Isolation, characterisation and in vitro evaluation of bacteriocins-producing lactic acid bacteria from fermented products of Northern Borneo for their beneficial roles in food industry

R Jawan1,3*, ME Kasimin1, SN Jalal1, AA Mohd. Faik1, S Abbasiliasi2 and A Ariff3,4

1Biotechnology Programme, Faculty of Science and Natural Resources, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah
2Halal Products Research Institute, Universiti Putra Malaysia, UPM Serdang, 43400 Selangor, Malaysia
3Bioprocessing and Biomanufacturing Research Centre, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM Serdang, 43400 Selangor, Malaysia
4Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM Serdang, 43400 Selangor, Malaysia

*Corresponding author: roslinaj@ums.edu.my

Abstract. In this study, lactic acid bacteria (LAB) isolated from traditional fermented foods namely coco bean, fermented cabbage, salted vegetable, tempeh, tempoyak, tapai ubi and tapai nasi were screened for production of bacteriocin. Characterisation and in vitro evaluation of them were carried out to assess their potential use in food industry. Towards these objectives, the inhibitory spectra of the isolates against Listeria monocytogenes ATCC13932, resistance to phenol, amylolytic and proteolytic activities, ability to produce acid and coagulate milk, antibiotic susceptibility and tolerance in the presence of various concentration of NaCl and at different temperatures were evaluated. Two out of 15 LAB strains were able to inhibit the growth of food-borne pathogen, L. monocytogenes ATCC 13932 and produce bacteriocin-like inhibitory substances. The strains were identified as Pediococcus acidilactici TN1 (from tapai nasi) and Lactobacillus farciminis TY1 (from tempoyak). Biochemical and physiological tests demonstrated that, both strains were able to grow at wide range of NaCl concentrations (0.5 - 5.0 %, w/v) and temperatures (28 - 70 °C), and capable to degrade protein. They lowered the pH level and coagulate milk after 24 h of incubation. Both strains showed intrinsic mechanisms of antibiotic resistance towards streptomycin, norfloxacin, erythromycin, amikacin and nalidixic acid. They also were able to grow in 0.3% (w/v) of bile salts and tolerate up to 0.5% (w/v) phenol. The findings from this study revealed that the presence of LAB strains in fermented foods of Northern Borneo which have an antimicrobial activity towards the food-borne pathogen. Even though this study had generated extensive information to validate Pediococcus acidilactici TN1 and Lactobacillus farciminis TY1 as potential probiotic strains for application in the food industry, the study is by no means comprehensive nor complete. More laboratory, particularly in vivo studies, are needed before this product could be accepted by the food industry and most importantly to explore its novel health promoting functions as well as its colonization behaviour in the gut.

Keywords: Lactic acid bacteria, isolation, characterisation, fermented foods, bacteriocins

1. Introduction

Massive development in food industry such as high processed, chemically preserved, high fat content and zero calories foods are available in the market that could lead to unhealthy lifestyle. An awareness by the community has brings a demand for the functional fermented foods that offer various benefit hence triggered the researchers to search for a newly isolated lactic acid bacteria (LAB) from the fermented products that will open the possibility to find new strains with health promoting characteristics. Various findings reveal the presence of potential probiotic strains that had been isolated from numerous fermented foods either from vegetables, fruits or animal-based products such as L. plantarum RYPR1 isolated from raabadi (fermented beverage) [1], Lactobacillus plantarum Bom 816
and *Lactobacillus pentosus* N3 from boza (cereal-based beverage) [2], various LAB strains from pastirma (air-dried cured beef) [3] and several *Lactobacillus* strains from lukanka (fermented and air-dried meat) [4]. Even though related findings with similar fermented foods had been published by Kormin *et al.* [5], the individuality and variance in fermented products preparation/ raw materials supported with dissimilarity in geographical areas and fermentation periods had become a key factor for discovering the new potential LAB with beneficial traits.

LAB are favourable microorganisms contributing to various industrial applications, extending from food trade for food and beverage fermentation, to pharmaceuticals and also nutraceuticals manufacturing. Benefits of LAB in controlling the development of foodborne diseases on fermented products were long before known caused by the production of various by-products such as lactic acid, acetic acid, ammonia, bacteriocins, ethanol, reuterin, hydrogen peroxide, and diacetyl. All these compounds are able to inhibit the growth of food spoilage and pathogenic organisms [6] as well as increasing the shelf life of the product [7]. Besides acting as natural food preservatives that delay the spoilage, LAB also contributed to the flavour and the aroma development in food and beverages [8] and increases the nutritional value of the product possibly containing health benefits [9]. One of the important compounds produced by LAB is bacteriocins. Bacteriocins are ribosomally synthesized antimicrobial peptides which provide promising technological alternative for food bio preservation, as they can avoid the growth of spoilage and pathogenic microorganisms [10]. The use of bacteriocin-producing bacteria is significantly more effective to improve human gut health as antimicrobial peptides produced by probiotic strains in the intestine, can interrelate directly with the sensitive pathogenic organisms in the intestine [11, 12].

In food industry, the strains have been selected for commercial use in foods must retain their characteristics for which they were originally selected including the characteristics of growth and survival during manufacture and, after consumption, during transit through the stomach and small intestine. Importantly, probiotic must retain these characteristics to provide various health benefits to the consumers [13]. Advancement in food manufacturing technology inspired the continuous search of newly isolated strains that able to endure the harsh conditions during food processing, coupled with the increase in antibiotic resistance incidence among the food-related microorganisms. Therefore, the objectives of this study are to isolate, characterise, and evaluate the bacteriocins-producing LAB from fermented foods of Northern Borneo to be applied in food industry such as in production of functional foods, starter cultures, bio preservatives, and flavour compounds.

2. Methods

2.1. Collection of food samples

Seven (7) types of locally fermented products namely coco bean, fermented cabbage, salted vegetable, *tempeh*, *tempoyak*, *tapai ubi* and *tapai nasi* were purchased from wet market at Kota Kinabalu, Sabah, Malaysia (Figure 1).

![Fermented food samples](image)

*Figure 1.* Fermented food samples. (a) Coco bean; (b) Cabbage; (c) Salted vegetable; (d) *Tempeh*; (e) *Tempoyak*; (f) *Tapai ubi*; (g) *Tapai nasi*. 
2.2. Isolation of lactic acid bacteria
Isolation of LAB was carried out based on Abbasiliasi et al. [14] with a slight modification. Briefly, for a solid sample, 5 g was mixed with 45 mL of NaCl solution (0.85%, w/v NaCl) and homogenised using a home blender to get a uniform sample. To enrich the growth of potential targeted LAB, 25 mL of food sample was added into 225 mL de Man Rogosa Sharpe (MRS) (Oxoid LTD, Basingstoke, Hampshire, England) and M17 (Oxoid LTD, Basingstoke, Hampshire, England) broth to obtain a 1:10 dilution and incubated at 37 °C for 24 h. The culture was then diluted (10-fold) in 0.85% (w/v) NaCl solution and 100 µL of diluted sample was spread-plated on M17 and MRS agar supplemented with 0.01% (w/v) sodium azide (as an inhibitor for growth of Gram-negative bacteria). The plates were incubated at 37 °C for 24 h under anaerobic conditions (AnaeroGen, Oxoid). Fifty colonies were selected randomly (based on their differences in colour, shape, elevation and size) and were streaked onto MRS and M17 agar media. The single colony was then sub-cultured twice to ensure the purity of the culture.

2.3. Biochemical and physiological characteristics of lactic acid bacteria
2.3.1. Gram staining and cell morphology
The identification of the isolates was performed by standard staining procedure. The shape morphology of the fresh grown cells was viewed under the light microscope.

2.3.2. Catalase activity and carbon fermentation test
A drop of 3% (v/v) hydrogen peroxide solution was placed on pure single colony. Immediate formation of bubbles (gas production) were consider as positive. In carbon fermentation test, nutrient agar was prepared with 1% (w/v) of glucose and 0.004% (w/v) of bromocresol purple (as a pH indicator). About 10 µL of culture was then spotted on the agar. After incubation at 37 °C for 24 h, positive result was shown by changing in purple colour to yellow zone around the culture as a result of reduced pH by acid production through the fermentation of glucose by the bacteria.

2.3.3. Effect of NaCl and temperature on growth of lactic acid bacteria
The isolate (1%, v/v) was inoculated into M17 or MRS broth containing different concentrations of NaCl (0.5, 2.0, 5.0 and 10 %, w/v) and 0.004 % (w/v) bromocresol purple prior to incubation at 37 °C for 24 h. In temperature test, culture and media preparation was same as previous (except no addition of NaCl) and culture was incubated at different temperatures (-20, -40, 14, 28, 37 and 70 °C). The tested temperatures representing the common range of temperatures in fermented food processing. After 24 h of incubation the growth was assessed as indicated by media colour changes from purple to yellow.

2.4. Antimicrobial activity against Listeria monocytogenes ATCC13932
The antimicrobial activity of the isolates against food-borne pathogens, Listeria monocytogenes ATCC13932, was determined by the agar well diffusion assay [15]. To prepare the cell-free culture supernatants (CFCS), the isolate was grown in M17 or MRS broth at 37 °C for 24 h and the cultures were centrifuged at 10,000 rpm for 10 min at 4 °C. The CFCS (100 µL) was then placed into 6 mm wells of agar plates that was earlier sowed with 1% (v/v) L. monocytogenes ATCC 13932. The plates were placed at 4 °C for 2 h to ensure a better diffusion of the CFCS through the agar media before incubated at 37 °C. After 24 h, the inhibition zones formed around the wells were measured using electronic caliper.

2.5. Determination of probiotic properties of lactic acid bacteria
The potential isolates with antimicrobial activity were selected and further characterised to evaluate their potentials use in food industry. The tests involved were design to mimic the in vitro gastrointestinal condition.
2.5.1. Bile salts and phenol tolerance test

Bile salts tolerance of the isolates was determined by the viable count method [16]. The overnight incubated culture (1%, v/v) was inoculated into 100 mL of M17 or MRS broth supplemented with 0.3% (w/v) bile salts. Media without addition of bile salts was used as control. The culture was agitated at 100 rpm and incubated at 37 °C for 4 h. An enumeration of viable cells was performed using pour plate technique at 0, 1, 2, 3 and 4 h of incubation. The colony number was count using a colony counter and compared with the control (0 h) to determine the bile salt tolerance. In conducting phenol tolerance test, the procedure was similar with that of bile salts test except the broth media added with different concentrations of phenol that usually applied in screening of probiotic strains (0.1, 0.3 and 0.5%, w/v). Inhibitory effect was determined by comparing the viable cells count of isolate at 0 and after 24 h of incubation. Negative sign of the equated viable cells count value indicating no inhibition occurs throughout the experiments.

2.5.2. Amylolytic and proteolytic activity tests

Starch hydrolysis test was conducted by inoculating a loop full of pure bacterial strains and streaking it on agar plates containing 2% (w/v) of soluble starch powder. After an overnight incubation at 37 °C, a small amount of iodine solution was poured onto the starch agar plates to detect starch hydrolysis. The presence of a clear halo zone around a tested colony was considered as indication of starch degradation and therefore the production of α-amylase. In proteolytic activity, the test was determined by inoculating the culture on agar supplemented with 1% (w/v) of skim milk [17]. The appearance of transparent halo-forming colonies was considered as positive reaction after an overnight incubation at 37 °C.

2.5.3. Acidifying activity

The acidification test was determined by the changes in pH of the skim milk solution and coagulation ability [18]. The cultures (1 mL) were inoculated into 100 mL of 10% (w/v) of skim milk and incubated at 37 °C for 24, 48 and 72 h. Skim milk without addition of inoculum was used as control. The physical properties of the skim milk such as the pH, aroma, coagulation and appearance were as described by Bodyfelt et al. [19].

2.5.4. Antibiotic sensitivity test

Antibiotic susceptibility was performed by the disc diffusion method of Abbasiliiasi et al. [14]. A single colony of the isolate was inoculated into 10 mL of M17 or MRS broth and incubated at 37 °C for 24 h. Bacterial suspension was adjusted to 0.5 McFarland and swabbed evenly onto Müller-Hinton agar plate. Commercially available disc (Oxoid) containing penicillin G, colistin sulphate, streptomycin, chloramphenicol, erythromycin, ceftriaxone, amikacin, norfloxacin, tetracycline, nalidixic acid and ampicillin were then placed on the surface of the dried agar plates. The experiment was performed in triplicate. All plates were incubated at 37 °C for 24 h before measuring the inhibition zones including the disc diameter. Isolates were categorized as sensitive (≥21 mm), intermediate (16-20 mm) or resistant (≤15 mm). Absence or presence of inhibition zones were defined as sensitivity or resistance, respectively.

2.6. Identification of isolates by 16S rRNA sequencing and phylogenetic analysis

DNA extraction and sequencing of the amplified fragments were carried out by Apical Scientific Sdn. Bhd. Selangor, Malaysia according to methods of [20]. Briefly, the bacterial 16S rDNA, full-length 1.5 kb, was amplified using universal primers 27F and 1492R. The total reaction volume of 25 uL contained genomic DNA purified using in-house extraction method, 0.3 pmol of each primer, deoxynucleotides triphosphates (dNTPs, 400 μM each), 0.5 U DNA Taq polymerase, supplied PCR buffer and deionised water. The PCR was performed as follow: 1 cycle (94 °C for 2 min) for initial denaturation; 25 cycles (98 °C for 10 sec; 53 °C for 30 sec; 68 °C for 1 min) for annealing and extension of the amplified DNA. The PCR products were purified by standard method and directly sequenced with primers 785F and 907R using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The fragments of
sequences were assembled and consensus sequences were compared with those deposited in the GenBank DNA database using the Basic Local Alignment Search Tool (BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi). Then, a phylogenetic tree based on 16S rRNA 137 genes was constructed to determine the closest bacterial species by using Neighbour Joining (Unrooted Tree) by NCBI Blast Tree Method using Molecular Evolutionary Genetics Analysis (MEGA) software version 10.0.5 [21]. Distances and clustering with the Neighbour-Joining method was determined using bootstrap values based on 1000 replications. Bacillus subtilis NCDO 1769 and Escherichia coli strain U 5/41 were used as an outgroup organism, respectively that serves as a reference group in evolutionary relationships determination of the ingroup.

3. Results

3.1. Isolation, morphological and biochemical characterisation of lactic acid bacteria

A total of 25 strains were isolated from 7 types of locally fermented foods. After preliminary identification, 15 out of 25 isolates were LAB as they were Gram-positive, catalase negative and able to ferment glucose and produce acid. Two of these LAB isolates, namely TN1 from tapai nasi, and TY1 from tempoyak were able to produce antimicrobial substances as they showed inhibitory activity against a food-borne pathogen, L. monocytogenes ATCC13932 with 9.0- and 10.0-mm diameter of inhibition zone, respectively (Table 1). Therefore, strain TN1 and TY1 were selected for further study. TN1 is coccic shape cell and appear as round, concave and white opaque colonies. TY1 has bacilli shape cell and seem round, concave with yellowish-white in colour.

| Characteristics                            | Coco bean | Fermented cabbage | Tapai nasi | Tapai ubi | Salted vegetable | Tempeh | Tempoyak |
|--------------------------------------------|-----------|-------------------|------------|-----------|------------------|--------|----------|
| No. of LAB isolates                        | 2         | 3                 | 3          | 2         | 3                | 1      | 2        |
| No. of isolates showing antimicrobial activity against L. monocytogenes ATCC13932 | 0         | 0                 | 1          | 0         | 0                | 0      | 1        |
| (TN1)                                      |           |                   | (TY1)      |           |                  |        |          |
| Cell morphology                            | Cocci     | Bacilli           | Cocci      | Cocci     | Bacilli          | Cocci  | Bacilli  |
| Gram stain reaction                        | +         | +                 | +          | +         | +                | +      | +        |
| Catalase activity                          | -         | -                 | -          | -         | -                | -      | -        |
| Glucose fermentation                       | +         | +                 | +          | +         | +                | +      | +        |

Note: Positive reaction (+), negative reaction (−)

3.2. Identification of isolates by 16S rDNA sequencing

The predicted size (1.5 kb) of exposed genomic DNA bands from PCR analysis for TN1 and TY1 are shown in Figure 2. The respective phylogenetic trees of partial 16S rDNA sequences as presented in Figure 3. The optimal tree with the sum of branch length for TN1 is 0.22820880 and 0.38133460 for TY1. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. This analysis involved 12 nucleotide sequences and codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). In addition, there were a total of 1606 (TN1) and 1574 (TY1) positions in the final dataset.
Figure 2. Agarose gel electrophoresis-PCR amplification products of 16S rDNA genes of lactic acid bacteria isolates. Lane M: 10kb GeneRuler DNA Ladder; Lane -ve: PCR without template control; Lane +ve: Positive control, Bacterial gDNA, 10 ng; Lane 1: TN1; Lane 2: TY1.

Figure 3. Phylogenetic tree of TN1 and TY1 isolates and related taxa based on partial 16S rDNA sequences. The phylogenetic tree was constructed by the Neighbour-joining method (MEGA X 10.0.5). Numbers in parentheses are accession number of published sequences. The numbers at the nodes are bootstrap confidence levels (percentage) from 1000 replicates. Bacillus subtilis NCDO 1769 and Escherichia coli strain U 5/41 were used as an outgroup organism, respectively (A) TN1; (B) TY1.

The obtained phylogenetic tree proved that strains TN1 (A_1515bp) and TY1 (B_1502bp) were most closely related to Pediococcus acidilactici and Lactobacillus farciminis strains supporting by 100 and 93% value from bootstrap analysis of the phylogenetic tree, respectively. Both strains show 99% similarity in its 16S rDNA gene sequences (Table 2).

Table 2. Analysis of 16S rDNA sequencing analysis (BLASTN) of TN1 and TY1.

| Sources      | Strains | Species             | % Similarity (BLASTN) | NCBI accession No |
|--------------|---------|---------------------|-----------------------|-------------------|
| Fermented rice | TN1     | Pediococcus acidilactici | 99                    | NR042057.1        |
| Tempoyak     | TY1     | Lactobacillus farciminis | 99                    | NR114396.1        |

3.3. Effect of NaCl and temperature on growth of lactic acid bacteria
P. acidilactici TN1 and L. farciminis TY1 were able to grow in MRS broth supplemented with up to 5% (w/v) of NaCl and there was no growth detected on 10% (w/v) of NaCl. Both strains can tolerate temperature ranged from 28 - 70 °C.

3.4. Probiotic characterisation of lactic acid bacteria
In starch hydrolysis and proteolytic activity test, only P. acidilactici TN1 was able to hydrolysed soluble starch by synthesising amylase indicating by a clear zone on starch agar. Both strains were capable to degrade protein by producing protease as clear zone was spotted on milk agar. Other than that, P. acidilactici TN1 and L. farciminis TY1 were able to acidify milk as they lowered the pH level (TN1:
pH 6.62 to 4.27; TY1: pH 6.65 to 4.24) prior to milk coagulation during 0 h until 24 h of incubation producing a rancid odour and cream colour curd (Table 3).

### Table 3 Milk acidification activity of TN1 and TY1.

| Strains | Incubation time (h) | pH       | Ability to coagulate milk | Aroma       | Colour        |
|---------|---------------------|----------|---------------------------|-------------|---------------|
| TN1     | 0                   | 6.62     | No                        | Flat        | White         |
|         | 24                  | 4.91     | Yes                       | Foreign     | Cream         |
|         | 48                  | 4.31     | Yes                       | Rancid      | Cream         |
|         | 72                  | 4.27     | Yes                       | Rancid      | Cream         |
| TY1     | 0                   | 6.65     | No                        | Flat        | White         |
|         | 24                  | 4.92     | Yes                       | Foreign     | Cream-white   |
|         | 48                  | 4.32     | Yes                       | Rancid      | Cream-white   |
|         | 72                  | 4.24     | Yes                       | Rancid      | Cream-white   |

*P. acidilactici* TN1 and *L. farcininis* TY1 were able to grow in 0.3% (w/v) of bile salts (Figure 4). TN1 had a higher survivability percentage (100.93-145.87%) (except at hour-3) as compared to control (MRS medium without addition of bile salt) (95.83-122.63%). Whereas, TY1 (111.12-154.49%) shows a comparable growth pattern as linked to control (105.31-149.76%) indicated by survivability percentage.

![Figure 4](image_url)

**Figure 4.** Survivability of TN1 and TY1 after 4 h of exposure to difference concentrations of bile salts

Phenol tolerance test has no inhibitory effect on growth of *P. acidilactici* TN1 and *L. farcininis* TY1, as indicated by negative inhibition value (Table 4). Negative value indicating an increase in cell number during the incubation time period as compared to the initial cell number, demonstrating the ability of the cells to grow with the presence of various concentrations of phenol in MRS medium. The inhibitory effect was decreased as phenol concentrations increase (except for 0.5%, w/v of phenol for TY1).

### Table 4. Effect of phenol on growth of TN1 and TY1 in MRS broth media supplemented with various concentrations of phenol

| Strain | Phenol (% , w/v) | Viable cell count (Log10 CFU/mL) | Incubation time (h) | Inhibition* |
|--------|------------------|----------------------------------|---------------------|-------------|
|        |                  |                                  | 0                   | 24          |
| TN1    | 0                | 6.49 ± 0.78                      | 7.95 ± 0.15         | -1.46       |
|        | 0.1              | 6.72 ± 0.01                      | 8.78 ± 0.04         | -2.06       |
|        | 0.3              | 7.14 ± 0.71                      | 9.0 ± 0.06          | -1.86       |
|        | 0.5              | 7.82 ± 0.15                      | 8.02 ± 0.18         | -0.20       |
| TY1    | 0                | 5.98 ± 0.01                      | 8.91 ± 0.07         | -2.93       |
|        | 0.1              | 6.94 ± 0.06                      | 9.01 ± 0.19         | -2.07       |
|        | 0.3              | 6.99 ± 0.19                      | 7.85 ± 0.16         | -0.86       |
|        | 0.5              | 8.04 ± 0.19                      | 8.01 ± 0.03         | 0.03        |

Note: *Inhibition= Viable cell count at 0 h – Viable cell count at 24 h*
Antibiotic sensitivity test showed that both strains (TN1 and TY1) exhibit intrinsic mechanisms of antibiotic resistance towards certain antibiotics such as amikacin, erythromycin, nalidixic acid, norfloxacin and streptomycin (Table 5). Additionally, TY1 was also resistance to colistin sulphate. Both strains were susceptible to β-lactams antibiotics (ampicillin and penicillin G) as well as ceftriaxone and chloramphenicol. Only TN1 susceptible to colistin sulphate. TN1 and TY1 strains showed an intermediate effect on tetracycline.

Table 5 Inhibitory response of TN1 and TY1 towards antibiotic sensitivity test

| Antibiotic     | Disc content | Inhibition zone diameter (mm) |
|----------------|--------------|-------------------------------|
|                | TN1          | TY1                           |
| Ampicillin     | 25 µg        | 24.5 ± 0.01 (S)               | 27.0 ± 0.07 (S)               |
| Amikacin       | 30 µg        | 0 (R)                         | 0 (R)                         |
| Ceftriaxone    | 30 µg        | 24.0 ± 0.03 (S)               | 25.0 ± 0.02 (S)               |
| Chloramphenicol| 30 µg        | 22.0 ± 0.03 (S)               | 22.5 ± 0.01 (S)               |
| Colistin sulphate | 10 µg    | 20.5 ± 0.01 (S)               | 0 (R)                         |
| Erythromycin   | 10 µg        | 0 (R)                         | 0 (R)                         |
| Nalidixic acid | 30 µg        | 0 (R)                         | 0 (R)                         |
| Norfloxacin    | 10 µg        | 0 (R)                         | 0 (R)                         |
| Penicillin G   | 2 units      | 25.0 ± 0.02 (S)               | 21.5 ± 0.01 (S)               |
| Streptomycin   | 10 µg        | 0 (R)                         | 0 (R)                         |
| Tetracycline   | 10 µg        | 16.0 ± 0.04 (I)               | 17.0 ± 0.04 (I)               |

Note: Results of zone of inhibition are triplicate and expressed as Mean ± S.D. Resistance (R) ≤15 mm; Intermediate (I) 16-20 mm; Susceptible (S) ≥21 mm.

4. Discussion
In this study, two isolates from fermented food products of Northern Borneo that able to produce antimicrobial substances against L. monocytogenes ATCC13932 were identified. Based on phylogenetic analysis, these two isolates were unambiguously recognised as P. acidilactici TN1 and L. farciminis TY1. Both strains were undergoing a testing based on experimental design that mimicking the gastrointestinal tract (GIT) of human-being to guarantee they are metabolically active within the GIT and biologically effective against the identified target. The ability of LAB to produce interesting inhibitory compounds (organic acids, H2O2, bacteriocins) that are important both in preventing the growth of spoilage and pathogenic bacteria fascinate their application in food industry. Bacteriocin-producing species and strains have been identified among all the genera that comprise the LAB, including Lactobacillus, Lactococcus, Streptococcus, Leuconostoc, Pediococcus and Carnobacterium as well as several Enterococcus spp. [22]. Bacteriocins produced by LAB have been the subject of extensive studies in recent years due to their potential use as novel, natural food preservatives also may play a role in the regulation of population dynamics within a fermenting ecosystem [23].

NaCl is one of the most extensively used additives in food manufacturing as it has a preservative and antimicrobial effect due to reduction of water activity values. It also has flavour enhancement effects by reducing or enhancing the enzymatic activity of some enzymes responsible for the development of organoleptic parameters or as a consequence of its effect on different biochemical mechanisms [24]. The current result showed that P. acidilactici TN1 and L. farciminis TY1 were able to grow in media supplemented with 0.5-5.0% (w/v) of NaCl. Our finding was in line with study conducted by Islam et al. [25], which stated the optimal growth of Lactobacillus spp was observed at 1-5% (w/v) NaCl. The notable effect of NaCl concentration was highlighted by Chin and Koehler [26]. They concluded that higher amine levels were found in low-salt (5%, w/v NaCl) formulations than in high-salt (10%, w/v NaCl). The variety in biogenic compounds production might relates to the inhibiting effect of higher salt concentration on the growth of many microorganisms, which in turn greatly decreases the likelihood of the production of decarboxylase enzymes responsible for the decarboxylation of amino acids to form amines. In a contrary, some of Lactobacilli species are more resistant to harsh conditions like higher NaCl concentration, anaerobic condition and reduced availability of nutrients [27].

The ability of the cultures to grow in a particular temperature range is an important physiological characteristic used for the identification of LAB [4]. Other than that, thermotolerance capacity of LAB
benefits in acceptance the thermal treatment as industrial production of probiotic foods generally involves processes taking place at high temperatures [28]. In this study, *P. acidilactici* TN1 and *L. farcininis* TY1 can tolerate temperature ranged from 28-70 °C. Thermotolerance trait of our strains may be advantageous if the bacteriocin produced is to be used as an antimicrobial agent in fermented foods or thermally processed foods as Mathew and Augustine [29] exposed that optimum level of bacteriocin was associated with an optimum temperature of growth. Fossi et al. [30] incorporated calcium and magnesium salts into the media to improve the thermotolerance of probiotic strains. The protective effect of the mineral salts is due to the fact that calcium and magnesium ions have the property of stabilizing certain structural proteins and even enzymatic ones thus may prevent the rapid denaturation of membrane proteins.

To reach the GIT in a viable form, probiotic strains have to overcome several biological barriers including the presence of lysozyme in the saliva, low pH in gastric juice and bile salts in the upper GI tract [31, 32]. Bacteria inhabiting intestinal tract must have intrinsic resistance mechanisms to cope with bile salts [33]. Result showed that *P. acidilactici* TN1 and *L. farcininis* TY1 can tolerate 0.3% bile salt thus they may survive under high acidity in the stomach and high concentration of bile components in human gastrointestinal tract. Our finding was concurred with the ability of *Bifidobacterium* and *Lactobacillus* species to cope with bile stress [33]. In addition, 0.3% of bile was used, as it corresponded to that found in the human intestinal tract and 0.3% bile is the maximum concentration that is present in healthy men [34].

To satisfy the probiotic criterion, the strain has to endure the action of toxic metabolites (primarily phenols) produced during the digestion process [35] because bacteria that are lenient to phenols may have better possibilities of persistence in the GIT. Furthermore, some aromatic amino acids derived from dietary or endogenously produced proteins can be deaminated in the gut by bacteria leading to the formation of phenols which have bacteriostatic properties [36]. *P. acidilactici* TN1 showed an exceptional tolerance to phenol up to 0.5% (w/v), whereas the tolerance of *L. farcininis* TY1 was limited to 0.3% (w/v). Results from this study is contradictory to *P. acidilactici* Kp10 that inhibited by as low as 0.2% (w/v) of phenol [37]. The mechanism of inhibitory effect of phenolic compound is simply by their ability to diffuse into the bacterial cell membrane and subsequently cause leakage of the intracellular membrane that destroys the bacterial cell membrane. In addition, most of the phenolic compounds remain in the gastrointestinal tract after consumption and this compound exert their inhibitory effects on the enzyme involved in degradation of proteins, lipids, and saccharides [38].

*P. acidilactici* TN1 and *L. farcininis* TY1 were able to express proteolytic and amylolytic enzymes. Fermentation with LAB is considered as an effective way to reduce whey protein antigenicity. Milk protein allergens can be degraded by a series of proteolytic enzymes produced during the microbial fermentation [39] in following steps: (i) Proteinases initially cleave the milk protein to peptides, (ii) Peptidases cleave the peptides into smaller peptides and amino acids, (iii) Small peptides and amino acids shift in the cellular uptake by transport systems [40]. Since probiotics require free amino acids for growth and survival, the release of essential amino acids and production of growth stimulators thereby assisted the growth and viability of probiotic in functional products [41].

Acidification capability of potential strains is one of the most important technological properties for potential starter cultures especially for milk-based probiotic products making. The reduction of pH can control the growth of a large number of pathogenic or undesirable microorganisms that cause spoilage of fermented products, and can improve the hygienic properties and storage of the final products [4]. In the present study, the pH of the milk decreased during fermentation, indicating increased acidity over the storage, but did not reach less than pH 4.0 (for both strains TN1 ad TY1) and resulted in a cream colour curd with rancid aroma after 24 h of incubation. This finding was in line with Senaka et al. [41], who claimed that pH below pH 4 is generally considered detrimental to the survival of probiotic organisms, even though sensitivity of probiotics to lower pH in functional food is species and strain specific. Theoretically, the acidification of milk directly impacts the stability of casein micelles, reducing their charge, dissolving some of the insoluble calcium phosphate crosslinks and modifying internal bonding between proteins. It is reported that the aroma and taste of soured milk products are
characterised by numerous bacterial metabolites (volatile or non-volatile acids and carbonyl compounds) such as acetaldehyde, acetone, acetoïn, and diacetyl in addition to acetic, formic, butanoic, and propanoic acids [42]. Furthermore, the strains that able to coagulate milk within 16 h were defined as fast coagulating strains, while slow variants require a longer period of time (more than 36 h) [43].

The human GIT ports 10^{13}–10^{14} bacterial cells in adults and this microbiota is often exposed to a variety of antibiotics, due to their routine use in clinical settings [44]. Consequently, the human GIT microbiota may serve as an important reservoir of antibiotic resistant strains that could act as opportunistic pathogens or as donors of resistance genes to other bacteria [45]. Authentication of virulence factors in LAB is necessary due to the risk of genetic transfer, since these genes are usually located in conjugative plasmids [46] hence antibiotic resistance genes can speedily move through bacterial populations and emerge in pathogenic bacteria via horizontal gene transfer [47]. Therefore, the safety assessment of potential probiotics strains towards antibiotics is highly needed. Our strains, *P. acidilactici* TN1 and *L. farciminis* TY1 show a resistance response towards amikacin, erythromycin, nalidixic acid, norfloxacin and streptomycin. Differences in resistance phenotypes may be due to non-functional and/or silent genes [48].

5. Conclusion

Results from this study revealed that bacteriocins producing-LAB were successfully isolated from fermented products of Northern Borneo identified as *P. acidilactici* TN1 (from *tapai nasi*) and *L. farciminis* TY1 (from *tempoyak*). Both strains showed an antimicrobial activity against food-borne pathogen, *L. monocytogenes* ATCC13932, grow at wide range of NaCl concentrations (0.5 - 5.0 %, w/v) and temperatures (28 - 70 °C). They also demonstrated a probiotic characteristic such as able to degrade protein, acidify skim milk, endure 0.3% (w/v) of bile salts, tolerate to phenol up to 0.5% (w/v) and show an intrinsic mechanism towards various antibiotic. In future, more *in vitro* assessment and *in vivo* evaluation should be carried out to unfold the novel potential of these two strains in food industry.

Acknowledgment

The authors wish to acknowledge the Faculty of Science and Natural Resources, Universiti Malaysia Sabah for providing research facilities and financial support (Undergraduate Scientific Project).

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