



Changes in lactic acid bacteria diversity during fermentation of sour pickled mustard green

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Abstract

Mustard green (*Brassica juncea* L var. *rugosa*) is popularly consumed in Thailand as salted or sour pickled vegetable. Naturally occurred lactic acid bacteria (LAB) from the raw ingredients play an important role in fermentation of sour pickled mustard green. Unsuccessful sour taste development is one of the major problems in the sour pickle industry. Therefore this study aims to study the LAB diversity and their changes during fermentation of the sour pickled mustard green, both in natural fermentation and in starter culture-added fermentation. This knowledge will help understanding and developing starter culture for successful fermentation. The experiment was conducted at the pickled mustard green factory with industrial-scale fermentation facility. A control batch without starter culture and two batches of starter culture-added at two different concentrations were fermented at room temperature for 12 days. LAB were isolated on de Man, Rogosa and Sharpe (MRS) agar supplemented with 0.5% CaCO₃ at day 0, 3, 7, and 12 of fermentation. Ten bacterial colonies were randomly selected from each time point and genomic fingerprint of each isolate was generated using repetitive element-based PCR (rep-PCR) technique. The control batch without starter culture showed sign of spoilage indicated by increase in pH from 3.33 to 4.44 at day 7 while in the starter culture-added batches the pH remained low throughout the fermentation period. Analysis of the bacterial diversity revealed succession of the LAB species from *Weissella* spp. to *Lactobacillus plantarum* in naturally fermented batch and from *Weissella* spp. to *Lactobacillus fermentum* in starter culture-added batches.

Keywords: Lactic acid bacteria (LAB), starter cultures, sour pickled mustard green, rep-PCR

1. Introduction

Mustard green or Chinese mustard (*Brassica juncea* L var. *rugosa*) is a vegetable that has large petioles, thick leaves, well wrapped and firm head. It is usually not consumed fresh because the taste of fresh produce is bitter and spicy. Preservation of the mustard green by fermentation can eliminate the undesired taste and improve flavor of the vegetable, which is desirable for several dishes. Sour pickled mustard green is one of the most consumed fermented vegetable in Thailand. The fermentation usually relies on microbial populations naturally presented in the raw materials, which may vary from batch-to-batch causing variations in the product quality and successful fermentation can not be guaranteed. Unsuccessful sour taste development is one of the major problems in the sour pickle industry. In this study, the key microorganisms, lactic acid bacteria (LAB), and their changes during fermentation in naturally fermented mustard green as well as in starter culture-added system were investigated. This knowledge will help understanding and developing of new starter culture for improved quality and consistency of the sour pickled mustard green product.

2. Materials and Methods

2.1 Sour pickled mustard green preparation and sampling procedures

In an attempt to ensure successful fermentation of sour mustard green in the factory scale, two levels of LAB starter culture (obtained from BIOTEC culture collection) were used. There were three treatments in this study, one tank of control (natural fermentation), two tanks inoculated with starter culture at low-inoculum level, and two tanks inoculated with high-inoculum level. Approximately 4,500 kg of mustard green were fermented in each experimental tank. Brines and other ingredients were added to the vegetable according to the factory recipe. The

fermentation process was conducted with minimum agitation at an ambient temperature for 12 days. The liquid suspension in the tanks were sampling for analysis periodically at day 0, 3, 7, and 12. Duplicate samples were collected from each tank, kept on ice and shipped to the laboratory for analysis in the next day.

2.2 Chemical analysis

The progress and success of the fermentation process was measured by pH of the samples. A pH meter with pin electrode (Sartorius PB-20, Germany) was used to measure pH of the solution.

2.3 Isolation of lactic acid bacteria

The brine samples were inoculated on MRS agar (Difco, USA) supplemented with 0.5% CaCO_3 and incubated at 30°C for 48 h. Colonies with clear zone were counted as LAB. Ten LAB colonies were randomly picked and purified from each sample for fingerprint analysis.

2.4 DNA extraction

Each bacterial isolate was grown in MRS broth at 30°C for 16-18 h. The bacterial cell pellet was collected by centrifugation. The whole cell DNA extraction was conducted as described by Chomczynski and Rymaszewski (1). In brief, 10 μl of the cell pellet was suspended in 0.2 ml alkaline-polyethylene glycol (alk-PEG) solution, boiled at 100°C for 10 min and centrifuged to discard the cell debris. The clear lysate was used as template for the fingerprint analysis.

2.5 Fingerprint analysis

The LAB isolates were analyzed for their genomic fingerprint profile using repetitive-element based PCR (rep-PCR) technique. A primer targeted for the tri-nucleotide repeat (GTG)₃ (5' GTGGTGGTGGTGGTG 3') was used (2). The amplification reactions were carried out in 25 μl reaction volume, using Takara Ex Taq DNA polymerase and buffer system (Takara Bio Inc., Japan). The final PCR mixture comprised of 1x Ex Taq buffer, 200 μM of each deoxynucleoside triphosphate, 800 nM primer, 1.25 units of Ex Taq DNA polymerase, and 2 μl of the

DNA template. The amplification cycles were initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 40°C for 1 min and extension at 65°C for 10 min, and additional final extension at 65°C for 20 min. Five microliters of the amplified products were analyzed in 1 % agarose gel electrophoresis with 0.5x TBE buffer using a Bio-Rad GT Subcell electrophoresis system (Bio-Rad Laboratories, USA). After electrophoresis, the gel was stained in ethidium bromide solution (5µg/ml) for 5 min and destained in tap water for 20 min. The gel images were captured using an image scanner Typhoon 9410 (Amersham Biosciences, USA). The DNA fingerprint patterns were analyzed by using fingerprint analysis software, GelCompar II, Version 5.1 (Applied Math, Belgium). Pearson product moment correlation coefficient was used to calculate similarities among patterns and a dendrogram was obtained by means of unweighted pair group method with arithmetic average (UPGMA)

2.6 Species identification

After fingerprint analysis, representative strains for each fingerprint patterns were selected for species identification. The identification was done by 16S rRNA gene sequence identification. The 16S rRNA gene of the representative isolates was amplified using universal primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The PCR amplification was carried out in 50 µl reaction volume, using Takara Ex Taq DNA polymerase and buffer system. The final PCR mixture comprised of 1 × Ex Taq buffer, 200 µM of each deoxynucleoside triphosphate, 400 nM of each primer, 1.25 units of Ex Taq DNA polymerase, and 2 µl of the DNA template. The amplification cycles were initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 2 min, and additional final extension at 72°C for 5 min. The 16S-PCR products were purified by using QIAquick PCR

Purification kit (QIAGEN, Germany), according to manufacturer's instruction. The purified PCR products were sequenced commercially. Homology searches of the 16S rDNA sequences were performed using the BLAST basic local alignment search tool in the Genebank (<http://www.ncbi.nlm.nih.gov/Blast/>) to determine the most likely identities of the isolates.

3. Results and Discussion

Results of the pH measurement of the samples were shown in Fig. 1. At the beginning of the experiment (day 0), the pH of all samples was similar in the range of 5.43-5.70. The pH in all five batches dropped rapidly to 3.24-3.33 on day 3 of fermentation. However, on day 7, pH of the naturally fermented batch was increased significantly from 3.33 to 4.44, while in all starter culture-added batches the pH remained low (3.22-3.27 for high-inoculum and 3.40-3.44 for low-inoculum). At the end of the fermentation on day 12, both batches of the high-inoculum samples had the lowest pH at 3.30-3.35, followed by the low-inoculum batches (pH 3.65-3.81), while the naturally fermented batch had the highest pH at 3.93 (Fig.1). Total LAB counts of the samples from all fermentation batches were shown in Fig. 2. The initial LAB population in naturally fermented batch was the lowest at 5.58 logCFU/ml, followed by the low-inoculum batches at 5.75-5.89 logCFU/ml. The high-inoculum batches had the highest number of LAB, at 6.11-6.16 logCFU/ml. The differences in the initial LAB population reflected the number of LAB starter culture added to each batch. For naturally fermented batch, the LAB population increased to the maximum value of 8.02 logCFU/ml on day 7 and then decreased to 6.95 logCFU/ml at day 12. For the low-inoculum batches, the increase in LAB population were highest on day 3 to an average of 7.57 logCFU/ml, then, slowly decreased to 7.25 logCFU/ml at day 12. For the high-inoculum batches, in contrast to other batches, the increase in LAB popula-

tion was small only ca. 0.5 log increased on day 3, then slowly decreased to ca. 6.06 logCFU/ml at the end of day 12. Lower LAB population found in the high-inoculum batches when compared to naturally fermented and

low-inoculum batches may be explained by ability of the starter culture to inhibit other LAB species and has advantages over other LAB with high initial inoculum.

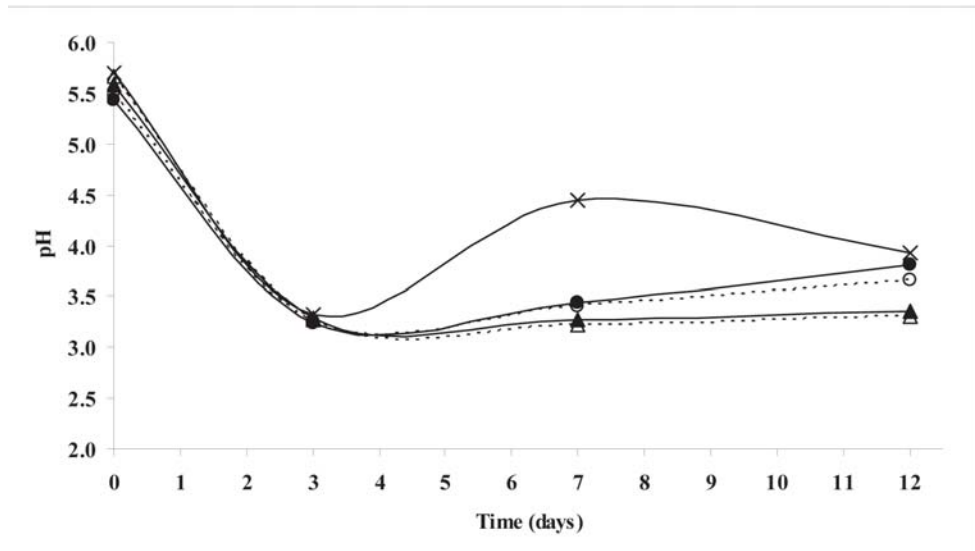


Figure 1. Changes in pH profiles during fermentation of sour pickled mustard green. —x—, Natural fermentation; —●—, Starter culture: low-inoculums batch 1; ...○... , Starter culture: low-inoculum batch 2; —▲—, Starter culture: high-inoculum batch 1; ...△... , Starter culture: high-inoculum batch 2.

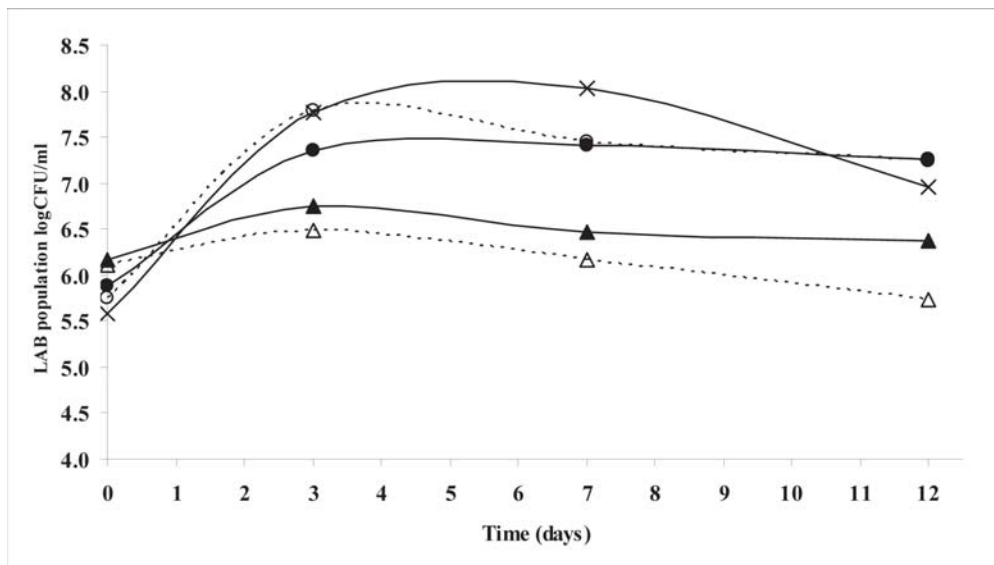


Figure 2. Changes in population of LAB during fermentation of sour pickled mustard green. —x—, Natural fermentation; —●—, Starter culture: low-inoculums batch 1; ...○... , Starter culture: low-inoculum batch 2; —▲—, Starter culture: high-inoculum batch 1; ...△... , Starter culture: high-inoculum batch 2.

Results of the cluster analysis and species identification of the 200 LAB isolates were shown in Fig. 3-5. In naturally fermented batch, bacteria in the genus *Weissella* were predominant in the beginning of the fermentation but then quickly replaced by *Lb. plantarum* and *Lb. fermentum* on day 3. Domination and succession of several *Lb. plantarum* isolates (demonstrated by different fingerprint patterns) occurred until the end of the fermentation period (Fig. 3). The result of LAB population analysis in the low-inoculum batches is shown in Fig. 4. In both tanks, similar to the naturally fermented batch, *Weissella* was the predominant bacteria at the beginning and was quickly replaced by other LAB on day 3. In tank A, the starter culture was successfully grown and become the major population on day 3 and then replaced by *Lb. fermentum* and *Lb. plantarum* on day 7 and day 12 (Fig. 4a). In tank B, small portion of the starter culture was successfully

established on day 3-7 and then also completely replaced by *Lb. fermentum* and *Lb. plantarum* on day 12 (Fig. 4b). Similarly, in the high-inoculum batches (Fig. 5), although the starter culture was added at high concentration and shown to be the major population at the beginning, the starter culture was eventually replaced by *Lb. fermentum* and *Lb. plantarum* at the later stage of the fermentation. Domination of *Weissella* spp. at the beginning of the fermentation was also reported in other fermented vegetable such as *mulkimchi* (3). Replacement of *Weissella* spp. by *Lactobacillus* spp. during fermentation was also reported in several studies such as in Taiwanese fermented mustard (4), *kimchi* (3), and sauerkraut (5). Domination of *Lb. plantarum* and *Lb. fermentum* in the final commercial products was also reported in several Thai fermented vegetables (6), and Vietnamese fermented mustard (*dua cai be muoi*) (7).

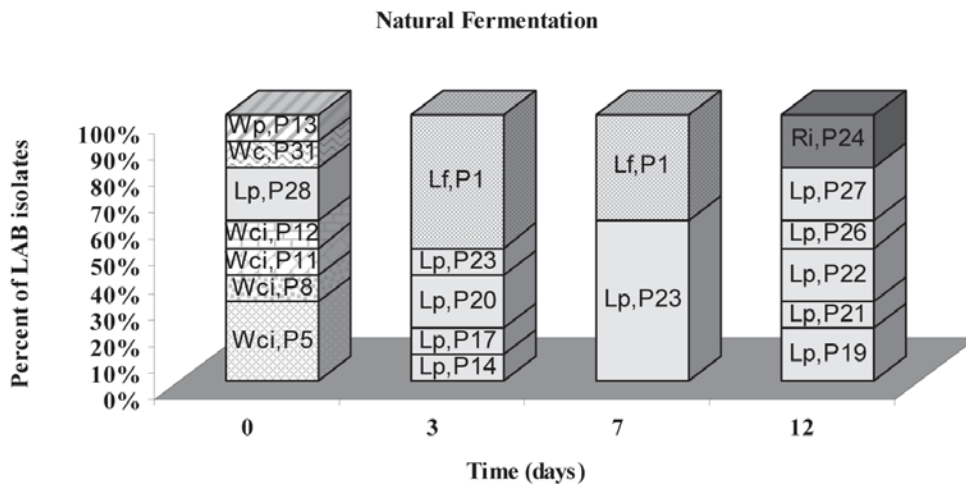


Figure 3. Distribution of the predominant LAB species from naturally fermented batch. Legends in the boxes are species abbreviation followed by fingerprint pattern number. The species abbreviations are Lf, *Lb. fermentum*; Lp, *Lb. plantarum*; Ri, *Ralstonia insidiosa*; Wc, *Weissella confuse*; Wci, *Weissella cibaria*; Wp, *Weissella paramesenteroides*.

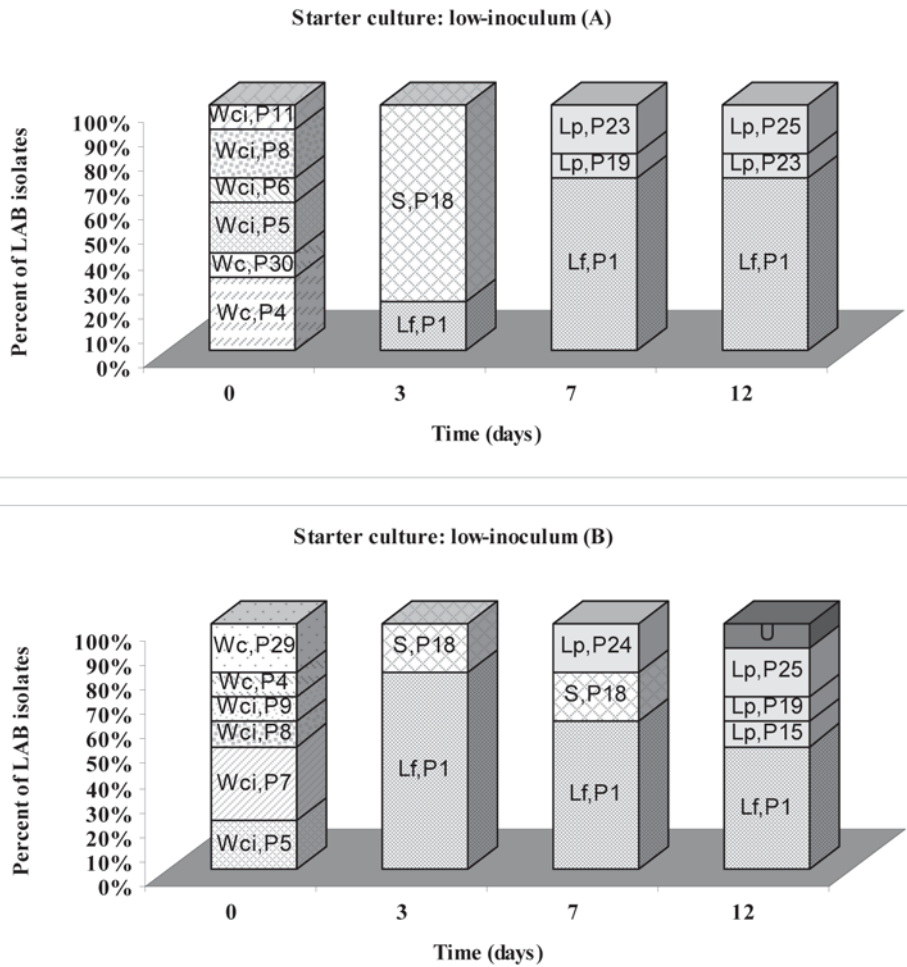


Figure 4. Distribution of the predominant LAB species from low-inoculum starter culture batches. (A), batch 1; (B), batch 2. Legends in the boxes are species abbreviation followed by fingerprint pattern number. The species abbreviations are Lf, *Lb. fermentum*; Lp, *Lb. plantarum*; S, Starter culture; U, Unidentified; Wc, *Weissella confuse*; Wci, *Weissella cibaria*.

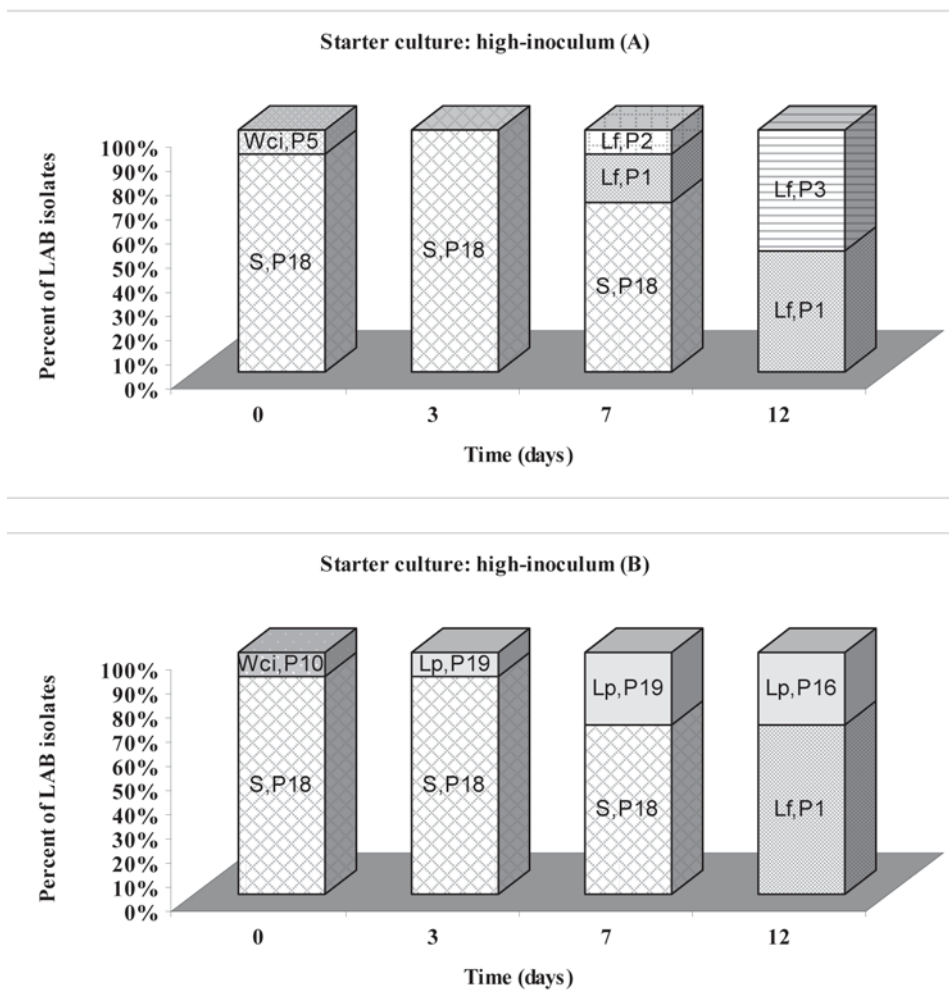


Figure 5. Distribution of the predominant LAB species from high-inoculum starter culture batches. (A), batch 1; (B), batch 2. Legends in the boxes are species abbreviation followed by fingerprint pattern number. The species abbreviations are Lf, *Lb. fermentum*; Lp, *Lb. plantarum*; S, Starter culture; Wci, *Weissella cibaria*.

4. Conclusion

The result from this study showed that in naturally fermented sour pickled mustard green, the fermentation was inconsistent indicated by increment in pH during fermentation and the higher final pH. Addition of the LAB starter culture was shown to help improving the consistency of the fermentation, especially at the high level of inoculum. Analysis of the LAB diversity revealed succession of the LAB species from *Weissella* spp. to *Lb. plantarum* in naturally fermented batch and from *Weissella* spp. to *Lb. fermentum* in starter culture-added batches. Therefore, it is suggested that *Lb. fermentum* may play an important role in the mustard green fermentation. This knowledge can be used in development of new starter culture that is more efficient in the pickled mustard green industries.

5. Acknowledgement

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6. References

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