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Physio-chemical, microbiological properties of tempoyak and molecular characterisation of lactic acid bacteria isolated from tempoyak

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ABSTRACT

This study aims to determine physio-chemical properties of tempoyak, characterise the various indigenous species of lactic acid bacteria (LAB) present at different stages of fermentation and also to determine the survival of selected foodborne pathogens in tempoyak. The predominant microorganisms present in tempoyak were LAB (8.88–10.42 log CFU/g). *Fructobacillus durionis* and *Lactobacillus plantarum* were the dominant members of LAB. Other LAB species detected for the first time in tempoyak were a fructophilic strain of *Lactobacillus fructivorans*, *Leuconostoc dextranicum*, *Lactobacillus collinoides* and *Lactobacillus paracasei*. Heterofermentative *Leuconostoc mesenteroides* and *F. durionis* were predominant in the initial stage of fermentation, and as fermentation proceeded, *F. durionis* remained predominant, but towards the end of fermentation, homofermentative *Lb. plantarum* became the predominant species. Lactic, acetic and propionic acids were present in concentrations ranging from 0.30 to 9.65, 0.51 to 7.14 and 3.90 to 7.31 mg/g, respectively. Genotyping showed a high degree of diversity among *F. durionis* and *Lb. plantarum* isolates, suggesting different sources of LAB. All tested *Lb. plantarum* and *F. durionis* (except for one isolate) isolates were multidrug resistant. *Salmonella* spp., *Listeria monocytogenes* and *Staphylococcus aureus* were not detected. However, survival study showed that these pathogens could survive up to 8–12 days. The results aiming at improving the quality and safety of tempoyak.

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1. Introduction

Durian (*Durio zibethinus*) fruit is one of the most important seasonal fruits cultivated in tropical Asian countries for its high price, exotic aromas and flavours. It is regarded as “King of fruits” in South East Asia. In most Asian countries, except for Thailand, durians are not harvested but allow to drop to the ground when it ripens. Durian has a short shelf-life ranging from two to three days post-harvest. Following harvesting, during retailing or storage, the durian undergoes rapid physio-chemical changes leading to dehiscence and softening of the pulp or the pulp becomes soggy or watery which leads to rapid decline in value (Paul and Ketsa, 2014). Durian pulp from low-quality durians is frozen and used as a flavouring ingredient in many desserts such as durian cake, ice cream and candies (Ho and Bhat, 2015).

Tempoyak is a popular acid-fermented condiment used with certain fish and vegetable dishes in ASEAN countries. The pH of tempoyak ranges from 3.96 to 4.08 (Amin et al., 2004; Wasnin et al., 2014). Tempoyak is usually produced using low-quality durian pulp obtained from crack, poor quality or over-ripen durian. Production of tempoyak allows for the salvage of durians which would be otherwise be discarded (Gandjar, 2000). Thus, fermentation of the over-ripen and poor quality fruit pulp is a cheap and efficient mean of preserving highly perishable climacteric fruits especially in developing countries where proper refrigeration or post-harvest handling facilities are lacking. Durian pulp is traditionally fermented through spontaneous and uncontrolled processes. Like other naturally fermented fruits, tempoyak produced is of variable quality as the indigenous microflora is not consistent (Chen et al., 2013; Nyanga et al., 2008). The fermentation relies on the

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microflora already present in the pulp. The microorganisms present in the pulp might originate from the environment, particularly the soil, rotting vegetation in the orchards, from the hands of vendors or customers, knives, retailing environment, baskets and cross contamination from other crack durians (Papalexandratou et al., 2011). Pulp from different durians is mixed with or without an addition of salt and allowed to ferment in a tightly sealed container (some cottage producers use earthen pots) at ambient temperature for a minimum of seven days for the development of acidity and flavour (Merican, 1977). From our observation while visiting small scale producers and households, we observed that tempoyak is allowed to ferment for months. Tempoyak is produced from one durian fruiting season to another and domestically produced for self-consumption.

Leisner et al. (2001) reported that LAB are the predominant microorganisms present in tempoyak, ranging from 8.4 to 9.2 log CFU/g of tempoyak. They had reported that *Lactobacillus plantarum* were the predominant members of the LAB flora in tempoyak. Other species of LAB present in tempoyak as reported by other researchers are *Lactobacillus brevis*, *Leuconostoc mesenteroides*, *Lactobacillus mali*, *Lactobacillus fermentum*, *Lactobacillus durianis* (Leisner et al., 2002), *Lactobacillus corynebacterium* (Wirawati, 2000), *Lactobacillus fersantum* (Ekowati, 1998), *Lactobacillus casei* (Mohd Adnan and Tan, 2007), *Fructobacillus durionis* (Endo and Okada, 2008; Leisner et al., 2005), *Weissella paramesenteroides and Pediococcus acidilactici* (Yuliani and Dixon, 2011). Among the species reported, *F. durionis* and *Lb. brevis* were reported to exhibit fructophilic characteristics. The term “fructophilic LAB” (FLAB) refers to a specific group of LAB that prefers fructose and sucrose as carbohydrate substrate. FLAB has been isolated from fructose-rich environments such as fruits, flowers, honey, and fermented foods and beverages as well flower- and fructose-related insects (Endo et al., 2009; Endo and Salminen, 2013; Neveling et al., 2012). They are characterised by comparatively rapid growth on fructose than glucose, requires external electron acceptors for glucose metabolism, and a limited number of carbohydrates (Endo et al., 2009).

Like many other fermented or acidified vegetable and fruit products which are consumed without heat treatment, tempoyak being a fermented product is also regarded as microbiologically safe. However, it is not unprecedented that foodborne pathogens were detected in fermented foods and beverages (Marty et al., 2012). Akaki et al. (2011) reported on the occurrence of various potential pathogenic bacteria (*Bacillus* spp., *Klebsiella pneumoniae* spp. *pneumoniae* and staphylococci) in traditional millet-based fermented gruels and *Bacillus* spp. were present at the end of the cooking (82–85 °C). They attributed the incidence to unhygienic practices and environmental conditions, as well as a possible adaptation of the bacteria to the new conditions. Outbreaks traced to contamination in fruits and products of fruit have also been reported (Laidler et al., 2013; Senkel et al., 2003). These researchers were of the opinion soil contaminated with faeces or use of fallen fruits for production as possible routes of contamination. In view of this, microbial safety of tempoyak which is mainly produced using fallen or cracked durian is of great concern.

Previous studies on tempoyak mainly focused on the isolation and identification of LAB present in tempoyak (Ekowati, 1998; Leisner et al., 2001, 2005; Yuliani and Dixon, 2011) as well as characteristics of tempoyak (Neti et al., 2011; Wasnin et al., 2014). The microbial changes of LAB during tempoyak fermentation and the safety aspects of tempoyak, however, have not been reported. As fermented foods possess diverse microflora, the presence of multidrug-resistant LAB is a concern as they pose a threat to food safety and human health (Hummel et al., 2007). The aims of this study were to study the biodiversity of LAB and microbial changes taking place during natural fermentation of tempoyak, as well as highlighting the presence of multidrug-resistant LAB isolates involved in the fermentation of tempoyak. In addition, the microbial safety of the naturally fermented tempoyak was evaluated by determining the survival of foodborne pathogens such as *Salmonella enterica* subsp. *enterica* serovar Enteritidis, *Listeria monocytogenes* and *Staphylococcus aureus* in naturally fermented tempoyak.

2. Materials and methods

2.1. Physio-chemical and microbiological properties of naturally fermented tempoyak

2.1.1. Preparation of tempoyak

The pulp of overripe, crack, unripe durians or durians of inferior quality is pooled by the farmers and usually purchased by individuals for tempoyak and durian candies production. We purchased durian pulp from a durian orchard in Balik Pulau, Penang. According to our observations, the orchard owner removes the pulp of unsold durians which are of inferior quality, over ripen, undergone dehiscence, crack or infested with larvae of fruit flies. The pulp from different durians is pooled and frozen and kept in a −18 °C freezer. The durian pulp used is obtained from different cultivars in the same orchard. The pulp was transferred to food processing laboratory within 1–2 h acquisition but the storage time in the orchard was unknown. The durian pulp was mixed well with a food mixer and separated into aliquots of 200 g each in tightly sealed glass jars. Three individual replicates of tempoyak were prepared. Tempoyak was produced by naturally fermenting (allowing the already present microorganism to grow) the durian pulp for 24 days at 30 ± 1 °C. No starter culture and preservative was added. The durian pulp was subjected to physio-chemical and microbiological analysis.

2.1.2. Physio-chemical analysis of naturally fermented tempoyak

Determination of pH, titratable acidity (TA), total sugar content and organic acid content of the naturally fermented tempoyak were carried out as described by Voon et al. (2006), with minor modifications. Measurements were performed in duplicate with three individual replicates of tempoyak on day 0, 1, 2, 4, 6, 8, 12, 16, 20 and 24.

Briefly, durian pulp (10 g) was homogenized in 90 mL of distilled water for 1 min using a Stomacher 400 Circulator (Seward, West Sussex, United Kingdom) and pH of the slurry was measured. Subsequently, TA of the slurry was determined by titration using 0.1 N NaOH to pH 8.1 and the results were expressed as the percentage of lactic acid.

$$\%\ lactic\ acid = \frac{mL\ of\ NaOH\ used \times Normality\ of\ NaOH \times 9}{Weight\ of\ sample}$$

Sugar extraction was performed as previously described (Hunt et al., 1977), with minor modifications. Briefly, 10 g of tempoyak was mixed with 85% methanol and allowed to stand for 30 min in a water bath (60 °C). The sample was centrifuged and the pellet was re-extracted twice with 75% methanol. The supernatant was evaporated using a rotary evaporator. Sample was diluted 100 folds with deionised water and cation exchange resin was added before filtration using muslin cloth. The same procedure was repeated using anion exchange resin. The sample was passed through a pre-activated Sep-Pak C_{18} disposable cartridge (Waters Corporation, Milford, USA) and subsequently filtered using a 0.45 μm membrane filter (Milipore, USA). Twenty microlitres of the extracted sample were injected to a Waters 2414 series HPLC system equipped with a

Sugar-Pak I Column (Waters Corporation, USA) and an RI-1530 detector. The mobile phase used was 0.1 mM Ca-EDTA, with a flow rate of 0.5 mL/min at 90 °C.

Organic acids were determined using a Waters 2414 series HPLC system (Waters Corporation, USA) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, California, USA) and an RI-1530 detector (Waters Corporation, USA). Briefly, 5 g of durian pulp were mixed in 20 mL of deionized water and centrifuged at 12,000 × g for 20 min at 4 °C. The supernatant was passed through a pre-activated Sep-Pak C_{18} disposable cartridge (Waters Corporation, Milford, USA) and subsequently filtered using a 0.45 μm membrane filter (Milipore, Milford, USA) prior to HPLC analysis. Isocratic elution was achieved at 45 °C using 5 mM H_2SO_4 as a mobile phase at a flow rate of 0.6 mL/min. Organic acids profile was analysed by comparing to organic acid standards.

2.1.3. Microbiological analysis of naturally fermented tempoyak

Microbial analyses were performed as previously described (Voon et al., 2006) on day 0, 1, 2, 4, 6, 8, 12, 16, 20 and 24. All the microbiological media used in this section was purchased from Merck, Darmstadt, Germany. Total Plate Count, LAB and Yeast and Mould counts were determined by homogenizing approximately 10 g of tempoyak in a stomacher bag containing 90 mL sterile peptone water. Appropriate serial dilutions were plated on plate count agar (PCA), MRS agar and Dichloran Rose Bengal Chloramphenicol (DRBC) agar. The PCA and MRS agar plates were incubated at 30 °C for 24–72 h while DRBC agar plates at 25 °C for 5–7 days. The experiment was performed in duplicate with three different tempoyak samples being analysed at each time point. Number of colonies was calculated from plates producing 25–250 well-separated single colonies and the results were expressed as log colony forming units (CFU) per gram of tempoyak. LAB were randomly isolated as described by Leisner et al. (2001), from both MRS agar and PCA supplemented with 0.5% (w/v) $CaCO_3$ every 24 h and purified for further analysis.

2.2. Identification and characterisation of lactic acid bacteria present in naturally fermented tempoyak

2.2.1. Identification using PCR amplification of 16S rRNA gene and sequencing

Genomic DNA of LAB isolates was prepared using Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) according to manufacturer's instructions. 16S rRNA PCR amplification was performed as described by Endo and Okada (2008), using two primers: 8F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 15R (5′-AAG GAG GTG ATC CAR CCG CA-3′). PCR amplification conditions were as follows: initial denaturation at 94 °C for 5 min; 30 cycles of denaturation of 94 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 1 min 15 s; and final extension 72 °C for 5 min. Subsequently, the PCR amplicons (approximately 1500 bp) were purified by Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The purified fragments were sequenced using the same primer sets of PCR (1st BASE laboratories, Selangor, Malaysia) and processed using BioEdit software. The closest recognized relatives of the isolates were determined by using BLASTN with the sequence of type strains available at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA).

2.2.2. Differentiation of LAB species by multiplex PCR using species-specific primers

For differentiation of *Lb. plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum*, a multiplex PCR amplification using species-specific primers was performed as described by Torriani et al. (2001). A set of four *recA* gene-based primers: paraF (5′-GTC ACA GGC ATT ACG AAA AC-3′), pentF (5′-CAG TGG CGC GGT TGA TAT C-3′), planF (5′-CCG TTT ATG CGG AAC ACC TA-3′), and pREV (5′-TCG GGA TTA CCA AAC ATC AC-3′) were used. The PCR products from multiplex PCR were subjected to electrophoresis in 2.0% (w/v) agarose gel in 1 × TAE. The PCR products were stained with EZ-Vision® One DNA dye (Amresco, USA) for direct visualization after electrophoresis.

2.2.3. Phenotypic characterisation of lactic acid bacteria

Gram staining, catalase production, cell morphology and motility were observed under a phase contrast microscope (Rax-Vision, USA) (Leisner et al., 2001). Lactic acid configuration produced from glucose was determined using D- and L-lactate dehydrogenase test kits (Megazyme, Ireland) according to manufacturer's instructions. To further differentiate FLAB, growth in Glucose Yeast Peptone (GYP) and Fructose Yeast Peptone (FYP) media were assessed. Gas production of the presumptively FLAB was also assessed using MRS broth with 1% (w/v) D-glucose combined with 1% (w/v) D-fructose (Leisner et al., 2005). The growth of presumptive FLAB in GYP media, supplemented with 1% (w/v) D-fructose and 1% (w/v) pyruvate, respectively, and FYP media supplemented with 30% (w/v) D-fructose were determined (Neveling et al., 2012). Carbohydrate fermentation pattern was determined using API 50 CHL kits (bioMerieux, Marcy-l'Etoile, France) according to manufacturer's instructions. All experiments were performed in duplicate.

2.2.4. Antibiotic resistance assay

The antibiotic resistance of 16 isolates each of *F. durionis* and *Lb. plantarum* were determined using the disc diffusion method as described by Bauer et al. (1966) and according to the guidelines of the CLSI (2005, 2013). Seventeen antibiotics classified as critically important and highly important based on the report of Joint FAO/WHO/OIE Expert Meeting were selected (FAO/WHO/OIE, 2008). Inocula were prepared by diluting overnight culture of each LAB isolate in 0.85% (w/v) NaCl solution to turbidity comparable to 0.5 McFarland standard (bioMerieux, Marcy-l'Etoile, France) (~1.5×10^8 CFU/mL). A sterile cotton swab was dipped into the bacterial suspension and spread evenly on Mueller-Hinton agar plates. Commercially available antibiotic discs (Oxoid, Basingstoke, UK) were placed on the lawn of the completely-diffused bacterial culture. The plates were then incubated at 30 °C for 48 h and the diameter of the inhibition zones inclusive of the diameter of the disc was measured to the nearest mm from the point of abrupt inhibition of growth. The antibiotic resistance was determined in triplicate. Results were interpreted referring to CLSI (2013), and those not covered by CLSI were adopted from Vlková et al. (2006). Multiple antibiotics resistance (MAR) index was calculated and interpreted as described by Krumperman (1983). LAB isolates classified as intermediate susceptible on the basis of inhibition zone were considered as sensitive for MAR as well as resistance spectrum.

2.2.5. Genotyping of lactic acid bacteria and data analysis

Composite analysis of Random amplified polymorphic DNA (RAPD) PCR and Pulse-field gel electrophoresis (PFGE) were performed for typing of *F. durionis* and *Lb. plantarum*. For RAPD PCR, random primer C11 (5′-AAA GCT GCG G-3′) and C19 (5′-GTT GCC AGC C-3′) synthesized by 1st BASE laboratories (Selangor, Malaysia) were used. PCR reactions and amplification conditions for *F. durionis* were as described by Szczuka and Kaznowski (2004), with modifications: initial denaturation at 94 °C for 6 min; 35 cycles of denaturation of 94 °C for 1 min, annealing at 34 °C for 2 min and extension at 72 °C for 2 min; and final extension 72 °C for

7 min. For *Lb. plantarum* isolates, similar PCR reactions and amplification conditions were performed with the annealing temperature of 40 °C. Gel electrophoresis, staining and visualization were performed as aforementioned, but in 1.5% (w/v) agarose gel. The experiment was repeated twice.

Pulse-field gel electrophoresis (PFGE) was conducted according to the method described by Coeuret et al. (2004), with the following modifications. Briefly, an overnight culture of *Lb. plantarum* and *F. durionis* in respective growing broth was adjusted to OD of 0.7 using a turbidity meter. The cells were suspended in suspension buffer containing 15 mg/mL lysozyme (Sigma, USA) and incubated at 37 °C for 1 h. The plugs were incubated at 54 °C in 2 mL of lysis buffer (50 mM Tris pH 8, 50 mM EDTA pH 8, 1% N-lauryl sarcosine) containing 1 mg/mL Proteinase K (Promega, Madison, USA) for overnight and washed with TE buffer for six times. DNA of both *F. durionis* and *Lb. plantarum* isolates were digested separately with 20 U of restriction enzyme *Sfi*I (Vivantis, Malaysia) at 50 °C for 4 h. DNA electrophoresis was performed on 1% (w/v) agarose gel in a CHEF Mapper system (BioRad, Hercules, CA) with 0.5 × TBE buffer for 22 h at 14 °C using a linear ramp of 5–10 s at 6 V/cm. PFG lambda marker (New England Biolabs, Ipswich, MA) and low range PFG marker (New England Biolabs, Ipswich, MA) were used as the DNA size marker. PFGE gel was stained with GelRed™ Nucleic acid gel stain (Biotium, USA) and viewed using Gel Doc XR + System (Bio-Rad, USA). The experiment was repeated twice.

RAPD and PFGE data were processed separately for *F. durionis* and *Lb. plantarum*, using Bionumerics software version 6.0 (Applied Maths, Kortrijk, Belgium), with the extent of variability determined by the DICE coefficient. Clustering of the fingerprints was based on the unweighted pair-group method using arithmetic averages clustering system (UPGMA) with a position tolerance of 0.10. Data from both genotyping methods were subjected to a composite analysis in a 1:1 weight ratio.

2.3. Survival of foodborne pathogens in naturally fermented tempoyak

All the microbiological media used in section 2.3 was purchased from Merck, Germany except for Brain Heart Infusion (BHI) broth which was purchased from Oxoid, Hampshire, United Kingdom.

2.3.1. Preparation of inoculum

Foodborne pathogens used in this study were *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076, *Listeria monocytogenes* ATCC 19115 and *Staphylococcus aureus* ATCC 6538P, which were stored in glycerol at −20 °C. *S. Enteritidis* and *Staph. aureus* cultures were revived using Tryptic Soy broth (TSB) and incubated at 37 °C for 24 h while *L. monocytogenes* was revived in BHI broth and incubated at 30 °C for 24 h. The working cultures of these pathogens were prepared by two consecutive 24 h loop transfers of each culture into 10 mL of their respective culture media.

2.3.2. Inoculation of durian pulp with Salmonella Enteritis, Listeria monocytogenes and Staphylococcus aureus

The durian pulp (200 g each) was inoculated separately with 2 mL of overnight broth cultures of *S.* Enteritidis, *L. monocytogenes* and *Staph. aureus* and mixed thoroughly with a Stomacher 400 Circulator (Seward, West Sussex, United Kingdom). The durian pulp not inoculated with foodborne pathogens served as untreated control. The containers were tightly sealed and incubated at 30 °C for 24 d. Two replicates each of inoculated durian pulp for each pathogen and the not inoculated control were prepared, and the experiments were repeated thrice.

S. Enteritidis, *L. monocytogenes* and *Staph. aureus* counts were determined by the spread plate method using Xylose Lysine Deoxycholate agar (XLD agar); PALCAM Listeria agar supplemented with selective supplements and Baird Parker agar supplemented with egg yolk tellurite emulsion, respectively, as described by Jeon et al. (2015). Enumeration was performed on day 0, 1, 2, 4, 6, 8, 12, 16, 20 and 24. Briefly, 10 g of tempoyak were aseptically transferred to stomacher bag containing 90 mL of buffered peptone water and homogenized for 1 min using a Stomacher 400 Circulator (Seward, West Sussex, United Kingdom). Hundred microlitres of the appropriate dilutions were plated on the respective selective culture media and incubated at 37 °C for 24–48 h. One ml of the homogenized tempoyak slurry of the lowest dilution was plated on the selective agars and absence of growth was reported as < 1 log CFU/g of tempoyak. Sample with pathogen count below the detection limit was further enriched for 18 h and subsequently plated on their respective selective agar for detection of presence/absence of the target pathogens.

3. Results

3.1. Characterisation of naturally fermented tempoyak

Fig. 1 shows the pH, TA and organic acid contents of tempoyak during natural fermentation for 24 days. The pH of naturally fermented tempoyak decreased rapidly from 7.13 on day 0, to 4.62 on day 1. The pH continued to decline during the fermentation period. The changes in pH during the fermentation period were consistent with the TA values (expressed as the percentage of lactic acid) and organic acids content. As the pH of tempoyak decreased, an increase in TA values and production of lactic acid was observed. Our results show that on day 0, the concentration of lactic, acetic and propionic acids present in the durian pulp was 0.30, 0.51 and 3.90 mg/g, respectively. On day 1, the concentration of lactic, acetic and propionic acids was 3.34, 2.78 and 4.22 mg/g, respectively. On day 2, lactic acid content increased drastically (7.66 mg/g) and during the rest of the fermentation period, lactic acid concentration ranged from 7.58 to 9.65 mg/g. On day 2–4, the acetic acid content ranged from 6.40 to 7.14 mg/g, respectively. As the fermentation proceeded, the acetic acid concentration began to decline from day 6 onwards. The microorganism present in tempoyak also produced propionic acid with concentration ranging from 3.90 on day 0, to 7.31 mg/g on day 20.

The concentration of sucrose, fructose and glucose present in durian pulp prior to fermentation were 68.38, 12.67 and 10.59 mg/g (Fig. 2). During the initial stages of fermentation (day 0–2), sucrose content decreased tremendously but it remained constant from day 4 onwards. Glucose and fructose showed similar trend throughout the fermentation period. The concentration of fructose and glucose increased from day 0–4, but decreased from day 4–12, and subsequently increased after day 12.

LAB were the predominant microorganisms present in tempoyak (Table 1). The tempoyak fermentation was characterised by high initial LAB counts on day 0–4 (8.88–10.42 log CFU/g) which decreased slightly as fermentation proceeded. Initially, yeasts were present (5.98 log CFU/g) but on day 2, yeasts were not detected. Moulds were absent.

3.2. Identification and characterisation of lactic acid bacteria present in naturally fermented tempoyak

A total of 177 isolates obtained from tempoyak were considered as LAB based on Gram reaction, non-motility, the absence of spore formation and negative catalase reaction (results not shown) and identified to species level (with sequence homologies of 99–100%) by 16S rRNA gene sequencing. The amplification of *recA* gene from

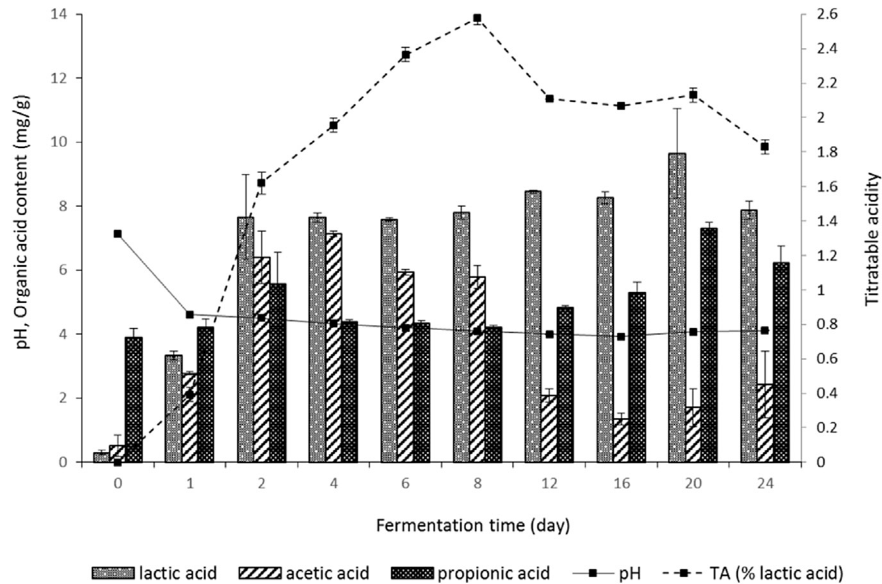

Fig. 1. pH, titratable acidity and organic acid contents of tempoyak fermented for 24 days.

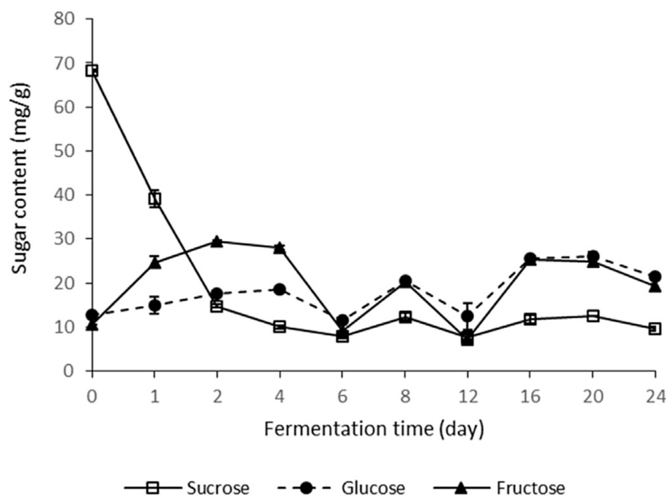

Fig. 2. Concentrations of sucrose, glucose and fructose in tempoyak fermented for 24 days.

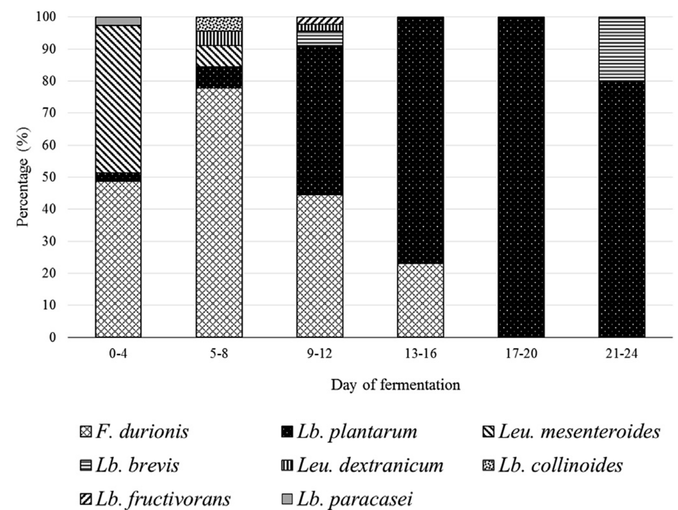

Fig. 3. Succession of indigenous LAB during fermentation of tempoyak for 24 days.

Table 1
Total plate count, LAB and yeast and mould counts during tempoyak fermentation.

Day	Total plate count (log CFU/g)	LAB count (log CFU/g)	Yeast and Mould count (log CFU/g)
0	9.13 ± 0.11	8.88 ± 0.10	5.98 ± 0.12
1	10.18 ± 0.09	10.22 ± 0.06	1.67 ± 0.22
2	10.37 ± 0.09	10.32 ± 0.06	N.D.
4	10.33 ± 0.04	10.42 ± 0.07	N.D.
6	10.23 ± 0.07	10.37 ± 0.13	N.D.
8	10.13 ± 0.08	10.13 ± 0.09	N.D.
12	9.78 ± 0.09	9.75 ± 0.10	N.D.
16	9.62 ± 0.11	9.53 ± 0.11	N.D.
20	9.57 ± 0.06	9.40 ± 0.03	N.D.
24	9.42 ± 0.11	9.38 ± 0.04	N.D.

Note: N.D., not detected (detection limit is < 25 CFU/mL). The experiments were conducted in triplicate and results were expressed as average ± standard error.

all *Lb. plantarum* isolates produced 318 bp (result not shown), confirming their identity.

Results in Fig. 3 shows that populations of different LAB genera dominated at different intervals or periods of fermentation. Initially, *Leu. mesenteroides* and *F. durionis* were predominant and as the fermentation progress, *F. durionis* became predominant (day 5–8) and subsequently both *F. durionis* and *Lb. plantarum* were predominant (day 9–12). This trend changed after day 12 as *Lb.*

plantarum became predominant until the end of fermentation. Occasionally, *Lb. brevis*, *Leu. dextranicum*, *Lb. collinoides*, *Lb. paracasei* and *Lb. fructivorans* were isolated from tempoyak at different stages of fermentation (Fig. 3 & Table 2).

Table 2 shows that all *F. durionis* and *Lb. fructivorans* isolates grew well (as reflected by profuse growth) in FYP broth and FYP media supplemented with 30% (w/v) fructose, but poorly in GYP. Moreover, *F. durionis* isolates fermented six to 11 carbohydrates while *Lb. fructivorans* utilised only fructose and glucose and was thus regarded as FLAB. The growth of FLAB was enhanced in GYP broth supplemented with D-fructose or pyruvate (results not shown).

Fig. 4 shows the genotypes and antibiograms of representative dominant LAB isolated from naturally fermented tempoyak. Composite analysis of RAPD and PFGE differentiated *F. durionis* isolates into two clusters (A and B) and six singletons. Both clusters consisted of five isolates each. *Lb. plantarum* isolates were differentiated into four clusters (A to D), and seven singletons. Three isolates belong to cluster A while Clusters B to D had two isolates each. For both *F. durionis* and *Lb. plantarum*, different antibiograms were observed among isolates grouped in the same cluster by composite analysis of RAPD and PFGE. All the tested isolates were multidrug resistant except for *F. durionis* d16p1, which was only resistant to vancomycin, nalidixic acid and sulphafurazole. *F. durionis* and *Lb. plantarum* exhibited distinct antibiograms and no common antibiogram was observed among these two species. *F. durionis* isolates were resistant to three to nine antibiotics (MAR index = 0.34) and produced 11 different resistance patterns. *Lb. plantarum* isolates were resistant to seven to 13 antibiotics (MAR index = 0.54) and 14 different resistance patterns were observed. All representative isolates of both *F. durionis* and *Lb. plantarum* were resistant to vancomycin, nalidixic acid, and sulphafurazole (except *F. durionis* d2m4) but susceptible to erythromycin, gentamycin, and chloramphenicol, kanamycin (except *F. durionis* d8p2 and *Lb. plantarum* d11m6) and amoxicillin/clavulanic acid (except *Lb. plantarum* d16m5).

3.3. Survival of foodborne pathogens in naturally fermented tempoyak

Salmonella spp., *Listeria* spp. and *Staph. aureus* were not detected in naturally fermented tempoyak (uninoculated control) throughout the fermentation (results not shown). Fig. 5 shows that *S.* Enteritidis, *L. monocytogenes* and *Staph. aureus* counts decreased drastically from day 0–12. *S.* Enteritidis and *Staph. aureus* were not detected on day 8 day, whereas *L. monocytogenes* was not detected on day 12 of fermentation.

4. Discussion

Research on tempoyak remains limited and the studies mainly focus on isolation and identification of LAB associated to tempoyak fermentation (Ekowati, 1998; Leisner et al., 2001, 2005; Yuliani and Dixon, 2011). To our knowledge, there is no study that reported on the succession of LAB present in tempoyak and also the safety aspects of naturally fermented tempoyak (antibiotic resistance of the indigenous LAB and survival of foodborne pathogens in tempoyak). We examined the microbiological and physio-chemical changes during 24 days of fermentation as we wanted to determine the microbial selection occurring during fermentation. In this study, the natural fermentation of tempoyak was carried out using one batch of durian obtained from a single orchard which have different cultivars of durians. Although this might be a limitation in comparing the prevalence of predominant LAB involved in tempoyak fermentation but in our opinion, the microflora of tempoyak might not have differed significantly as other researchers have also reported that *Lb. plantarum* strains were predominate organisms in fermented tempoyak (Leisner et al., 2001; Yuliani and Dixon, 2011). In term of diversity of LAB during fermentation, various researchers have also reported on the presence of diverse LAB species in tempoyak (Ekowati, 1998; Endo and Okada, 2008; Leisner et al., 2002, 2005; Mohd Adnan and Tan, 2007; Wirawati, 2000; Yuliani and Dixon, 2011). In this study, we report the presence of *Leu. dextranicum*, *Lb. collinoides*, *Lb. paracasei* and *Lb. fructivorans* in tempoyak for the first time. In addition, we also for the first time, report the presence of a novel fructophilic strain of *Lb. fructivorans*. FLAB which previously have been reported by other researchers consist of all members of *Fructobacillus* (*F. fructosus*, *F. ficulneus*, *F. durionis*, *F. pseudoficulneus* and *F. tropaeoli*) (Endo and Okada, 2008; Endo et al., 2009, 2011) and three members of *Lactobacillus* (*Lb. kunkeei*, *Lb. florum* and *Lb. brevis*) (Endo et al., 2010; Neveling et al., 2012).

Physio-chemical and microbiological properties of tempoyak observed in this study are in agreement with other studies (Amin et al., 2004; Leisner et al., 2001; Wasnin et al., 2014). Lactic and acetic acid were reported as major organic acids produced by LAB microflora in previous studies (Leisner et al., 2001; Wasnin et al., 2014). In this study, we detected the presence of propionic acid (up to 7.31 mg/g) besides lactic and acetic acids. The rapid decline in pH and increase in lactic, acetic and propionic acids content can be

Table 2
Isolation, identification and characterisation of LAB isolated from tempoyak.

LAB species (no. of isolates)	Metabolism	Growth in			Isomer of lactate	Carbohydrate fermentation profile
		FYP	GYP	FYP30		
F. durionis (80)	Heterofermentative	+	W	+	D	Glu-Fru-Gal*-Mal*-Ara*-Man*-Mel*-Rib*-Suc-Tre-Xyl*
Lb. plantarum (65)	Homofermentative	+	+	+	DL	Glu-Fru-Gal-Lac-Mal-Ara*-Cel-Man-Mn-Mel-Raf-Rha*-Rib*-Sal-Sor*-Suc-Tre-Esc
Leu. mesenteroides subsp. *mesenteroides* (21)	Heterofermentative	+	+	+	D	Glu-Fru-Gal-Lac-Mal-Ara*-Cel-Man*-Mn-Mel-Raf-Rib*-Sal-Suc-Tre-Xyl*-Esc
Lb. brevis (4)	Heterofermentative	+	+	−	DL	Glu-Fru-Gal-Mal-Ara-Man*-Mel-Rib-Suc-Xyl-Esc*
Leu. mesenteroides subsp. *dextranicum* (3)	Heterofermentative	+	+	+	N.T.	Glu-Fru-Mal-Man-Mn-Rib-Suc-Tre-Esc
Lb. collinoides (2)	Heterofermentative	+	+	+	DL	Glu-Fru-Gal-Mal-Ara-Rib-Xyl
Lb. paracasei subsp. *paracasei* (1)	Homofermentative	+	+	−	N.T.	Glu-Fru-Gal-Lac-Mal-Cel-Man-Mn-Rib-Sal-Sor-Suc-Tre-Esc
Lb. fructivorans (1)	Heterofermentative	+	W	+	N.T.	Glu-Fru

Note: GYP, Glucose Yeast Peptone; FYP, Fructose Yeast Peptone; FYP30, Fructose Yeast Peptone media supplemented with 30% (w/v) D-fructose; +, positive reaction; −, negative reaction; W, weak positive reaction; N.T., not tested; Glu, Glucose; Fru, Fructose; Gal, Galactose; Lac, Lactose; Mal, Maltose; Ara, Arabinose; Cel, Cellobiose; Man; Mannitol; Mn; Mannose; Mel, Melibiose; Raf, Raffinose; Rha, Rhamnose; Rib, Ribose; Sal, Salicin; Sor, Sorbitol; Suc, Sucrose; Tre, Trehalose; Xyl, Xylose; Esc, Esculin; *, LAB isolates produced a combination of positive, negative and/or weak positive reaction in carbohydrate fermentation.

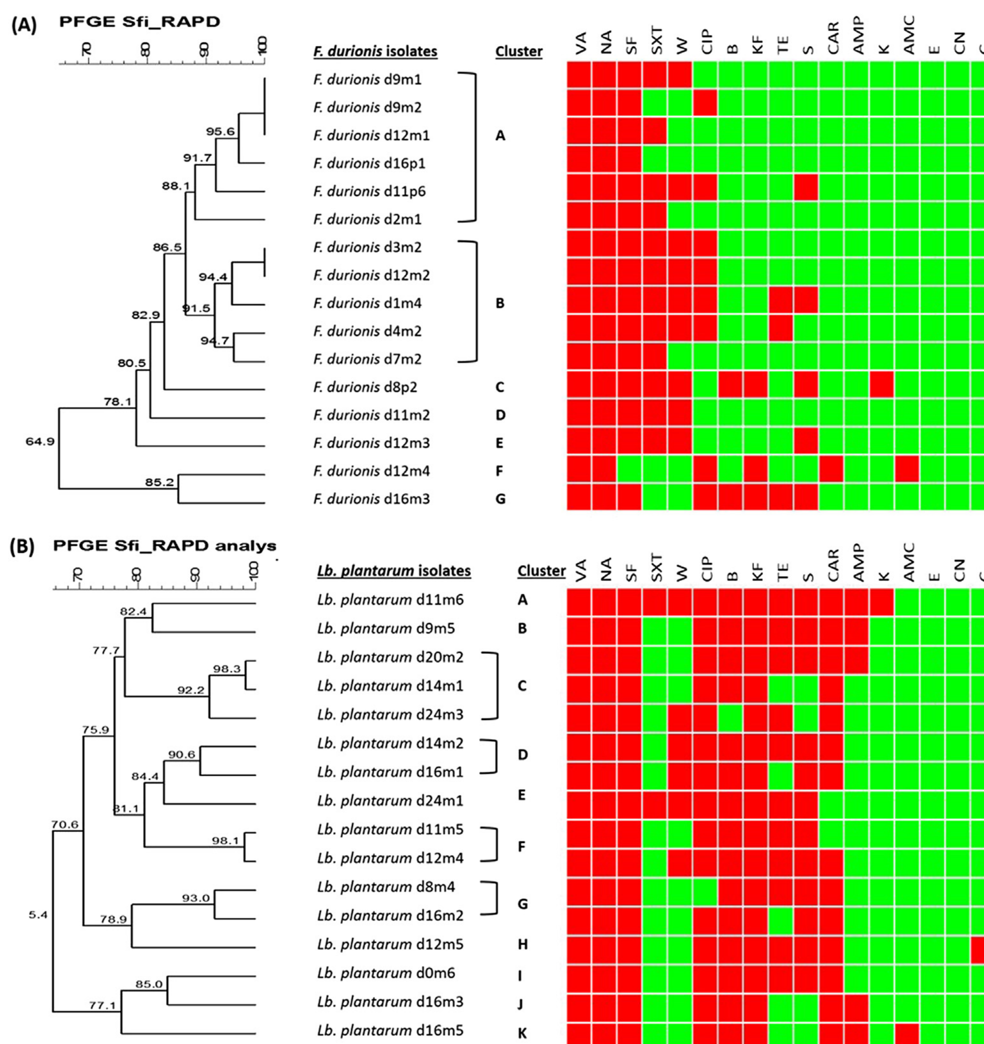

Fig. 4. Genetic relatedness of representative dominant LAB isolates and the corresponding antibiograms. **(A)** *F. durionis*; **(B)** *Lb. plantarum*. The dendrograms were obtained by composite analysis of RAPD and PFGE fingerprints. Grouping of fingerprints were performed by the unweighted pair group method using arithmetric averages clustering algorithm (UPGMA). For antibiogram, resistance is denoted by red rectangle and susceptibility by green rectangle. Abbreviations: vancomycin (VA), nalidixic acid (NA), sulphafurazole (SF), sulphamethoxazole/trimethoprim (SXT), trimethoprim (W), ciprofloxacin (CIP), bacitracin (B), cephalothin (KF), tetracycline (TE), streptomycin (S), carbenicillin (CAR), ampicillin (AMP), kanamycin (K), amoxycillin/clavulanic acid (AMC), erythromycin (E), gentamicin (CN), and chloramphenicol (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

attributed to high initial LAB counts. Various studies have reported on the occurrence of yeast in association with LAB during fermentation (Argyri et al., 2013; Arroyo-López et al., 2012; Nyambane et al., 2014). In this study, however, yeast population decreased tremendously as LAB population increased during fermentation. Yeasts were not detected on the second day of fermentation. The rapid decrease in yeast population was also observed in the spontaneous fermentation of leek (Wouters et al., 2013). Previous studies have reported on the inhibition of yeasts by acetic and propionic acid (Savard et al., 2002), which are in agreement with our results as increased production of acetic and propionic acid by LAB were observed during natural fermentation.

The major differences between our study and those reported by other researchers are on the prevalence of predominant LAB species and the diversity of indigenous LAB population. Previous studies have attributed the fermentation of tempoyak to different LAB species and these studies conclude that *Lb. plantarum* is the predominant organism responsible for the fermentation (Leisner et al., 2001; Yuliani and Dixon, 2011). They have reported on isolation of LAB from tempoyak that was already fermented or the samples obtained from retailers while this study reports on isolation of LAB from different time points during fermentation. We observed that *F. durionis* was also present in large numbers in tempoyak together with *Lb. plantarum*. In our opinion, the ecology from where the durian pulp is obtained influences the type of microorganisms that are present and responsible for the fermentation of tempoyak. Several researchers have also reported that indigenous microflora present on the skin of the fruits, the equipment and utensils used during the fermentation process and other environmental sources were able to grow extensively (7–9 log CFU/g) and are responsible for the fermentation (Fleet, 2003; Nyanga et al., 2007). Different geographical locations, fruit cultivars and the fruit developmental stages may also contribute to the diversity of microflora present on the durian. Similar findings were reported for masau (*Ziziphus mauritiana*) fruits and their fermented fruit pulp (Nyanga et al., 2007) and Tempranillo wine produced by spontaneous malolactic fermentation (Ruiz et al., 2010). Media used for the isolation for LAB can also influence the type of LAB that is recovered. In the routine isolation of LAB, MRS agar is used and this might not favour isolation of all LAB. Endo et al. (2009) were able to

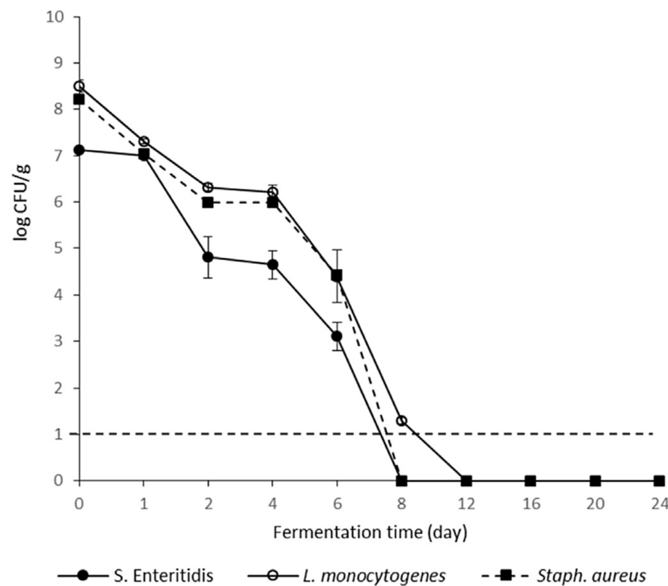

Fig. 5. Survival of *Salmonella* Enteritidis, *Listeria monocytogenes* and *Staphylococcus aureus* in artificially-spiked durian pulp (tempoyak) during fermentation for 24 days. Dotted line: detection limit of the enumeration method.

isolate FLAB by using media containing fructose.

Various studies have reported on the role of *Lb. plantarum*, *Leu. mesenteroides* and *Lb. brevis* in fermented vegetable products such as cucumber, sauerkraut and kimchi (Breidt, 2004; Jung et al., 2014), hence the association of these LAB species with tempoyak is not surprising. This study reports on the succession of LAB microflora which have not being reported by previous researchers. We observed a sequential succession of indigenous LAB microflora during tempoyak fermentation, which is similar to sauerkraut or other plant-based fermented foods (Breidt, 2004; Jung et al., 2014). The dominance of heterofermentative *Leu. mesenteroides* at during the early stages of vegetable fermentation is well recognised. They produce a significant amount of acetic acid in addition to lactic acid, as well as carbon dioxide which rapidly lower the pH and creating an anaerobic condition, which favours the growth of more acid-tolerant homofermentative lactobacilli such as *Lb. plantarum* (Breidt, 2004; McDonald et al., 1990). The role of *F. durionis* in natural fermentation, however, is not well understood. In our study, the predominance of heterofermentative *F. durionis* was observed at the early stage of fermentation. Durian pulp is rich in sucrose and fructose, hence a favourable environment for the fructophilic *F. durionis* (Charoenkiatkul et al., 2016; Voon et al., 2006). We are of the opinion that heterofermentative *F. durionis* plays a similar role to the closely-related *Leuconostoc* species. *F. durionis* do not produce gas in the presence of glucose but produce gas in abundance in the presence of sucrose and fructose (Endo et al., 2009). We observed a drastic decrease in sucrose but an increase in fructose and glucose concentration at the early stage of fermentation. This observation suggests the hydrolysis of sucrose by heterofermentative *F. durionis* and hence production of carbon dioxide leading to an anaerobic condition. In addition, the rapid increase in lactic and acetic acids content from day 0–4 was in agreement with the predominance of heterofermentative LAB in the early stage of tempoyak fermentation (Endo et al., 2009).

To determine the genetic relatedness among the indigenous LAB population of tempoyak, representative strains of the dominant LAB were subjected to genotyping. Composite analysis of RAPD and PFGE demonstrated a high degree of polymorphism within each of the two dominant LAB species isolated from tempoyak. A similar observation was reported by Lucena-Padrós and Ruiz-Barba (2016) on a high degree of diversity within dominant LAB involved in Spanish-style green table-olive fermentations. Genetic diversity observed among LAB isolates might be due to the different sources of the durian pulp. The durian pulp was pooled from durian fruits obtained from different trees in the same orchard. Variability was also observed in their antibiotic resistance profile as different antibiograms were observed among LAB isolates grouped in the same cluster. The results suggest diversity among the dominant LAB species present in naturally fermented tempoyak. Similar observations were made by Xu et al. (2011). Our results suggest that the organoleptic properties of tempoyak were due to diverse consortia of LAB microflora.

As tempoyak fermentation is due to indigenous microflora and not due to pure culture (starter culture), the type of microorganism responsible is not well characterised. Antibiotic-resistant organisms may be introduced in tempoyak during fermentation. A study conducted by Oguntoyinbo and Okueso (2013) showed a high prevalence of multidrug-resistant enterococci in two Nigerian traditional fermented dairy products. In this study, *Lb. plantarum* isolated from tempoyak were resistant to multiple antibiotics of various classes. Our findings were in agreement with the study reported by Nawaz et al. (2011) who also reported on multidrug-resistant *Lb. plantarum* isolated from fermented foods. Information on antibiotic resistance in *Fructobacillus* spp., however, remains scarce. Although many LAB strains are not pathogenic, they could serve as a reservoir of antibiotic resistance genes with the potential to be transferred to humans, animals and pathogenic microbes via the food chain (Mathur and Singh, 2005). Numerous studies have reported on a wide range of antibiotic resistance detected in lactobacilli as being intrinsic, hence, safety issue is not of concern (Ashraf and Shah, 2011). Antibiotic resistance becomes a safety concern when the resistance is transferrable, especially to pathogenic bacteria (Gueimonde et al., 2013). Detection of plasmid-borne antibiotic resistance genes, especially involved in tetracycline, erythromycin, chloramphenicol and gentamicin were frequently reported among LAB (Gueimonde et al., 2013; Ouoba et al., 2008). Antibiotic resistance to chloramphenicol, erythromycin and gentamicin are plasmid-mediated, but in this case, all *Lb. plantarum* isolates are susceptible to these antibiotics, thus, the risk of horizontal transfer of antibiotic resistant genes is at a minimum. As most of *Lb. plantarum* isolates and few isolates of *F. durionis* are resistant to tetracycline, there is a potential risk for the horizontal transfer of antibiotic resistance genes as *Tet* genes are plasmid-mediated.

Despite its high acidity, microbial safety of naturally fermented foods which requires no heat treatment prior to consumption is of concern. Sainz et al. (2001) reported the presence of *Escherichia coli* in an acid-fermented maize beverage in Mexico with pH values ranging from 3.7 to 4.7. Microbial safety of naturally fermented tempoyak, however, have not been reported by previous researchers. Our results showed that *Salmonella* spp., *L. monocytogenes* and *Staph. aureus* were not detected in the un-inoculated tempoyak, suggesting that the naturally fermented tempoyak does not support the growth of these bacteria. We further studied the survival of *S.* Enteritidis, *L. monocytogenes* and *Staph. aureus* in the naturally fermented tempoyak by artificially spiking the durian pulp with these pathogens. A similar study was conducted by Cho et al. (2011) who reported that the survival of *E. coli* and *L. monocyogenes* in artificially-spiked kimchi was greatly reduced but not totally inhibited. They reported that the gradual decrease in pH of fermented kimchi may lead to acid adaptation of *E. coli* and *L. monocytogenes* and hence, the prolonged survival of these pathogen. In this study, rapid reduction in pH and substantial production of lactic, acetic and propionic acids at the early stage of

fermentation contributed to the inhibition of *S.* Enteritidis, *L. monocytogenes* and *Staph. aureus*. Complete inhibition was observed on day 8–12 of fermentation suggesting that the rapid reduction in pH and organic acid production by the LAB microflora play a critical role in ensuring the safety of the naturally fermented tempoyak.

In conclusion, the present study reports that diverse LAB species were involved in the fermentation and hence the organoleptic properties of tempoyak. We for the first time report a strain of *Lb. fructivorans* with fructophilic properties, and the presence of *Leu. dextranicum*, *Lb. collinoides*, *Lb. paracasei* and *Lb. fructivorans* in tempoyak. This study reports on the succession LAB microflora which have not being reported by previous researchers. Previous studies have reported on the presence of certain LAB that were isolated from tempoyak that was already fermented or the samples obtained from retailers. In this study, the LAB succession was monitored and thus in our opinion using one batch of durian sample from a single orchard might be of no consequence on the final microflora that is responsible for the fermentation of tempoyak. We reveal a sequential succession of indigenous LAB microflora during natural fermentation of tempoyak. Heterofermentative *Leu. mesenteroides* and *F. durionis* were predominant in the initial stage of fermentation, and as fermentation proceeded, *F. durionis* remained predominant, but towards the end of fermentation, homofermentative *Lb. plantarum* became the predominant species. Diversity among the dominant LAB species in tempoyak was further verified by the results we obtained from genotyping. As different species of LAB are involved in the fermentation of tempoyak it would be very difficult to develop or produce starter cultures for tempoyak. In our opinion, tempoyak is fermented by indigenous microorganisms, namely species belonging to LAB, which also influences the sensory characteristics of the final product. We suggest that tempoyak production via natural fermentation might be more favourable as compared to fermentation using a starter culture. It is of great concern that LAB isolated from foods are resistant to multiple antibiotics as they pose a threat to food safety and human health. Hence, it is of paramount importance that antibiotic resistant determinants present in the LAB isolates are determined for foods produced by spontaneous fermentation. Our present study for the first time shows that naturally fermented tempoyak does not support the growth of pathogens. However, survival study showed that the pathogens survive up to 8–12 days if there was contamination at any stage of fermentation. The diverse indigenous LAB microflora provides a prospective consortium for product development in future. Even though we do not anticipate the selection of starter culture for tempoyak fermentation, further research on investigating the potential probiotic properties of LAB microflora is ought to be undertaken.

Conflict of interest

The authors declare that they have no conflict of interest.

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