

Diversity and distribution of culturable lactic acid bacterial species in Indonesian Sayur Asin

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ABSTRACT

Background and Objectives: Lactic acid bacteria (LAB) play important roles in processing of Sayur Asin (spontaneously fermented mustard). Unfortunately, information about LAB in Indonesian Sayur Asin, prepared by traditional manufactures which is important as baseline data for maintenance of food quality and safety, is unclear. The aim of this study was to describe the diversity and distribution of culturable lactic acid bacteria in Sayur Asin of Indonesia.

Materials and Methods: Four Sayur Asin samples (fermentation liquor and fermented mustard) were collected at harvesting times (3-7 days after fermentation) from two traditional manufactures in Tulung Agung (TA) and Kediri (KDR), East Java provinces, Indonesia. LAB strains were isolated by using MRS agar method supplemented with 1% CaCO₃ and characterized morphologically. Identification of the strains was performed based on *16S rDNA* analysis and the phylogenetic tree was drawn to understand the phylogenetic relationship of the collected strains.

Results: Different profiles were detected in total count of the plates, salinity and pH of fermenting liquor of Sayur Asin in TA and KDR provinces. A total of 172 LAB isolates were successfully isolated and identified based on their *16S rDNA* sequences. Phylogenetic analysis of 27 representative LAB strains from Sayur Asin showed that these strains belonged to 5 distinct species namely *Lactobacillus farciminis* (N=32), *L. fermentum* (N=4), *L. namurensis* (N=15), *L. plantarum* (N=118) and *L. parafarraginis* (N=1). Strains D5-S-2013 and B4-S-2013 showed a close phylogenetic relationship with *L. composti* and *L. paralimentarius*, respectively where as the sequence had slightly lower similarity of lower than 99%, suggesting that they may be classified into novel species and need further investigation due to exhibition of significant differences in their nucleotide sequences. *Lactobacillus plantarum* was found being dominant in all sayur asin samples.

Conclusion: Lactobacilli were recognized as the major group of lactic acid bacteria in Sayur Asin including 5 known and 2 novel candidate species. The distribution of LAB species was associated with the manufactures where Sayur Asin is produced.

Keywords: *16S rDNA*, East Java, Lactic acid bacteria, Sayur Asin

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INTRODUCTION

Sayur asin, spontaneously fermented mustard (*Brassica juncea* (L.) Czern), is commonly consumed in many areas in Indonesia in particular in East Java province (1). Fermentation of sayur asin is performed over 3-7 days at ambient temperature (28-30 °C) in the presence of brine and coconut water and an anaerobic treatment of the mustard (2). Salt addition to the mustard results to the growth of epiphytic lactic acid bacteria (LAB) and fermentation until developing acidic and unique characteristics within the mustard (3).

The quality and food safety is related to the development of microbes during fermentation procedure. There is still limited information available on the diversity of microbes in Sayur Asin. Puspito and Graham reported slight variations in manufacturing of mustard fermentation in some regions in Indonesia (1). It was probably due to the diverse topographies, tropical climate and other physical condition of Indonesia. Combination of these environmental factors has produced a large diversity of microbes. Therefore, local fermented mustard from different regions in Indonesia become an interesting source for exploration of indigenous LAB diversity. In other regions, many new species have been reported from fermented foods including fermented mustard. For example, four new species of LAB were reported in Taiwan from fermented mustard (4) and different new species of *Lactobacillus* from kimchi was also found in Korea (5) and Tiongkok (6). The genetic variability and high diversity of LAB from fermented foods were possibly caused by divergence in manufacturing processes, varying conditions of concentration of salts, anaerobiosis, moisture levels and temperature. These factors can determine and select the composition and community structure of LAB in the fermentation final product.

Information regarding microbiology and diversity of LAB species in fermented mustard from different regions in Indonesia is still limited and is mainly obtained from a study applied conventional methods in identification of LAB (1). Current LAB taxonomy and identification studies involve combination of phenotypic (physiological and biochemical) and genotypic (DNA sequence) analyses. Molecular identification method is more accurate, sensitive, rapid, reproducible, and reliable which is not influenced by

environmental factors (7). This method supports the phenotypic methods suffer from lack of reproducibility due to its specific conditions such as its association to the culture and the diversity of strains (8). In bacteria, nucleotide sequences related to the *16S rDNA* have been useful and informative to provide information about the genus and species. *16S rDNA* is a common housekeeping genetic marker present in almost all the bacteria. The length of this region is relatively large enough (approximately 1540 bp) for genetic based identification and characterization purposes (9, 10).

The aim of this study was to describe the diversity and distribution of culturable lactic acid bacteria in Sayur Asin of Indonesia.

MATERIALS AND METHODS

Samples of sayur asin. Four samples (contain fermenting liquor and fermented mustard) were collected from traditional manufactures located at Tulung Agung (TA) and Kediri (KDR) in East Java province, Indonesia (Table 1). Sayur Asin from two manufactures was prepared by spontaneous fermentation of *Brassica juncea* (L.) Czern at room temperature (28-30 °C) for 3-7 days. Samples were collected twice at different periods of the harvesting time. For the analyses of Sayur Asin, samples were taken aseptically and packaged into a plastic bag and stored at 4 °C in ice boxes. Analyses and isolation were immediately performed in the laboratory.

Salinity and pH measurement. Salinity of fermenting liquor was determined as described previously (11). pH values of fermenting liquor was measured by a pH meter.

Enumeration and isolation of LAB. One gram fermented crushed mustard or 1 ml fermenting liquor was mixed with 9 ml 0.85% (w/v) NaCl solution. Each sample was serially diluted (10^{-3} to 10^{-6}). The serial dilutions were spread directly on the surface of MRS agar plates supplemented with 1% CaCO_3 , 1% NaCl and 6% NaCl (for halophilic lactic acid bacteria). Samples were incubated under anaerobic conditions in anaerobic jar at 28 °C for 4 days (2, 12). Colonies surrounded by a clear zone were counted. Colonies of acid-producing bacteria with distinct morphologies in terms of color,

shape and size were further purified by streaking at least twice on MRS agar plates containing 1% NaCl (4). Only Gram-positive, catalase-negative strains were selected (13). The selected strains were stored at -80°C in MRS broth containing 10% glycerol (145).

DNA extraction, PCR amplification and sequencing. Genomic DNA from each LAB strain was isolated according to the method described before (15). Amplification of *16S rDNA* was carried out using primer pair of 27F (5'-agagtttgatcctggctcag-3') and 1492R (5'-ggttacctgttacgactt-3') (16). PCR amplification was performed in a final volume of 50 µl. Each reaction mixture contained 25 µl Go Taq® Green Master Mix (2X) (Promega), 4 µl each primer (10 µM) and 1 µl DNA template (10-100 ng). The amplification program was set as follows: 94 °C (90 sec), 30 cycles of 95 °C (30 sec), 50 °C (30 sec), 72 °C for (90 sec) and a final extension at 72°C for 5 min. PCR products were electrophorized on 1% agarose gels at 100 V for 20 min and stained by ethidium bromide and visualized using UV transilluminator. Single-pass sequencing was performed on each PCR product using the forward primer. Full-length sequencing of *16S rDNA* was performed with the following universal primers: 27F, 518F, 800R, and 1492R.

Phylogenetic analysis. Homology of the sequences was examined by comparing the obtained *16S rDNA* sequences with those available in the nucleotide databases of the GenBank (<http://www.ncbi.nlm.nih.gov/BLAST>) using the Basic Local Alignment Search Tool (BLAST) program. 'Sequence from type material' was selected in the BLAST option to find the homolog sequences only from the type strains materials. About new sequences of LAB strains from sayur asin were aligned with eight sequences of the LAB sequences from type strains using Multiple Sequence Comparison by Log-Expectation (MUSCLE) implemented in MEGA (Molecular Evolutionary Genetics Analysis) version 5.2 (17). Phylogenetic analysis was performed using the neighbor-joining method (18) in MEGA. *Escherichia coli* strain JMC 1649^T (X80725) was used as the outgroup. The stability of the tree was assessed by bootstrap method (20) using 100 replications. Genbank accession numbers of the sequences used in this study was shown in Fig. 1.

RESULTS

Determination salinity, pH and enumeration of lactic acid bacteria. Our study demonstrated different profiles of Sayur Asin produced by two traditional manufacturers, Tulung Agung and Kediri. Salinity of fermenting liquor from four samples ranged from 3.60 to 5.67% (w/v). pH value of sayur asin samples were also variable from 3.50 to 4.57. LAB colony counts based on the total plate count (TPC) method in four samples of sayur asin was also variable from 20×10^5 to 26×10^7 CFU/ml. The highest LAB population was found on the sayur asin sample from Tulung Agung (TA2) (26×10^7 CFU/ml) while the lowest colony count was found in the sayur asin from Kediri (KDR1). All sayur asin samples from Tulung Agung (TA1 and TA2) had lower salinity and higher pH than those from Kediri (KDR1 and KDR2). Detailed information regarding properties of these samples has been presented in Table 1.

***16S rDNA* sequence data.** A total of 172 LAB strains were successfully isolated from sayur asin origin East Java. All isolates were subjected to *16S rDNA* sequence analysis. Sequence analysis using BLAST showed that all the isolates belonged to the genus *Lactobacillus*. Based on the 99% cut off homology similarity, 32 isolates were determined as *L. farciminis*, four isolates as *L. fermentum* 15 isolates as *L. namurensis*, 118 isolates as *L. plantarum* and a single isolate as *L. parafarraginis*. *Lactobacillus* sp. strain D5-S-2013 showed 98% similarity to *L. paralimentarius* while *Lactobacillus* sp. strain B4-S-2013 showed 97% DNA sequence similarity to *L. compositi*. According to the results of a published study (20), if the rate of similarity between *16S rDNA* sequences of two organisms is lower than 98.7-99.0% they will belong to separate species. Due to the observed similarity rates lower than 99%, the isolates in the present study could be classified into novel species.

Lactic acid bacteria (LAB) composition and assemblage in four samples of sayur asin have been presented in Tabel 2. *Lactobacillus plantarum* and *L. farciminis* were found as common LAB used in sayur asin origin from East Java. Two unknown species (*Lactobacillus* sp. strain D5-S-2013 and B4-S-21) were found in sayur asin from Tulung Agung and Kediri, respectively. Those strains are rarely found in samples TA or KDR. Distribution of species was varied amongst the samples. Culturable *L. planta-*

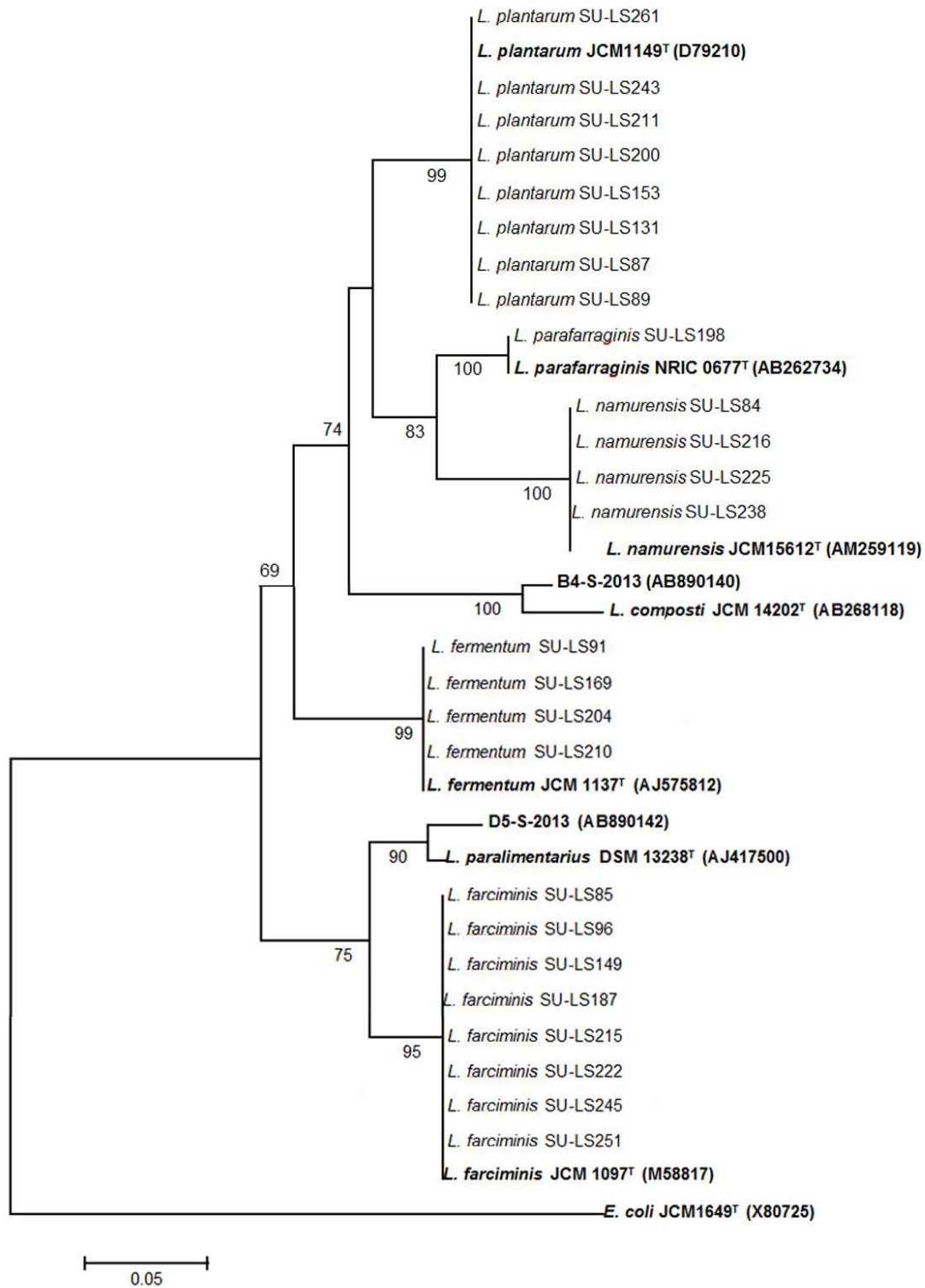


Fig. 1. Phylogenetic tree generated from NJ analysis of *16S rDNA* sequences from representative LAB isolates from sayur asin collected in Tulung Agung and Kediri (East Java). *Escherichia coli* JCM1649^T (X80725) was used as the outgroup. Bootstrap values $\geq 50\%$ have been shown below the branch length and the scale bar represents 0.05 sequence divergence.

Table 1. Profile of sayur asin collected from Tulung Agung and Kediri (East Java) in this study

| Parameter | Sample Code | | | |
|-----------------------|-------------------------------------|----------------------|--------------------------------|---------------------|
| | TA1 | TA2 | KDR1 | KDR2 |
| Sample location | Tulung Agung, East Java Province | | Kediri , East Java Province | |
| Colony count (CFU/ml) | 29 x 10 ⁶ | 26 x 10 ⁷ | 20 x 10 ⁵ | 25x 10 ⁵ |
| % Salinity (w/v) | 3.77 | 3.60 | 5.67 | 5.55 |
| pH | 4.25 | 4.57 | 3.50 | 3.72 |

Table 2. Distribution of LAB assemblage in four samples of sayur asin

| Species | Tulung Agung | | Kediri | | No. of isolates |
|--|--------------|-----------|-----------|-----------|-----------------|
| | TA1 (%) | TA2 (%) | KDR1 (%) | KDR2 (%) | |
| <i>L. farciminis</i> | 3 (6.7) | 3 (4.6) | 6 (28.6) | 20 (48.8) | 32 |
| <i>L. fermentum</i> | 1 (2.2) | 1 (1.5) | 2 (9.5) | 0 | 4 |
| <i>L. namurensis</i> | 4 (8.9) | 0 | 0 | 11 (26.8) | 15 |
| <i>L. plantarum</i> | 37 (82.2) | 60 (92.3) | 12 (57.1) | 9 (21.9) | 118 |
| <i>L. parafarraginis</i> | 0 | 0 | 1 (4.8) | 0 | 1 |
| <i>Lactobacillus</i> sp. strain D5-S-2013 | 0 | 1 (1.5) | 0 | 0 | 1 |
| <i>Lactobacillus</i> sp. strain B4-S-2013 | 0 | 0 | 0 | 1 (2.4) | 1 |
| Total No. of isolates | 45 | 65 | 21 | 41 | 172 |

rum was found to predominate Sayur Asin TA (82.2-92.3%), whereas spread evenly LAB species was recognized in Sayur Asin KDR. *L. namurensis* were found in both samples TA and KDR but not always appeared at the time of harvesting time of Sayur Asin.

Phylogenetic affinity of LAB from sayur asin based on 16S rDNA sequence analysis. From a total 172 isolated LAB strains from sayur asin, 27 representative 16S rDNA sequences were selected for phylogenetic studies. The remaining 145 LAB sequences were excluded from the analyses due to their high similarity with 27 representative LAB sequences (data not shown). Full-length 16S rDNA sequence analysis was performed for *Lactobacillus* sp. strains B4-S-2013 and D5-S-2013. The generated phylogenetic tree showed that isolated LAB strains could be divided into 7 independent clades, viz, *L. plantarum* (Orla-Jensen) Bergeys et al. (99% BS), *L. parafarraginis* Endo and Okada (100% BS), *L. namurensis* Scheirlinck et al. (100% BS), *L. fermentum* Beijer-

inck (99% BS), *L. farciminis* Reuter (95% BS), *L. composti* (100% BS), and *L. paralimentarius* (90% BS). Sequences categorized within *L. plantarum*, *L. parafarraginis*, *L. namurensis*, *L. fermentum* and *L. farciminis* clades showed almost identical sequences in each clade which is represented by the length of each branch (no difference was observed in the branch length within these clades). However in the *L. composti* and *L. paralimentarius* clades a significant branch length was observed between *Lactobacillus* sp. strain B4-S-2013 with *L. composti* and *Lactobacillus* sp. strain D5-S-2013 with *L. paralimentarius*, respectively. Further analysis is necessary to reveal the identity of B4-S-2013 and D5-S-2013 strains.

DISCUSSION

The microbiology of mustard fermentation is similar to the other fermented vegetables because the microorganisms responsible for this fermentation process are mainly belonging to LAB (1, 21). Activity of

LAB during fermentation can cause a decrease in pH value of the fermented vegetables. The final pH resulted from the fermentation process is possibly related to the incubation time. Our data showed that average pH of sayur asin from Tulung Agung and Kediri were different. Sayur asin samples from Kediri possessed in more acidic conditions than those from Tulung Agung (Table 1). Although the incubation time of sayur asin fermentation process in Tulung Agung and Kediri was 3 days but sayur asin in Kediri was maintained by backslopping and the acidic conditions from the previous batch probably will affect the pH. Any changes in pH of sayur asin by the LABs can also be considered as one of the main indicators for a good fermentation.

A previous study has reported that the variety of LAB population in the fermented mustard was influenced by different fermentation processes and treatments especially the salt concentration (4). In this study the highest LAB population was found within the lowest salt concentration. It seems that low salt concentration can be supportive for the growth of LAB strains in sayur asin than higher salt concentration. During the fermentation process, LAB are initially present in low numbers, however, they immediately proliferate because the growth of other microorganisms are inhibited by the initial addition of salt. Rapid growth of LAB strains decreases the pH of the medium which result to the slow growth of other microorganisms (21). The growth of lactic acid bacteria is also influenced by the nutrient movement from plant material into the surrounding liquid (3). Among LAB species found in this study, *L. plantarum* was found predominant which is probably due to its acid tolerance and in particular because of its ability in transportation and metabolism of different carbohydrates (22).

Phylogenetic analyses of a total 172 *16S rDNA* sequences showed that these LAB strains belong to a single genus, *Lactobacillus*, and five species (*L. farciminis*, *L. fermentum*, *L. namurensis*, *L. plantarum* and *L. parafarraginis*). These five species have been found in different fermented foods (23), reported as non pathogenic bacteria for humans or animals (24). Identification of five *Lactobacillus* species from sayur asin is not unpredictable since members of *Lactobacillus* have commonly been found and used in various fermentation processes in developing various foods and drinks. Genus *Lactobacillus* has been known as the largest group of lactic acid bacteria with over than 145 recognized species (25). Members of

this genus are also very heterogenous, encompassing species with a large variety of phenotypic, biochemical and physiological properties. Many species have been found in many spontaneous lactic fermentation procedures such as vegetable and silage fermentations (26).

The phylogenetic affinity of LAB from sayur asin showed a close relationship among particular LAB species (Fig. 1). *Lactobacillus parafarraginis* showed a close relationship with *L. namurensis* (83% BS). This clade formed a monophyletic clade with *L. plantarum* and *L. composti* clades (74% BS). This data indicates that *L. parafarraginis*, *L. namurensis*, *L. plantarum* and *L. composti* were originated from recent common ancestor species. *Lactobacillus parafarraginis* and *L. composti* were firstly described from compost of sochu (27, 28) while *L. namurensis* from sourdough (29) and *L. plantarum* from fermented vegetables (30). In addition members of *L. farciminis* formed a sister clade to *L. paralimentarius* with 75% BS while *L. fermentum* formed independent clade closed to the large clade containing *L. parafarraginis*, *L. namurensis*, *L. plantarum* and *L. composti* clades. *Lactobacillus farciminis* was originated from sausage while *L. paralimentarius* was isolated from sourdough (31). The phylogenetic analysis of *Lactobacillus 16S rDNA* sequences showed that there is no relationship between these species based on the source of isolation. Species from the same source such as *L. parafarraginis* and *L. composti* from sochu compost and *L. namurensis* and *L. paralimentarius* from sourdough nested in distantly related clades (Fig. 1).

The results from this study has revised the previous report (1) in which *L. confusus* Sharpe et al., *L. curvatus* Abo-Elnaga and Kandler, and *L. plantarum* as members of LAB from sayur asin in Indonesia. Several species which were not identified by Puspito et al. (1), have been determined in the current study which included *L. farciminis*, *L. fermentum*, *L. namurensis* and *L. parafarraginis*. It showed that sayur asin is potential resource for the exploration of unique and distinct species of LAB in Indonesia. This study is the first report on *L. parafarraginis* from sayur asin in Indonesia. Diversity of LABs from Central Java reported by others (2) is higher than those from East Java, eleven species of LAB viz, *L. farciminis*, *L. fermentum*, *L. namurensis*, *L. plantarum*, *L. helveticus*, *L. brevis*, *L. versmoldensis*, *L. casei*, *L. rhamnosus*, *L. fabifermentans* and *L. satsumensis*.

CONCLUSION

Five distinct species of *Lactobacillus* were determined from sayur asin collected from Tulung Agung and Kediri (East Java Provinces, Indonesia) based on phylogenetic analysis of *16S rDNA* sequences. These species include *L. farciminis*, *L. fermentum*, *L. namurensis*, *L. plantarum* and *L. parafarraginis*. Two *Lactobacillus* isolates (strains D5-S-2013 and B4-S-2013) showed slightly lower similarity rates of lower than 99% and had clade with significant branch length in phylogenetic tree which was observed with their closely related species, viz, *L. paralimentarius* and *L. composti*, respectively suggesting that they may be classified into novel species. Therefore more investigation is needed to exhibit significant differences in their nucleotide sequences.

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