acid and sodium chloride, it was revealed that two LAB isolates including ITY11 and ITS14 were the best two isolates that can flourish at temperatures of 30 and 37 °C. Also, they could be prosperous at 45 °C which was good in the case of making products that were fermented at elevated temperatures such as yogurt. Yogurt was made by inoculation a combination of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in equal ratio 1:1 (18 hours old) directly into pasteurized milk and incubated at 42°C for 6-8 hours in the incubator (Bashiti, 2010; Corrieu and Beal, 2016).

For acid tolerance at pH 3.0 acidified with HCl, it was shown that two LAB isolates including ITY11 and ITS14 could survive at certain numbers compared to control (pH 6.2). However, both of them could not survive at pH 2.0 owing to the excessive high acidic conditions. Microorganisms can remain viable for several hours at pH as low as 2.5 in a stationary phase, mainly owing to their Acid Resistance Mechanisms (ARMs; the survival systems used in acidic conditions) (Waterman and Small, 2003). The two general ARMs adopted by microbes for maintaining their pH homeostasis during growth are as follows: i) use of H+ antiport system such as H+-ATPase activity, acid end-product efflux, and decreased proton permeability to maintain a low intracellular concentration of protons; and ii) synthesis of alkaline products to neutralize acid generated during extracellular metabolism. Several bacteria have evolved diverse resistance or tolerance mechanisms against the normally lethal pH values of ≤ 2.5, mainly through acid-tolerance responses (ATRs) which provide the ability to sense, respond and adapt to an acidified environment, an ARMs (Spector and Kenyon, 2012).

For bile tolerance, all LAB isolates could survive well at both levels of 0.5 and 1% bile compared to control (0% bile). A potential probiotic must overcome the acidic conditions of human stomach and resist in presence of bile salts to colonize the small intestine (Babic *et.al.*, 2011). Hence, the resistance of lactic acid bacteria to bile and acid pH are prerequisites for bacteria to be used as probiotics (Fontana *et al.*, 2013). The pH of the stomach generally varies from pH 2.5 to pH 3.5 and approximately the concentration of bile salts in the small intestine range from 0.2% to 2.0% (w/v), in relation to the individual, the type and the amount of food (Ashraf and Smith, 2016). For *in vitro* screening, 0.3% is considered a critical concentration for bile tolerance (Hyronimus *et al*., 2000; Zhou *et al*., 2007) and pH 2.5 is set as standard for acid tolerance (Sahadeva *et al*., 2011).

However, no LAB isolate could withstand lactic acid at high concentrations (2.5-15%). Acid tolerance is an important property of lactic acid bacteria as the producer of high concentration of lactic acid. Experiments revealed the different tolerances of tested LAB to acid medium, thus requires the different amounts of neutralizing agent. The most tolerant strains to acid medium were *Pediococcus pentosaceus* KTU05-9 and *Pediococcus acidilactici* KTU05-7 (Juodeikiene *et al*., 2016).

Varying concentrations of sodium chloride at 0, 1.5, 2.5, 5, 7.5 and 10% were simulated and it was found that LAB isolates including ITY11 and ITS14 could tolerate salt concentrations up to 5%. Chikthimmah *et al.*, (2001) had reported that the presence of a higher level of NaCl (5%) in Lebanon bologna (a moist, smoked, fermented sausage manufactured from lean beef) inhibited the growth of lactic acid bacteria (LAB), which yielded product with higher pH (~5.0). Conversely, lower concentrations of NaCl (0, 2.5%) yielded Lebanon bologna with higher LAB counts and lower pHs (4.4-4.5), compared to product with 5% NaCl. Shockey and Borger (1991) had reviewed that common NaCl had been used for pickling and preserving food by inhibiting the growth and proteolytic activity of *Clostridium* but not the more salt-tolerant lactic acid bacteria.

Lactic acid bacteria (LAB) are non-sporulating, catalase–negative, Gram-positive, rod-or coccus-shaped and strictly fermentative organisms, with lactic acid as the major metabolic end product of carbohydrate fermentation (Holzapfel *et al*., 2001). LAB could be found in different habitats such as soil,water, animal and human gastrointestinal tract, as well as in food and fermented products (Zacharof and Lovitt, 2012). Several members of LAB, pure or mix cultures, are commonly used as probiotics. Thus, the evaluation of probiotics is essential to optimize their use (Awad *et al*., 2010).

However, the effectiveness of the probiotics is species or strain dependent; therefore, each probiotic strain should meet a number of requirements, including safety (i.e. isolation from suitable habitats, correct identification), functional

(i.e. resistance to gastrointestinal environment) and beneficial (i.e. lactic acid production, antagonism against pathogens) (FAO/WHO, 2002).

The selection of a functional starter culture with some desired properties is a complex process; the critical point of this process is the management of a very complex dataset and the definition of the cut-off points and the criteria of decision (Corbo *et al*., 2017).

A total of 45 lactic acid bacteria isolates isolated from Naem-Neua samples obtained from three sampling locations (SN1, SN2 and SN3 was screened until the best two LAB isolates were achieved and then identified by 16S rDNA sequence analysis. For ITS14 sample code isolate, it was identified as *Lactobacillus pentosus* with 100% similarity whereas for ITY11 sample code isolate, it was identified as *Lactobacillus plantarum* with 99.86% similarity.

The 16S rDNA method is sequence based taxonomy and in the presence scenario, molecular characterization based on RAPD fingerprinting is additionally used to study the microbial diversity or variability and their ecological distribution. RAPD is a very convenient and cost effective method employed for bacterial identification and variability estimation (Kannan and Vincent, 2011). The PCR based method of gene typing based on genomic polymorphism is a recent approach which is widely used for the assessment of inter and intraspecific genetic variation and uses a single short random oligonucleotide primer (Williams *et al*., 1990). In most cases of bacterial genetics, RAPD assay generated the best DNA pattern for differentiation of bacteria. A number of studies have reported success in using RAPD assays to distinguish bacterial strains among diverse species (Rains*et.al*., 1997; Babalola *et al*., 2002; Desai and Varadaraj, 2010; El-Hamshary *et al*., 2012).

Xiao *et al*. (2018) had reported that meat safety has attracted increasing attention world-wide because of the potential carcinogenicity of red meat and processed meat, as described by the International Agency for Research on Cancer (IARC) on October 26, 2015 (IARC, 2015). N-Nitrosamines (NAs) areone of the most important carcinogens and are present in various meat products (Lu *et al*., 2017; Yurchenko and Molder, 2007). N-Nitrosodimethylamine (NDMA) and N-nitro-sodiethylamine (NDEA) are classified as Group 2A carcinogens (probably carcinogenic) whereas other NAs commonly found in meat products are classified as Group 2B (possibly carcinogenic) to humans (IARC, 1978). However, Xiao *et al.* (2018) have shown that microbial fermentation can be used to inhibit NA formation as a natural and sustainable method. For instance, *Lactobacillus* spp. are commonly used as starter cultures in fermented sausage production and have been observed to reduce nitrite residue and inhibit the accumulation of biogenic amines and NAs (Sun *et al*., 2016; Sun *et al*., 2017). Lactic acid bacteria (LAB) from milk and Korean kimchi were found to directly degrade NDMA during cultivation in NDMA-containing Mann-Rogosa-Sharp (MRS) broth (Kim *et al*., 2017) and found that *Lactobacillus pentosus* R3, a strain isolated from a Chinese dry sausage, decreased NA content in MRS broth (Xiao *et al*., 2018). Another research, *Lactobacillus plantarum* can decreased total plate count in raw minced chicken meat which analyzed for 0 as well as 24 h of fermentation. This signifies the role of *L. plantarum* in preventing the growth of spoilagemicro-organism in the same environment (Ajit and Yadav, 2017). Yilmaz and Velioglu (2009) also concluded in their study that prevention of spoilage microorganisms in the product is the main effect of lactic acid bacteria.

## CONCLUSION

Forty-five LAB isolates were isolated from Naem-Neua products and then screened for the potential ones with antimicrobial effects against bacterial pathogens. Sixteen out of forty-five isolates were then chosen for their great pathogen inhibition potential. Furthermore, sixteen isolates were challenged to high temperatures, acidic pHs, bile, high concentrations of lactic acid and sodium chloride. Two LAB isolates were eventually achieved with high tolerance capabilities and they were then identified based on 16s rDNA sequencing as *Lactobacillus pentosus* and *Lactobacillus plantarum* with 100 and 99.86 % similarity, respectively.

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