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Identification of microflora and lactic acid bacteria in pado, a fermented fish product prepared with dried *Pangium edule* seed and grated coconut

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Abstract

Pado is a unique fermented fish product of West Sumatera Indonesia, prepared using dried *Pangium edule* Reinw seed in combination with grated coconut, with or without salt. Changing of microflora during fermentation has not been extensively reported. This study observed the changes of microflora, especially lactic acid bacteria, during fermentation for 7 days at room temperature (25-30 °C) of pado without the addition of salt. No microorganisms were found on dried *P. edule* seed. Enterobacteriaceae and spore-forming bacteria decreased and were not found at the end of the fermentation, while the yeast and mold counts were relatively constant at a low level. Instead, lactic acid bacteria increased along with the fermentation process, in line with the decreasing of pH, water contents, and water activity of the fish product. Apparently, few species of lactic acid bacteria are involved during fermentation, most likely due to the use of *P. edule* seed (as no salt was used). Partial sequencing based on the 16S rRNA gene showed that *Lactobacillus plantarum* and *Lactobacillus pentosus* were consistently identified at the various fermentation steps. This study reports that although lactic acid bacteria predominate, only a few species are involved in pado fermentation.

Keywords: fermented fish; grated coconut; *L. plantarum*; *L. pentosus*; *pado*; *Pangium edule*.

Practical Application: Use of *Pangium edule* seed and grated coconut for fish fermentation.

1 Introduction

Pado is a popular and unique fermented fish product from West Sumatra, Indonesia. The use of dried *Pangium edule* and grated coconut covering the fish during the fermentation process has been assumed to be an important factor for enhancing the shelf-life of the product. Usually, small pelagic fish, such as *Rastrelliger* sp, are used and mixed with dried *Pangium edule* seed and grated coconut, with or without salt, and stacked in a closed container for 5 to 7 days at room temperature. The fermented pado fish visually remains as whole fish, with blackish-brown color, slightly dry, brittle, and has a unique taste and aroma (Figure 1). After 14 weeks of storage at room temperature, this product still has a semi dried normal texture and odor without surface mycelia (Hasbullah et al., 2016a). Previous studies have also shown that pado fish were safe and did not contain pathogenic bacteria, such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, and *Vibrio parahaemolyticus* (Hasbullah et al., 2016b).

Pangium edule is a native tropical plant within the Flacourtiaceae family. *P. edule* seed has been used as spices in some South East Asia countries, including Indonesia. The dried *P. edule* seed has also been used as a constituent of fermentation media for the pado fish process in West Sumatera. Meanwhile, the fresh *P. edule* seed has been used for *picungan* fish fermentation in West Java. *P. edule* seed has been reported to show antibacterial and antioxidant properties due to its phenolic and alkaloid compound (Chye & Sim, 2009; Heruwati et al., 2009). Previous study has shown that water extract of *P. edule* seed can inhibit the growth of *Aspergillus flavus* (Listyorini et al., 2021). According to Ismail et al. (2021) lactic acid bacteria that isolated from fish fermented *Tilapia nicolicus* incorporated with spices such chili, turmeric and black pepper have antimicrobial activity against *B. cereus*, *S. aureus*, *E. coli* and *Salmonella enterica* serovar Thyphimurium.

Lactic acid bacteria are the most common and predominant bacteria found in fermented products. A previous study showed that 62 isolates from 74 existing isolates in the fermented fish product, bekasam, belong to lactic acid bacteria (Desniar et al., 2013). Lactic acid bacteria have also been predominantly found in fermented fish gajami shikae from Korea (Kim et al., 2014). Lactic acid bacteria have been identified in fermented fish, including *Lactobacillus sakei*, *Lactococcus (Lc.) garvieae*, *Lc. lactis*, *Lc. raffinolactis*, *Enterococcus hermanniensis*, *Streptococcus parauberis* (Dai et al., 2013), *Enterococcus gallinarum*, *Lactobacillus brevis*, *Lactobacillus plantarum*, *Pediococcus acidilactici*, and *Treptococcus spp* (Kuley et al., 2020).

Previous studies have reported that the presence of microorganisms in fermented food generally varies depending on the type of products, production processes, environmental conditions, and duration of fermentation (Rezac et al., 2018).

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Figure 1. Pado as fermented fish product prepared with dried *P. edule* seed and grated coconut.

However, the microflora in pado fish during fermentation has never been well documented. It was assumed that lactic acid bacteria also play an important role in the fermentation and preservation of the product. Hence, the present study aimed to determine the microflora as well as identify the predominant lactic acid bacteria during fermentation and from the pado fish available in the market. The changes in water contents, pH, and water activity of the product were also observed.

2 Materials and methods

2.1 Samples

Pado samples were collected from Tandikek market in Padang Pariaman (Market A) and Malalak Market in Agam (Market B), West Sumatra Regency, Indonesia. Five samples were taken from each producer. The producer in market A made the pado fish without the addition of salt, whereas the producer of market B added 3% salt (w/w to fish weight) at the beginning of the fermentation process.

2.2 Pado fermentation

Production of pado fish was also conducted in the laboratory according to the procedure used by the producer from market A. Two hundred grams of *sarai fish (Rastrellinger* sp*)*, 200 g of dried roughly-chopped *P. edule* seed, and 100 g of grated coconut were prepared. The dried *P. edule* seed and grated coconut (as fermentation medium) were mixed and placed in a food-grade plastic bag, then the fish were placed above the medium and covered again with the medium. This step was done layer-by-layer until all fish were covered. Four-plastic bags were prepared and placed in a closed container (15 cm long x 10 cm wide x 5 cm high) allowing fermentation for 7 days at room temperature (25-30 °C). Microflora enumeration, as well as water contents, pH, and water activity measurements, were conducted during the fermentation process, i.e., at the beginning (day 0) and days 3, 5, and 7 of fermentation. The experiments were replicated three times.

2.3 Microflora enumeration

Since the fermented pado fish consisted of fish and medium (*P. edule* seed and grated coconut) parts, the analysis was carried out for each part. A total of 25 g of sample, either fish or medium, was diluted in 225 mL of buffered peptone water (Oxoid, UK) and homogenized in a Stomacher (Seward, US) to obtain a 10^{-1} dilution. A serial dilution was then prepared for each sample.

Enumeration of the total plate count and spore-forming bacteria was conducted using plate count agar media (PCA, Merck, Germany). For spore-forming bacteria analysis, the initial suspension was heated for 5 min at 80 °C. The enumeration was carried out using the pour plate technique with incubation at 30 °C (International Organization for Standardization, 2013). Furthermore, de Man Rogosa Sharpe Agar (MRSA, Merck, Germany) was used for enumeration of lactic acid bacteria following the standard method for the enumeration of mesophilic lactic acid bacteria, colony-count technique at 30 °C (International Organization for Standardization, 1998), and Violet Red Bile Glucose Agar (VRBGA, Merck, Germany) for total *Enterobacteriaceae* (International Organization for Standardization, 2017). Enumeration of yeast and mold was carried out according to the enumeration of yeast and mold method for products with water activity less than or equal to 0.95 by the spreading on 18% dichloran glycerol agar (DG18, Merck Germany) (International Organization for Standardization, 2008).

2.4 Measurement of water content, pH and water activity

The water content of the product was determined following the standard method (Association of Official Analytical Chemists, 2016). The pH was measured with a digital tester (Eutech, Singapore) after 10 g of sample was homogenized in 90 mL aquadest, whereas water activity was measured by a_w meter (Shibaura, Japan) (Ly et al., 2020).

2.5 Characterization of bacterial isolates

The colonies of lactic acid bacteria and spore-forming bacteria were sub-cultured on MRS agar and Tryptone Soy Agar (TSA, Merck, Germany), respectively. Selected lactic acid bacteria isolates were prepared for microscopy analysis by Gram staining, catalase test using 3% H_2O_2, and gas production test (CO_2) using MRS broth media containing Durham tubes at 30 °C for 48 h. Proteolytic activities of the isolates were tested using 1.5% skim milk agar (SMA), with the composition of 1.5% skimmed milk and bacteriological agar (Merck Germany), incubated at 37°C for 48 h.

Selected isolates of spore-forming bacteria were also prepared for microscopy analysis by Gram staining and spore staining, as well as for catalase test using 3% H_2O_2. Lipolytic activities of the isolates were tested using phenol olive oil with phenol red (Singh et al., 2006).

2.6 Molecular identification based on 16S rRNA gene

Ten selected isolates of lactic acid bacteria from the experiment samples (i.e., four isolates from days 3, two isolates from days 5, and four isolates from days 7) and four selected isolates from the market samples (i.e., two isolates from market A and two isolates from market B) were prepared for molecular identification based on the 16S rRNA gene. Each isolate that was grown in MRS broth at 30 °C for 24 h was placed in 3 mL tubes, then collected by centrifugation (Hermle Germany) at 280 *g* (5000 rpm) for 5 min.

The remaining biomass was used for DNA extraction using a Quick-DNA™ Miniprep Kit (Zymo Research Corp. the US). The primers used were the universal primers 27F (5'-AGAGTTTGATCCTGG CTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Dai et al., 2013; Kim et al., 2014; Nurhikmayani et al., 2019). The PCR conditions were set at 95 °C for pre-denaturation for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, 55 s annealing at 55 °C, extension at 72 °C for 1 min, and followed by a final extension at 72 °C for 10 min (Tajabadi et al., 2013). The product was visualized by electrophoresis using 1% (w/v) agarose, followed by partial sequencing. The DNA sequencing is conducted using a DNA sequencer (ABI SoliD, Applied biosystem; by BigDye terminator cycle sequencing kit v3.1). The sequenced data were edited with the ClustalX program to get complementary sequences (contig) and formatted to FASTA format. DNA sequence data in FASTA was analyzed using the BLAST program to identify homology using the NCBI database. Analysis of phylogenetic tree was carried out using MEGA 6 with the Neighbor-Joining (NJ) method.

2.7 Data analysis

Microbial population, water contents, pH, and water activity of pado fish were analyzed using the IBM SPSS software, with a general linear model, while the significant difference was analyzed with the Duncan Multiple Range Test (DMRT).

3 Results and discussion

3.1 Microbial population

The total plate counts and lactic acid bacteria increased along with the fermentation process (Table 1), while the yeast and mold were found in low numbers. In freshly grated coconut, however, the yeast and mold were found at approximately 4 $\log_{10}$ cfu/g. This result was higher than that reported by Ismail (2001) in desiccated coconut (2.9 $\log_{10}$ cfu/g). The presence of a high number of spore-forming bacteria (i.e., 5 $\log_{10}$ cfu/g) in pado fish from market B was assumed to be triggered by the use of salt during fermentation. The use of salt during fermentation was reported to increase the *Bacillus* sp. counts (Anihouvi et al., 2007).

In general, lactic acid bacteria dominated the microbial population of pado samples from the experiment and the market, although the spore-forming bacteria were also found in a relatively high number in fish products from market B. Total lactic acid bacteria increased over time, from 3.48 $\log_{10}$ cfu/g to 8.45 $\log_{10}$ cfu/g after 7 days of fermentation. Surprisingly, lactic acid bacteria were not detected in grated coconut nor dried *P. edule* seed at the beginning of the fermentation process. However, they were found in fresh fish, as well as in the fermented products after days 3, 5, and 7 of fermentation.

The fact that no microbial growth was found in dried *P. edule* seed was likely caused by the bioactive compounds in the seed, as well as the low moisture content and low water activity value. Polyphenol and phytochemical compounds in *P. edule* have been reported to inhibit the growth of microorganisms (Chye & Sim, 2009; Heruwati et al., 2009).

Enterobacteriaceae were not detectable after 3, 5 and 7 days of fermentation, although at the beginning of the fermentation they were detected in fresh fish at a low level and in grated coconut for approximately 4 $\log_{10}$ cfu/g. Enterobacteriaceae were likely inactivated and/or inhibited during the fermentation process. *P. edule* likely also contributed to the inhibition of the Enterobacteriaceae. Chye & Sim (2009) reported that phenolic

Table 1. Microbial population of fish and fermentation medium of pado samples prepared in laboratory (prepared without salt), from market A (prepared without salt) and market B (prepared with salt).

Sample	Origin	TPC ($\log_{10}$ cfu/g)	LAB ($\log_{10}$ cfu/g)	Yeast and Mold ($\log_{10}$ cfu/g)	*Enteobacteriaceae* ($\log_{10}$ cfu/g)	Spore forming bacteria ($\log_{10}$ cfu/g)
Fish E	Exp-day 0	4.25 ± 0.92^{b}	3.48 ± 0.40^{b}	1.18 ± 0.00^{b}	0.50 ± 0.71^{a}	0.94 ± 1.33^{a}
	Exp-day 3	5.71 ± 0.37^{c}	6.27 ± 0.38^{d}	1.24 ± 0.34^{b}	< l.00^{a}	0.59 ± 0.83^{a}
	Exp-day 5	6.45 ± 0.57^{d}	7.56 ± 0.09^{e}	< l.00^{a}	< l.00^{a}	< l.00^{a}
	Exp-day 7	7.64 ± 0.15^{e}	8.45 ± 0.48^{f}	1.39 ± 0.13^{b}	< l.00^{a}	< l.00^{a}
Grated coconut E	Exp-day 0	5.54 ± 0.27^{b}	< l.00^{a}	3.94 ± 0.15^{c}	4.26 ± 0.08^{b}	1.06 ± 1.49^{a}
P. edule seed E	Exp-day 0	< 1.00^{a}	< 1.00^{a}	< 1.00^{a}	< 1.00^{a}	< 1.00^{a}
medium*	Exp-day 3	5.63 ± 0.51^{c}	5.15 ± 0.16^{c}	0.50 ± 0.71^{ab}	< l.00^{a}	0.74 ± 1.05^{a}
	Exp-day 5	5.42 ± 0.14^{c}	6.40 ± 0.01^{d}	< l.00^{a}	< l.00^{a}	< l.00^{a}
	Exp-day 7	6.49 ± 0.46^{d}	6.78 ± 0.52^{d}	< l.00^{a}	< l.00^{a}	< l.00^{a}
Fish A	Mark A-wk 2	5.38 ± 0.16^{c}	6.60 ± 0.52^{d}	1.55 ± 0.21^{b}	0.72 ± 1.01^{a}	0.87 ± 1.22^{a}
	Mark A-wk 4	5.65 ± 0.16^{c}	6.56 ± 0.52^{d}	1.45 ± 0.42^{b}	0.43 ± 0.75^{a}	< l.00^{a}
Medium*A	Mark A-wk 2	5.59 ± 0.33^{c}	6.59 ± 0.48^{d}	1.39 ± 0.13^{b}	< l.00^{a}	0.65 ± 0.92^{a}
	Mark A-wk 4	5.45 ± 0.55^{c}	6.73 ± 0.23^{d}	1.54 ± 0.49^{b}	0.96 ± 0.95^{a}	< l.00^{a}
Fish B	Mark B-wk 2	5.25 ± 0.21^{c}	6.35 ± 0.42^{d}	1.38 ± 0.54^{b}	0.50 ± 0.71^{a}	5.41 ± 0.15^{b}
	Mark B-wk 4	5.28 ± 0.11^{c}	6.56 ± 0.29^{d}	1.37 ± 0.37^{b}	0.83 ± 0.76^{a}	5.34 ± 0.13^{b}
Medium*B	Mark B-wk 2	5.19 ± 0.27^{c}	6.49 ± 0.33^{d}	0.50 ± 0.71^{ab}	0.50 ± 0.71^{a}	5.26 ± 0.15^{b}
	Mark B-wk 4	5.65 ± 0.15^{c}	6.35 ± 0.17^{d}	1.19 ± 1.05^{b}	1.15 ± 1.00^{a}	5.40 ± 0.41^{b}

*Fermentation medium consisting of grated coconut and dried *P. edule* seed. Exp = experiment; Mark = market; wk = week. Numbers followed by the same letter of each column is not significantly different ($p > 0.05$).

extract of *P. edule* seed inhibited the growth of and killed *S.* Typhimurium that belongs to the Enterobacteriaceae. However, low levels of Enterobacteriaceae were found in samples from market A and B. These facts possibly caused by contamination during selling. The growth of microorganisms in fermented products depends on the handling, process, conditions, and duration of fermentation (Rezac et al., 2018; Zang et al., 2020).

The trends in changes of the total plate count, lactic acid bacteria, and Enterobacteriaceae during the fermentation process in this study were similar to that found in *Enam Ne-Setaakye* fermented fish (Asiedu & Sanni, 2002). The total plate counts and lactic acid bacteria increased during the fermentation process until the 5th day of fermentation. Likewise, the growth of *Enterobacteriaceae* declined and was undetectable after 4 days of fermentation.

3.2 Water content, pH and water activity

The fermentation process resulted in a decrease in the water content of fresh fish from 73.02 ± 0.29% to 62.66 ± 0.38% after 7 days (Table 2). The decrease in water content of fresh fish after the fermentation process was most likely caused by evaporation as well as by absorption by grated coconut and *P. edule* seed as the fermentation medium. The water content of pado fish in this study was slightly higher than that studied by Kasim & David (2013) since they used dried grated coconut as a raw material next to the dried *P. edule* seed.

Different production processes of pado fish at markets A and B led to significantly different results ($P < 0.05$) with regard to their water content, pH, and water activity. The addition of salt during the production process by the producer in market B resulted in lower water content and water activity of the end products. The water content of pado fish in this study, however, was higher than salted-fermented fish products reported by Gassem (2019), which had an average water content of 47.96%, and those of Kumar et al. (2017) with a water content of 54.9%.

The pH of pado fish products decreased along with the fermentation process. This was assumed to be caused by increases in the numbers of lactic acid bacteria, which resulted in increased production of organic acids, as also reported by Dai et al. (2013). Decreasing the pH of the product prevent the growth of spoilage or pathogenic microorganisms and lead to increasing the shelf life of the product. The pH value also be used as an indicator of the quality of fermented products (Nofiani et al., 2019). The pH of pado fish in this study, however, was lower than hout-kasef fish, a traditional salted-fermented fish product of the Jazan Region, Saudi Arabia, that showed an average pH value of 6.39 (Gassem, 2019). *P. edule* seed, as a raw material used for pado fermentation, was presumed to also contribute to the low pH of the product since it had an average pH value of 5.44.

Furthermore, the growth of microorganisms was influenced by the a_w of the product. Since the a_w of pado fish products in experiments and both market products were higher than 0.85, it was expected that the growth of microorganisms in the product to be dominated by bacteria. The a_w value of the product from market B that was much lower than that from market A and laboratory experiment, was likely due to the addition of salt that led to the lowering of the a_w value (Zang et al., 2020).

3.3 Characteristics of bacterial isolates

Despite all samples exhibiting high numbers of lactic acid bacteria, the appearance of colonies on MRS agar was relatively similar. Thirty-six isolates of suspected lactic acid bacteria were observed. All suspected lactic acid bacteria isolates were Gram-positive with rod shape, catalase-negative, and did not demonstrate gas production (Table 3).

Table 2. Water content, pH and water activity of pado fish product.

Sample	Origin	Water content (%)	pH	Aw
Fish E	Exp-day 0	73.02 ± 0.29^{i}	6.30 ± 0.10^{i}	0.95 ± 0.00^{gh}
	Exp-day 3	65.03 ± 0.71^{ij}	5.77 ± 0.00^{h}	0.93 ± 0.00^{defgh}
	Exp-day 5	65.38 ± 0.35^{j}	5.68 ± 0.10^{gh}	0.92 ± 0.00^{de}
	Exp-day 7	62.66 ± 0.38^{gh}	5.46 ± 0.11^{def}	0.93 ± 0.00^{def}
Grated coconut E	Exp-day 0	69.19 ± 0.61^{k}	6.48 ± 0.00^{j}	0.95 ± 0.00^{h}
P. edule seed E	Exp-day 0	5.95 ± 0.28^{a}	5.46 ± 0.57^{def}	0.66 ± 0.28^{a}
Medium*	Exp-day 3	36.37 ± 0.29^{b}	5.35 ± 0.01^{cde}	0.92 ± 0.05^{bcd}
	Exp-day 5	38.79 ± 0.38^{c}	5.30 ± 0.03^{cd}	0.91 ± 0.00^{cd}
	Exp-day 7	38.72 ± 0.42^{c}	5.19 ± 0.01^{c}	0.93 ± 0.00^{def}
Fish A	Mark-wk2	63.86 ± 1.02^{hij}	4.60 ± 0.01^{b}	0.94 ± 0.00^{fgh}
	Mark-wk4	63.31 ± 1.17^{hi}	4.35 ± 0.03^{a}	0.93 ± 0.01^{efgh}
Medium*A	Mark-wk2	58.89 ± 0.11^{e}	4.69 ± 0.02^{b}	0.94 ± 0.00^{fgh}
	Mark-wk4	61.14 ± 1.58^{fg}	4.53 ± 0.05^{b}	0.94 ± 0.01^{def}
Fish B	Mark-wk2	60.11 ± 0.57^{ef}	5.74 ± 0.57^{h}	0.89 ± 0.01^{b}
	Mark-wk4	61.00 ± 0.10^{fg}	5.60 ± 0.13^{fgh}	0.88 ± 0.02^{b}
Medium*B	Mark-wk2	54.68 ± 1.31^{d}	5.63 ± 0.08^{fgh}	0.89 ± 0.01^{bc}
	Mark-wk4	54.39 ± 0.82^{d}	5.52 ± 0.18^{efg}	0.89 ± 0.01^{b}

*Fermentation medium consisting of dried *P. edule* seed and grated coconut. Exp = experiment; Mark = market; wk = week. Numbers followed by the same letter each column is not significantly different ($p > 0.05$).

The differences among isolates were apparently found only on the colony size. Moreover, all suspected lactic acid bacteria isolate also showed proteolytic activity, confirming that they have extracellular protease enzymes, as characterized by the formation of clear zones on skim milk agar media.

Furthermore, fifteen isolates of spore-forming bacteria were found as Gram-positive rod bacteria, catalase-positive, formed endospores, and showed moderate lipolytic activity (Table 4). The differences among isolates were also apparently found only on the colony size found on Nutrient Agar media. Since the catalase test resulted in positive results, the spore-forming bacteria isolates found in pado fish were most likely belong to *Bacillus* sp.

The presence of *Bacillus* species with moderate lipolytic activity in the fermented fish product has also been reported in another study. For example, *Bacillus subtilis* and *Bacillus licheniformis* were isolated during the fermentation and persisted up to the end of fermentation of cassava fish, as reported by Anihouvi et al. (2007). Their presence was suggested to play important roles during the fermentation.

3.4 Lactic acid bacteria

Table 5 shows the alignments of partial gene sequencing results of selected lactic acid bacteria isolates to the data available at NCBI (BLASTN). The data were summarized from 100 selected sequences by the program, with E value = 0 and Query coverage 100%. Almost all isolates showed the closest relationship with *Lactiplantibacillus plantarum*, previously known as *Lactobacillus plantarum*, with a high number of hits, and with *Lactiplantibacillus pentosus*, previously known as *Lactobacillus pentosus*, with a low number of hits. The taxonomy of the genus *Lactobacillus* has currently been reclassified as *Lactiplantibacillus* (Zheng et al., 2020). The results of phylogenetic tree construction analysis performed using a neighbor-joining tree model with a bootstrap of 1000 × showed that the bacterial isolates were closely related to the *L plantarum* and *L. pentosus* (Figure 2).

The *lactobacillus* genus is a group of lactic acid bacteria often found in fermented fish products. *L. plantarum* has been found in shidal fermented fish product from India (Ahmed et al., 2015), pekasam fermented fish product from Malaysia (Muryani et al., 2017), lamea fermented fish from Bengkulu Indonesia (Okfrianti et al., 2018), and chao fermented fish from North Sulawesi (Nurhikmayani et al., 2019). *Lactobacillus pentosus* has also been found in fermented fish products, such as in pla-ra (Rodpai et al., 2021), and pla-chom (Tanasupawat et al., 1998), a fermented fresh fish product from Thailand.

Furthermore, the same strain of *L. plantarum* (strain 3334 and 8268) and *L pentosus* (strain HBUAS5624, SKB1211, SKB1212, NGI17, NGI19, NGI06) consistently found during fermentation at day 3, 5 and 7 (Table 5). The same strains have also been found in the product from market A. These results indicate that *L. plantarum* and *L. pentosus* play important roles during the fermentation of pado. These bacteria were assumed to originate from the fresh fish since no lactic acid bacteria were found in *P. edule* seed nor grated coconut.

Lactic acid bacteria have been found as one of the normal microflora in fresh fish. Lactic acid bacteria group such as *Lactococcus* sp, *Carnobacterium* sp, *L. plantarum* and *L. pentosus* have been isolated in puffer fish (Yang et al., 2007). Nair & Surendran (2005) also found lactic acid bacteria from the fresh fish, i.e., *Streptococcus* sp, *Leuconostoc* sp, *Pediococcus* sp and *Lactobacillus* sp, with *L. plantarum* as the most predominant species. The result of this study was slightly different with the study from Kopermsub & Yunchalard (2010) on fermented fish

Table 3. Some characteristics of suspected isolates of lactic acid bacteria from MRSA.

Sample origin	Number of isolates	Cell shape	Gram	Catalase	Gas production	Proteolytic activity	Colony diameter (mm)
Fish-experiment	6	Rod	+	-	-	+	0.1-0.3
Medium*-experiment	8	Rod	+	-	-	+	0.1-0.5
Fish-marketA	5	Rod	+	-	-	+	0.1-0.2
Medium*-marketA	5	Rod	+	-	-	+	0.1-0.3
Fish-marketB	6	Rod	+	-	-	+	0.1-0.4
Medium*-marketB	6	Rod	+	-	-	+	0.1-0.2

*Fermentation medium consisting of dried *P. edule* seed and grated coconut. MRSA means MRS Agar (de Man, Rogosa, Sharpe Agar).

Table 4. Some characteristics of suspected isolates of spore forming bacteria.

Sample origin	Number of isolates	Cell shape	Gram	Catalase	Spore production	Lipolytic activity	Colony diameter (mm)
Fish-experiment	2	Rod	+	+	+	+	0.3-0.6
Medium*-experiment	3	Rod	+	+	+	+	0.1-0.3
Fish-marketA	1	Rod	+	+	+	+	0.2
Medium*-marketA	1	Rod	+	+	+	+	0.3
Fish-marketB	4	Rod	+	+	+	+	0.2-0.4
Medium*-marketB	4	Rod	+	+	+	+	0.2-0.3

*Fermentation medium consisting of dried *P. edule* seed and grated coconut.

Table 5. Isolates identified from pado fish by 16S rRNA gene sequencing based on the data available at NCBI (the data were summarized from 100 selected sequences by the program, with E value = 0 and Query coverage 100%).

Isolate	Origin	Identified microorganism	Score	% Ident	Number of hits	Strain
Id3_1U2	Fish E,	..*Lactiplantibacillus plantarum*	2654	100	99	8268, 6401, 6093, MLG5-5, 6349, 6324, l168, etc.
	Exp-day3	..*Lactiplantibacillus pentosus*	2654	100	6	HBUAS5624, SKB1211, SKB1212, NGI17, NGI19, NGI06
		..*Lactiplantibacillus paraplantarum*	2654	100	1	B
Id3_2U2	Fish E,	..*Lactiplantibacillus plantarum*	2643	100	89	3334, 1417, 8276, 8268, 6706, 6401, 6093, etc.
	Exp-day3	..*Lactiplantibacillus pentosus*	2643	100	6	HBUAS5624, SKB1211, SKB1212, NGI17, NGI19, NGI06
Id5_2U1	Fish E,	..*Lactiplantibacillus plantarum*	2652	100	85	3334, 8276, 8268, 6706, 6401, 6093, MLG5-5, etc.
	Exp-day5	..*Lactiplantibacillus pentosus*	2652	100	6	HBUAS5624, SKB1211, SKB1212, NGI17, NGI19, NGI06
		..*Lactiplantibacillus paraplantarum*	2652	100	1	B
Id7_1U2	Fish E,	..*Lactiplantibacillus plantarum*	2651	100	88	3334, 247, 8276, 8268, 6401, S-24, 6093, etc.
	Exp-day7	..*Lactiplantibacillus pentosus*	2651	100	6	HBUAS5624, SKB1211, SKB1212, NGI17, NGI19, NGI06
Id7_2U2	Fish E,	..*Lactiplantibacillus plantarum*	2651	100	88	3334, 247, 8276, 8268, 6401, S-24, 6093, etc.
	Exp-day7	..*Lactiplantibacillus pentosus*	2651	100	6	HBUAS5624, SKB1211, SKB1212, NGI17, NGI19, NGI06
APd3_2U1	Medium E,	..*Lactiplantibacillus plantarum*	2634	100	90	3334, 2991, 2097, 1417, 247, 8276, 8268, etc.
	Exp-day3	..*Lactiplantibacillus pentosus*	2634	100	6	HBUAS5624, SKB1211, SKB1212, NGI17, NGI19, NGI06
APd3_3U1	Medium E,	..*Lactiplantibacillus plantarum*	2658	100	98	3334, 8276, 8268, 6401, 6093, MLG5-5, 6349, etc.
	Exp-day3	..*Lactiplantibacillus pentosus*	2658	100	6	HBUAS5624, SKB1211, SKB1212, NGI17, NGI19, NGI06
		..*Lactiplantibacillus paraplantarum*	2658	100	1	B
APd5_2U1	Medium E,	..*Lactiplantibacillus plantarum*	2645	100	99	3360, 3358, 3331, 2964, 2759, 2587, 1996, etc.
	Exp-day5	..*Lactiplantibacillus pentosus*	2645	100	1	YL-R2
		.*Lactilactobacillus sakei*	2645	100	1	GBL7
APd7_1U2	Medium E,	..*Lactiplantibacillus plantarum*	2652	100	99	3334, 8276, 8268, 6401, 6093, MLG5-5, 6349, etc.
	Exp-day7	..*Lactiplantibacillus pentosus*	2652	100	6	HBUAS5624, SKB1211, SKB1212, NGI17, NGI19, NGI06
		..*Lactiplantibacillus paraplantarum*	2652	100	1	B
APd7_2U2	Medium E,	..*Lactiplantibacillus plantarum*	2651	100	88	3334, 247, 8276, 8268, 6401, S-24, 6093, etc.
	Exp-day3	..*Lactiplantibacillus pentosus*	2651	100	6	HBUAS5624, SKB1211, SKB1212, NGI17, NGI19, NGI06
A2Aw4	Medium A	.*Lactiplantibacillus plantarum*	2588	99.65	89	3334, 2991, 2097, 1417, 247, 8276, 8268, etc.
	MarktAweek4	.*Lactiplantibacillus pentosus*	2588	99.65	6	HBUAS5624, SKB1211, SKB1212, NGI17, NGI19, NGI06
A5Iw2	Fish A,	.*Lactiplantibacillus plantarum*	2621	99.72	100	3360, 3358, 3335, 3333, 3331, 2964, 2954, etc.

Table 5. Continued...

Isolate	Origin	Identified microorganism	Score	% Ident	Number of hits	Strain
	MarktAweek2	*.Lactilactobacillus sakei*	2621	99.72	1	GBL7
B1Aw4	Medium B	*.Lactiplantibacillus plantarum*	2601	99.79	99	3360, 3358, 3331, 2964, 2759, 2681, 2587, etc.
	MarktBweek4	*.Lactilactobacillus sakei*	2601	99.79	1	GBL7
B3Iw4	Fish B,	*..Lactiplantibacillus plantarum*	2623	99.65	95	7232, 1797, 3156, 2993, 2979, 2952, 2877, etc.
	MarktBweek4	*..Lactiplantibacillus pentosus*	2623	99.65	4	9700, 10261, NGI01, 4591
		.Lacticaseibacillus paracasei	2623	99.65	1	L1

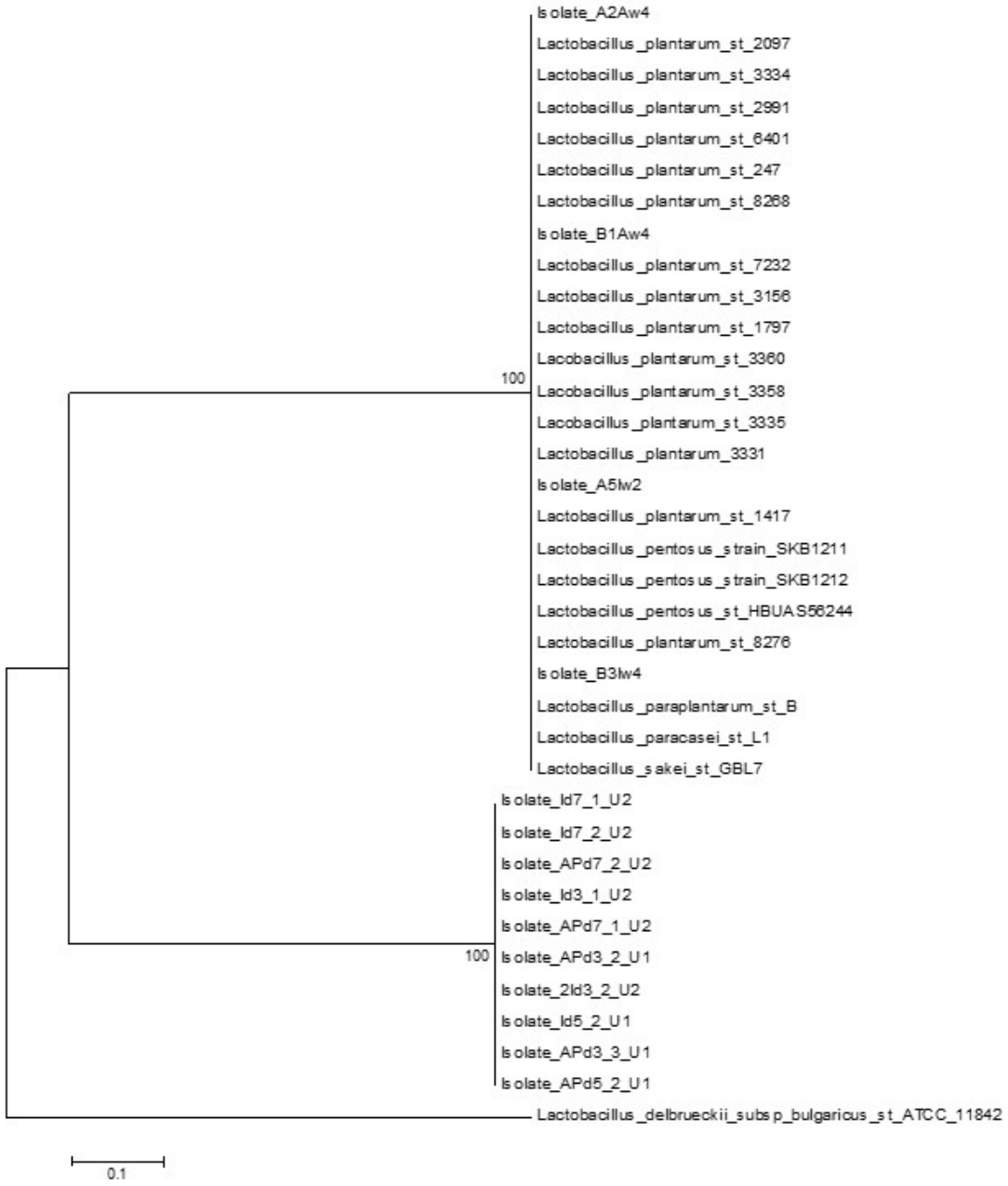

Figure 2. Phylogenetic tree of 16S rRNA gene of lactic acid bacteria isolates constructed with MEGA 6 program.

product named plaa-som from Thailand, which reported that *L. plantarum* was not found at the beginning of the fermentation process but became the predominant after the third to sixth day of fermentation.

4 Conclusions

During fermentation of pado fish by utilizing dried *P. edule* seed and grated coconut without salt, at room temperature (25-30 °C) for 7 days, apparent changes in microbial population were observed. Generally, the total plate count and lactic acid bacteria increased, the total Enterobacteriaceae and spore-forming decreased, whereas the mold and yeast count remained relatively constant at a low-level during fermentation. A total of 51 isolates were found under this study, consisting of 36 isolates of lactic acid bacteria and 15 isolates of spore-forming bacteria suspected to be *Bacillus* sp. Lactic acid bacteria were the predominant bacteria found in the final product of pado fish. *L. plantarum* and *L. pentosus* were consistently found along the fermentation process, indicating the important roles of these bacteria. The use of *P. edule* seed during fermentation is assumed to play an important role as natural selective agents that determine the microbial diversity of the product.

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