

Phenotypic Identification of Lactic Acid Bacteria Isolated from *Tempoyak* (Fermented Durian) Made in the Philippines

Neti Yuliana (Corresponding author)

Department of Agro industrial Technology (THP), Faculty of Agriculture,
University of Lampung, Sumantri Brojonegoro#1, Bandar Lampung, Indonesia
Tel: 62-0721-781-498 E-mail: yuliana_thp@unila.ac.id

Erlinda I. Dizon

Institute of Food Science and Technology, College of Agriculture
University of the Philippines Los Baños, College, Laguna, Philippines - 4031
Tel: 63-049-536-2312 E-mail: ei_dizon@yahoo.com

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Abstract

Nine (9) Gram-positive bacteria, isolated from 8-day old fermenting durian (tempoyak) made in the Philippines were classified and subjected to phenotypic analyses. Based on morphological and biochemical characteristics, the lactic acid bacteria (LAB) isolates were identified as *Lactobacillus plantarum*, *Lactobacillus sp.*, *Weissella paramesenteroides* and *Pediococcus acidilactici*. This is the first report for the presence of *Weissella sp.* and *Pediococcus sp.* in tempoyak. Live cells of LABs in tempoyak makes it a functional food and probably with potential as probiotic product.

Keywords: Lactic acid bacteria, *Tempoyak*, Phenotypic analysis, *Lactobacillus*, *Weissella*, *Pediococcus*

1. Introduction

Tempoyak is the fermented pulp of durian fruit that has distinctive durian smell and a creamy yellow color and is widely consumed in both Malaysia and Indonesia as side dish and condiment (Battcock and Ali, 1998; Irwandi and Che-Man, 1996; Gandjar, 2000). As a condiment, tempoyak is used with certain fish and vegetables dishes. This condiment is made by mixing durian pulp with salt and fermented under partially anaerobic condition at ambient temperature in a closed container. Fermentation usually takes about 4-7 days and the texture of durian pulp changes from a solid to a semisolid consistency with acid odor and dominant taste. The acidity of tempoyak was reported as approx. 2.8 to 3.6% (Amin *et al.*, 2004, Leisner *et al.*, 2002).

The sour taste of tempoyak is attributed to the acid produced by lactic acid bacteria (LAB) during fermentation. Earlier studies showed that LAB were the predominant microorganism in tempoyak (Leisner *et al.*, 2001; Amiza *et al.*, 2006). The chemical composition of the durian fruit, with 15-20% total sugar (Ketsa and Daengkanit, 1998) is expected to support the growth of lactic acid bacteria. Species of *Lactobacillus* have been reported as the main LAB isolated from tempoyak made in Indonesia and Malaysia. It is expected that strains of LAB and other microorganisms vary depending on the place where the product is prepared. *Lb. plantarum*, *Lb. brevis*, *Lb. mali*, *Lb. fermentum* were found in tempoyak from Malaysia (Issa 2000, Leisner *et al.*, 2001), while Wirawati (2002) and Ekowati (1998) isolated *Lb. plantarum*, *Lb. casei*, *Lb. corynebacterium* and *Lb. fersantum*, *Lb. casei*, respectively, from tempoyak in Indonesia. Leisner *et al.*, (2002) reported the new species of *Lactobacillus*, *L. durianis sp.*, isolated from Malaysian tempoyak. Other LAB present in tempoyak from Malaysia was *Leuconostoc mesenteroides* (Leisner *et al.*, 2001).

In general, LABs are the most important microorganisms in fermented fruits and vegetables. There are several potential health and nutritional benefits from LAB which are sometimes known as “probiotics”. Bacteria that

lives in the human intestine and control the balance of intestinal microflora finally elicit physiological and beneficial effects on health of the host have been recently named as “probiotics” (Saito, 2004). In 2002, Tannock defined probiotics as ‘living microorganisms, which upon ingestion in certain number, exert health benefits beyond inherent basic nutrition’. Probiotics have been suggested to have the following properties and functions: adherence to host epithelial tissues; acid resistance and bile tolerance; elimination of pathogens or reduction in pathogen adherence; production of acids, hydrogen peroxide and bacteriocin antagonistic to pathogen growth; safety, non-pathogenic and non-carcinogenic; and improvement of intestinal microflora.

Unlike other neighboring countries, Filipinos are just beginning to appreciate the taste of durian fruit due to its strong, strange flavor. Durian can be eaten as fresh, used as flavoring agent for ice cream, and processed into candies and other delicacies. Lactic acid fermented durian is not known in the Philippines. However, with the consumers’ clamoring for natural and healthy foods nowadays, isolation of useful microorganisms from traditional fermented products becomes the target of several investigations. Thus, this study aimed to identify the predominant lactic acid bacteria present in tempoyak made in the Philippines based on the LAB phenotypic characteristics.

2. Materials and Methods

Fully ripened durian (*Durio zibethinus* Murr.) fruits were obtained from local grower of durian in Los Baños, Laguna, Philippines. Microbiological media were purchased from Merk Company and all other chemicals were of analytical grade.

2.1 Fermented Durian Preparation

Prior to this study, the author observed actual commercial processing of tempoyak in Indonesia. In the laboratory, different salt concentrations were tested in the preparation of tempoyak to identify the optimum formulation. The optimum salt concentration was selected in the succeeding experiment. Tempoyak was prepared in triplicates by mixing durian pulp with salt (3% by weight). The mixtures were placed in sealed plastic containers and allowed to naturally ferment for 8 days at ordinary room temperature (28 to 34°C).

2.2 Isolation and Purification of LAB

Samples of an 8-day old fermented durian (tempoyak) were analyzed microbiologically for the presence of LAB. The standard pour plating technique was used for the enumeration of acid producing bacteria. Briefly, a 25-gram sample was added with 225 ml of 0.1% sterile peptone water (as diluents), homogenized and series of dilutions were performed. An aliquot (1ml) of the appropriate dilutions were plated out (duplicates) using Glucose Yeast Peptone (GYP) agar medium plus calcium carbonate (1.0%). The plates were incubated upside down at 30 C for 48 hrs. Acid formers were identified by the presence of a zone of clearing the CaCO₃ around the colonies. Plates containing separated colonies were selected for isolation. The colonies of acid forming microorganisms were transferred by stabbing into tubes of GYP agar. Purification of the isolates was done by repeated pour plating technique using the same agar medium until pure cultures were obtained. Pure cultures were transferred and maintained in de Man Rogosa and Sharpe (MRS) agar stabs. Duplicate tubes of the isolates were prepared, one tube was stored in refrigerator as stock culture, and the other tube was used for identification studies.

2.3 Identification of LAB Isolates

Cultural, morphological, physiological and biochemical tests were done following the method developed by Kozaki, *et al* (1992) to identify the LAB isolates. Morphological characteristics, i.e. shape, form and cell arrangement, were elucidated by scanning electron microscopy (SEM). In addition, motility test, spore formation, and Gram reaction (Harrigan, 1998) were performed as taxonomic indices. Oxygen requirement, catalase test and cultural growth on GYP soft agar stab were conducted. The ability to ferment different type of carbohydrates was performed using API 50 CH test kit. On the other hand, gas production of the isolates was used as an index of type of fermentation grown in broth culture with inverted Durham tubes. Motility was observed on soft agar medium. Genotypic characteristic of selected isolate was elucidated by 16S rRNA sequencing analysis at Nodai Culture Collection Center, Tokyo University of Agriculture, Tokyo, Japan. Sequence alignment was conducted using the BLAST software from the Gen Bank.

3. Results

Out of 30 isolates, 9 acid forming bacteria were initially chosen based on their growth appearance on GYP soft agar and MRS agar media. All isolates were found non-motile and microaerophilic, where optimum growth was observed just below the surface of the media. Gram staining revealed that all isolates were Gram-positive. Spore formation was also observed and all isolates were found to be non-spore formers. Isolates were then grouped based on their form, cell arrangements, Gram reaction, catalase production, motility, spore formation, and gas

production from glucose (Table 1), as well as the growth characteristics of isolates at different temperatures, pH and NaCl concentrations (Table 2).

Based on the shape, form and cell arrangement observed under the scanning electron microscope, the acid-forming microorganisms were then eventually grouped according to cell shape, as cocci and rods. Seven (7) of nine (9) isolates were found to be the rod-shaped strains with long and rounded ends mostly appeared as chains of 4-5 cells, pairs or single cells and these could presumptively determined as derivatives of the genus *Lactobacillus*. The rest of the isolates (2) were cocci, single, tetrads cell arrangement therefore they tentatively referred to *Pediococcus* (Figure 1a). Physiological test in relation to fermentation type for the rod-shaped strains (7) showed 5 out of 7 strains do not produce gas from glucose (Table 1).

In API CH assay, all of LAB isolates were screened for their performance regarding growth characteristics in 49 carbon sources and the results of carbohydrates fermentation are shown in Table 3. All isolates fermented L arabinose, ribose, galactose, D-glucose, D-fructose, D-mannose, N acetyl glucosamine, amygdaline, lactose, trehalose, saccharose, trehalose and β gentiobiose. Only isolates of *Pediococcus* group could utilize D-tagatose. This physiological test was able to identify species of homofermentative *Lactobacillus* (2 strains) and *Weissella* (2 strains) while the three (3) remaining strains of heterofermentative *Lactobacillus* were not clearly identified down to species level on phenotypic tests only.

4. Discussion

A total of 9 acid producing bacterial strains isolated from tempoyak were considered as presumptive LAB because they were Gram positive, catalase-negative, microaerophilic, non-motile and non-spore forming. Of the 9 strains, 2 were tetrad-forming cocci classified under the genus *Pediococcus*. The *Pediococcus* isolates selected for species identification grow at pH 6.5 and 50°C. Following the scheme presented in LAB identification manual of Kozaki *et al.* (1992), the isolates were identified as *Pediococcus acidilactici*, the only species of *Pediococcus* that can grow at 50°C and pH 6.5. This strain fermented arabinose, and tagatose but did not utilize sorbitol, melibiose, melezitose, or xylose (Table 3). From industrially –useful LAB point of view, the ability to grow at high temperature is a desirable trait as it could translate to increased rate of growth and lactic acid production. Presence of this thermophilic *Pediococcus* is probably caused by high temperature especially during summer months in the Philippines when the tempoyak was made for this study. This existence of thermophilic, homofermentative *P. acidilactici*, implies that tempoyak product has enough organic acid (lactic acid) for flavor and shelf stability against spoilage microorganisms.

The remaining isolates (7) were rods, and were subjected to the type of fermentation to determine whether the isolates are homofermentative or heterofermentative. LABs that produce substantial amount of gas (CO₂) are termed as heterofermentative (heterolactics) while those that produce only trace amount or no gas formation on glucose as carbon source are known as homofermentative (homolactics). Gas formation is indicated on the inverted Durham tubes after the desired incubation time during the test for sugar fermentation. The five isolates producing gas from glucose were identified as either heterofermentative *Lactobacillus* or *Weissella* (Table 1). The remaining two (2) isolates that were not able to produce gas from glucose were identified as homofermentative *Lactobacillus*. The homofermentative *Lactobacillus* isolates were identified based on their ability for carbohydrate fermentation following the LAB identification scheme of Kozaki *et al.*, (1992). The 2 selected isolates were found positive for gluconate and arabinose fermentation, but negative for xylose. Hence, these isolates were tentatively identified as *Lactobacillus plantarum* (Figure 1d). The results of 16S rRNA sequencing analysis of the heterofermentative *Lactobacillus* isolate (Figure 2) further confirmed this species with 99% similarity when compared to nucleotide data in Gene Bank using BLASTN sequence alignment from NCBI for the nucleotide comparison. Association of *Lb. plantarum* with tempoyak, acid-fermented durian, is not surprising. It is well known that this isolate has role in some acid-fermented vegetable foods such as sauerkraut, Korean kimchi and fermented bamboo tender shoots (Steinkraus *et al.*, 1983; Battcock and Ali, 1998; Tamang *et al.*, 2008). Products containing live and useful microorganisms like LABs are considered “probiotics” since consumption of these foods benefited the host by improving the properties of indigenous intestinal microflora (Havenaar and Veld, 1992).

Members of the genus *Weissella* may be distinguished readily from homofermentative *Lactobacilli*, *Pediococci*, *Enterococci*, *Lactococci* and *Streptococci* by the formation of gas from carbohydrates. A cell wall murein based upon lysine with an interpeptide bridge containing alanine, or alanine plus serine or glycine distinguishes *Weissella* from heterofermentative *Lactobacilli* (Collins *et al.*, 1993). Additional test with regards to their ability to grow at 45°C was also done to differentiate *Lactobacillus* and *Weissella*. Following the taxonomic studies by Collins *et al.*, (1993), growth does not occur at 45°C for *Weissella* (with the exception of *W. confusa*). Based on

this characteristic and differences of cell form, the three isolates are probably heterofermentative *Lactobacillus* and the two remaining isolates belong to *Weissella*.

The capability of strains for carbohydrate fermentation was used to identify species of heterofermentative *Lactobacillus*. These isolates were found positive for fructose, ribose and mannose fermentation. Based on the scheme described by Kozaki *et al.*, (1992), these isolates were probably *Lactobacillus viridans*, *Lb. confusus*, *Lb. halotolerans*, *Lb. minor*, *Lb. bifermetans* or *Lb. divergens*. To further confirm the identity of the isolates, determination of peptidoglycan cell wall type and other test is needed.

The *Weissella* group was identified down to the species level through the production of acid anaerobically from glucose, L arabinose, cellobiose, galactose, maltose, melibiose, raffinose, ribose, sucrose, trehalose and xylose (Collins *et al.*, 1993). The isolate tested were found positive for acid production from glucose, L- arabinose, cellobiose, galactose, maltose, melibiose, raffinose, ribose, sucrose, trehalose but negative for xylose, hence, the isolates were identified as *Weissella paramesenteroides* (Figure 1c).

Overall, the prevalent LAB belonged predominantly to genera *Lactobacillus*, consisted of *Lactobacillus sp* and *Lactobacillus plantarum*, while others were identified as *Weissella paramesenteroides* and *Pediococcus acidilactici*. A similar study on the biodiversity of lactic acid bacteria from Malaysian and Indonesian tempoyak also found that *Lactobacillus* strains were predominant members of LAB flora (Leisner *et al.*, 2001; Wirawati, 2002). This is the first report on the presence of *Pediococcus sp* and *Weissella sp* isolated from tempoyak that is probably due to the differences in microflora as affected in general by variety of raw materials, ingredients, and other environmental conditions

5. Conclusion

On the basis of phenotypic and genotypic (for *Lb. plantarum*) properties, the lactic acid bacterial population in tempoyak prepared in the Philippines consisted of: *Lactobacillus sp*, *Lactobacillus plantarum*, *Weissella paramesenteroides*, and *Pediococcus acidilactici*. The majority of the acid forming bacteria belongs to the genera *Lactobacillus*, 40% of which were *Lactobacillus plantarum* while the remaining strains were still unidentified. For the unidentified strains, there is a need further examination with regards to phylogenetic determination because of the inability of biochemical tests to differentiate strains among *Lactobacillus*. Presence of live LABs suggests that this resulting product has a potential to be included as a probiotic food, since its consumption could possibly benefit the host by improving the properties of indigenous intestinal microflora.

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Table 1. Morphological and cultural characteristics of isolates

Genus	Characteristics				
	Morphology	Gram reaction	Catalase	Motility	Gas production/ Fermentation type
(I) <i>Lactobacillus</i>	Short rods, rounded edge, 0.3x0.5um, no spore formation	+	-	-	+ Heterofermentative
(II) <i>Weissella</i>	Short rods, rounded edge, around 0.2x0.5um, no spore formation	+	-	-	+ Heterofermentative
(III) <i>Lactobacillus</i>	Short rods, straight edge, 0.3x0.6um, no spore formation	+	-	-	- Homofermentative
(IV) <i>Pediococcus</i>	Cocci, tetrads and pairs, around 0.5um diameter, no spore formation	+	-	-	- Homofermentative

Table 2. Growth characteristics of isolates at different temperature, pH and NaCl concentrations.

Characteristics	Isolate Groups			
	I	II	III	IV
Growth at 10°C	+	-	+	+
Growth at 37°C	+	+	+	+
Growth at 45°C	+	-	±	+
Growth at 50°C	-	-	-	+
Growth at pH 4.8	+	+	+	+
Growth at pH 6.5	+	+	+	+
Growth at pH 9.6	+	+	+	+
Growth at 3% NaCl	+	+	+	+
Growth at 6.5% NaCl	+	+	+	+

+ good growth; ± very weak; - no growth

Table 3. Carbohydrate fermentation characteristics of acid-forming bacteria.

Sugar:	Hetero. <i>Lactobacillus</i> (Group I)	<i>Weissella</i> (Group II)	Homo. <i>Lactobacillus</i> (Group III)	<i>Pediococcus</i> (Group IV)
Glycerol	-	-	-	-
Erythritol	-	-	-	-
D-arabinose	-	-	-	-
L-arabinose	+	+	+	+
Ribose	+	+	+	+
D-xylose	-	-	-	-
L-xylose	-	-	-	-
Adonitol	-	-	-	-
β -methyl-xylose	-	-	-	-
Galactose	+	+	+	+
D-glucose	+	+	+	+
D-fructose	+	+	+	+
D-mannose	+	+	+	+
L-sorbose	-	-	-	-
Rhamnose	-	-	-	-
Dulcitol	-	-	-	-
Inositol	-	-	-	-
Mannitol	+	+	+	-
Sorbitol	+	+	+	-
α Methyl D manoside	+	+	+	-
α Methyl D glucoside	+	+	+	-
N acetyl glucosamine	+	+	+	+
Amygdaline	+	+	+	+
Arbutin	+	+	+	+
Esculin	+	+	+	+
Salicin	+	+	+	+
Cellobiose	+	+	+	+
Maltose	+	+	+	+
Lactose	+	+	+	+
Melibiose	+	+	+	-
Saccharose	+	+	+	+
Trehalose	+	+	+	+
Inuline	+	+	+	-
Melezitose	+	+	+	-
D raffinose	+	+	+	-
Amidon	-	-	-	-
Glycogene	-	-	-	-
Xylitol	-	-	-	-
β gentibiose	+	+	+	+
D turanose	+	+	+	-
D lyxose	-	-	-	-
D tagatose	-	-	-	+
D fucose	-	-	-	-
L fucose	-	-	-	-
D arabitol	-	+	-	-
L arabitol	-	-	+	-
Gluconate	-	-	+	-
2 Cetogluconate	-	-	-	-
5 Cetogluconate	-	-	-	-

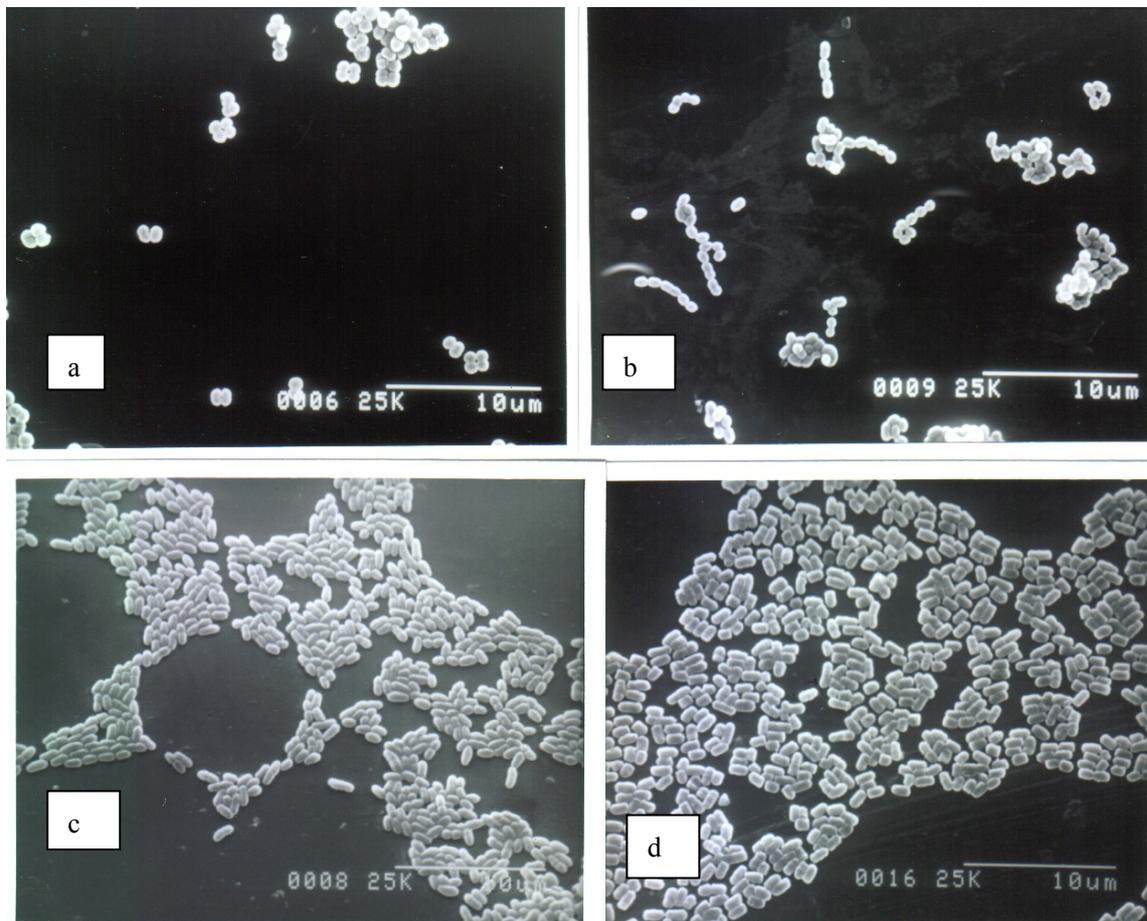


Figure 1. Identified Lactic Acid Bacteria Isolated from Tempoyak: (a) *Pediococcus acidilactici*, (b) *Lactobacillus* sp., (c) *Weissella paramesenteroides*, and (d) *Lactobacillus plantarum*

CAAGNCGAACGAACTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCCGAAC
 GGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGATAACACCTGGAAACAGATGCTAATACC
 GCATAACAACCTGGACCGCATGGTCCGAGTTTGAAGATGGCTTCGGCTATCACTTTTGGATGGTCC
 CGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAG
 GGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACCTCTACGGGAGGCAGCAGTAGGGAATCT
 TCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAgAAGGGTTTCGGCTCGTAAAA
 CTCTGTTGTTAAGAAAACATATCTGAGAGTACTGTTTCAGGTATTGA

Figure 2. 16S rRNA, gene sequence of *Lactobacillus plantarum*