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PHENOLIC CONTENT, ANTIOXIDANT ACTIVITY AND BIODIVERSITY CHANGES DURING SPONTANEOUS FERMENTATION OF CARICA PAPAYA LEAF

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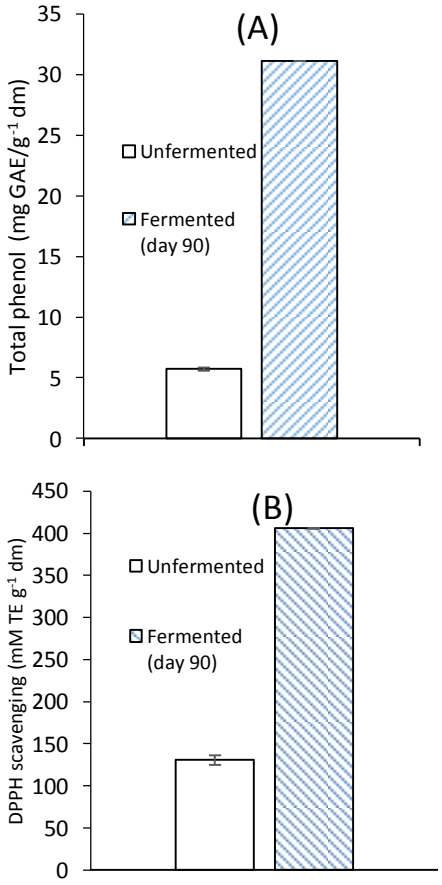
Graphical abstract



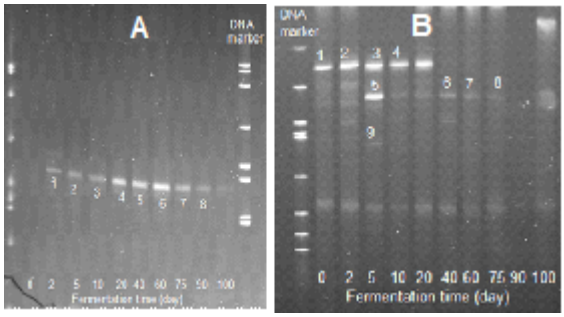
Abstract

The spontaneous fermentation was carried out on *Carica papaya* leaf (CPL) in view of its potential improvement on antioxidant functionality and cultivation of lactic acid bacteria. The effect of the spontaneous fermentation on the total phenolic content and antioxidant activity of CPL, as well as biodiversity profiling were evaluated in this study. Total phenolic content and antioxidant capacity of the fermented CPL were 31.14 mg GAE g^{-1} and 405.8 mM TE g^{-1} respectively, higher than the unfermented CPL (5.71 mg GAE/g and 130.5 mM TE g^{-1}) respectively. Microbial community was predominantly lactic acid bacteria (LAB) and yeasts, both populated at 10^4 to 10^8 CFU/mL during most part of the fermentation. Presumptive *Enterobacteriaceae* showed up briefly at the onset of the fermentation before disappearing. PCR-DGGE fingerprinting revealed *Lactobacillus plantarum* (*Lb. plantarum*) as the sole dominant bacterial species. More diverse yeasts community was detected by PCR-DGGE where succession of *Zygosaccharomyces*, *Saccharomyces*, *Candida* and *Aspergillus* genera were detected along fermentation time. Spontaneous fermentation successfully enhanced the total phenolic content and antioxidant capacity of the CPL. The cultivation of lactic acid bacteria was indicated by the presence of *Lb. plantarum*, whereas the disappearance of *Enterobacteriaceae* conferred a safe consumption of the fermented CPL.

Keywords: Spontaneous fermentation, *Carica papaya* leaf, total phenolic content, antioxidant, lactic acid bacteria

Abstrak

Kaedah penapaian spontan telah dijalankan ke atas daun *Carica papaya* (CPL) kerana potensi kaedah ini dalam meningkatkan fungsi antioksida dan pengkulturan bakteria laktik asid (LAB). Kesan penapaian spontan ke atas CPL yang diukur dari sudut jumlah kandungan fenolik, aktiviti antioksida serta pemprofilan kepelbagaian biologi di dalam ekosistem penapaian telah dijalankan dalam kajian ini. Jumlah kandungan fenolik dan aktiviti antioksida dalam CPL yang ditapai adalah msing-masing 31.14 mg GAE g^{-1} and 405.8 mM TE g^{-1}, iaitu lebih tinggi daripada CPL yang tidak ditapai, iaitu masing-masing



DGGE fingerprints of bacteria (A) and yeasts (B)

pada 5.71 mg GAE/g dan 130.5 mM TE g^{-1}. Komuniti mikrobiologi didominasi oleh spesis LAB dan yis di mana populasi kedua-duanya pada 10^4 to 10^8 CFU/mL sepanjang tempoh penapaian. Spesis *Enterobacteriaceae* hanya muncul di awal proses penapaian tetapi kemudiannya menghilang. Keputusan PCR-DGGE menunjukkan hanya spesis bakteria *Lactobacillus plantarum* yang hadir semasa proses penapaian manakala kepelbagaian spesis yis ditonjolkan melalui kehadiran *Zygosaccharomyces*, *Saccharomyces*, *Candida* dan *Aspergillus*. Kesimpulannya, kaedah penapaian spontan telah berjaya meningkatkan jumlah kandungan fenolik dan aktiviti antioksida CPL. Pengkulturan bakteria laktik asid pula dibuktikan melalui kehadiran spesis *Lactobacillus plantarum,* manakala ketiadaan spesis *Enterobacteriaceae* memastikan keselamatan pengambilan CPL yang ditapai.

Kata kunci: Penapaian spontan, daun *Carica papaya*, jumlah kandungan fenolik, antioksida, bakteria laktik asid

1.0 INTRODUCTION

The medicinal properties of *Carica papaya* leaf (CPL) in forklore medicine to treat wound, burn, infection and fever [1] are well known. Recent evidences asserted the therapeutic properties of CPL in treating myriad illnesses; diabetes, cancer, autoimmune disorder, inflammation [2] and dengue [3]. Despite these properties, the bioactive compounds of CPL which consist of polyphenols such as saponin, tannins ferulic acid, caffeic acid, rutin, quercetin, protocatechuic acid, quercetin and kaempferol are not easily absorbed at the digestive tracts in their native form [4, 5]. Only a tiny fraction of polyphenols, mostly dimeric and monomeric structures, are directly absorbed at the small intestine, whereas most polyphenols (90-95%) are digested by gut microbiota [6].

The benefit of fermentation in enhancing the medicinal functionality of plant materials by digesting the polymeric polyphenols into lower molecular weight has been demonstrated by fermented papaya (Immun'Age®)[7, 8] and other phenolic-rich plants [9, 10]. Moreover, the technique is also known for enrichment of essential amino acids, vitamins, minerals as well as promoting the growth of beneficial microorganisms such as lactic acid bacteria (LAB) which promotes healthy effect such as immunomodulation and inhibition of pathogen [11]. Apart from papaya fruit, fermentation on other parts of the *Carica papaya* plant such as its root or leaf is nonexistence. In view of the benefit of fermentation in enhancing the functionality of fermented materials as well as diverse species, geography, cultivars and the farming practice of the CPL, spontaneous fermentation technique is a preferred choice to allow the complex 'wild strain' to colonise the materials and performs diverse metabolic functions and products in which available starter culture may in deficient at [12]. Spontaneous fermentation has been applied on Spider flower (*Gynandropsis gynandra*) [13], leek [14], carrot juice [15], garlic [16] and Cornelian cherry [17] to enhance the bioactivities or cultivating the probiotic element of the respective material.

In this study, the changes of phenolic content, antioxidant activity and biodiversity changes during spontaneous fermentation of CPL were investigated.

2.0 METHODOLOGY

2.1 Fermentation

CPL was purchased from a farm in Banting, Selangor, Malaysia. The leaf was processed instantly after harvesting to maintain its original characteristics. The fresh CPL was washed to remove physical dirt, shredded into smaller pieces using kitchen blender and loaded at 10 %w/v into a 60 L food grade, high density polyethylene barrel. Ten %w/v of unrefined sugar was added as supplement, followed by distilled water to add to 50 L working volume. The fermentation was carried out in anaerobic condition for 100 days at room temperature. Fifteen milliliters (15 mL) of liquid samples were collected at day 0, 2, 5, 10, 20, 40, 60, 75, 90 and 100. The pH of the samples were instantly measured in duplicate (Mettler-Toledo) and later proceeded to chemical and microbiological analyses.

2.2 Sample Extraction

The extract from fermentation broth was prepared according to Curiel *et al*. [9]. The CPL suspension collected at day 0 (fresh CPL), day 40 and day 90 were centrifuged at 10,000 g for 20 minutes and 5 ˚C to remove solid debris. Then, the supernatant was collected and dried by rotary evaporator at 30 ˚C for 45 minutes to remove water content and later re-

suspended with 80% of methanol (MeOH) (R&M) at 1:1 (v/v) to yield methanolic extract (ME). The ME was purged with nitrogen for 30 minutes, followed by re-centrifugation (4,600g) for 20 minutes to remove the residue. Finally, the supernatant was collected and purged with nitrogen for 45 minutes.

2.3 Total Phenolic Content (TPC)

Extraction of total phenolic content (TPC) was carried out according to Madaan *et al.* [18] and Curiel *et al.* [9] with some modifications. For the construction of gallic acid equivalent (GAE) standard curve, 10 mg of gallic acid (Sigma-Aldrich) was dissolved in 100 mL of 50% MeOH to yield (100 µg/mL) of stock solution and further diluted into 0, 6.25, 12.5, 25 and 50 µg/mL of working solutions. One mililitre of each working solution was added into 10 mL of distilled water. Then 1.5 mL of Follin & Ciocalteu's (Sigma-Aldrich) reagent was added into each working solution and incubated at room temperature for five minutes, followed by addition of four milliliter of 20% w/v Na_2CO_3 (Sigma-Aldrich). Then, distilled water was added to bring to 25 mL volume and the assay was left to stand for 30 minutes at room temperature. The absorbance value of standard assays was measured at 765 nm using UV-vis spectrophotometer. Gallic acid standard calibration curve was constructed by plotting absorbance against known concentrations of gallic acid.

For the estimation of TPC of the fermented CPL, 20 mg of dried ME was added into 10 mL of 50% MeOH. Then, 1 mL of mixture was added into 10 mL of distilled water and 1.5 mL of Follin & Ciocalteu's reagent followed by incubation for five minutes at room temperature. Next, four mililitre of 20% w/v Na_2CO_3 was added into the mixture followed by addition of distilled water to bring 25 mL volume. The assay was left for 30 minutes at room temperature prior to absorbance reading at 765 nm. All readings were done in triplicate.

Total phenolic content (TPC) of samples was calculated in terms of mg gallic acid equivalent per dry mass (dm) of ME in gram (g) of ME i.e. mg GAE g^{-1} [19].

2.4 Antioxidant Activity

The antioxidant capacity was estimated according to Vuong *et al.* [20] with some modifications. For the construction of standard calibration curve, 10 mg of (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (Sigma-Aldrich) as antioxidant model, was added into 20 mL of absolute methanol and further diluted into 0 to 800 uM of working solutions. Meanwhile, the free radical model was assayed by dissolving 24 mg of DPPH (Sigma-Aldrich) in 100 mL of MeOH and stored at -20 °C until needed. The DPPH working solution was prepared by mixing 10 ml of stock solution with 45 mL of MeOH which gave 1.1 ± 0.02 absorbance unit at 515 nm with UV-vis spectrophotometer.

Free radical scavenging activity was assayed by mixing 0.15 mL of each working solution of Trolox with 2.85 mL of DPPH solution. The reaction of Trolox and DPPH was allowed for 24 h under darkness absorbance reading at 515 nm. Free radical scavenging activity of ME was assayed by dissolving 20 mg of dry ME in 10 mL of methanol. Then, 0.15 mL of sample was added into 2.85 mL of DPPH assay. Finally, the absorbance reading at 515 nm was taken using UV-vis spectrophotmeter after 24 h incubation under darkness. All readings were done in duplicate. It was expressed in terms of milimolar Trolox equivalent (mM TE) per dry mass (dm) in gram (g) of ME i.e. mM TE g^{-1} dm.

2.5 Microbial Plating

Exactly 0.1 mL of broth collected at day 0, 2, 5, 10, 20, 40, 60, 75, 90 and 100 was homogenized in 0.9 mL of sterile saline-peptone water, then serially diluted into appropriate dilution factors and cultivated onto the following selective media in duplicate: Man Rogosa Sharpe agar (MRS) for lactic acid bacteria (LAB), plate count agar (PCA) for total bacteria, Dichloran Bengal Chloramphenicol (DRBC) agar for yeasts and MacConkey agar for *Enterobacteriaceae*. Each agar solution was supplemented with L. cysteine of 0.05% w/v as reducing agent to improve the anaerobic condition. Cultivated MRS, PCA and DRBC media were incubated at 30 °C for 1-2 days, while MacConkey medium was incubated at 37 °C for 1-2 days in candle jar. After incubation, the number of viable colonies on the media plates were enumerated.

2.6 PCR-DGGE

The total genomic DNA of the microorganisms from each broth sample was extracted using bead beating method according to protocol by GenElute™ Soil DNA Isolation Kit (Sigma). Prior to DGGE, PCR amplification and purification of bacteria and yeast were carried out.

Amplification of V3 region of 16S rRNA gene of bacterial DNA by PCR was carried out according to Muyzer *et al.* [21] and Shobaky & Montet [22] using a set of universal primer; forward gc338f (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3') and reverse 518r (5'-ATT ACC GCG GCT GCT GG-3') resulting approximately 200-250 bp DNA fragments. Forty GC nucleotide length (underlined) was clamped to 5' position of the forward primer to retain partial double stranded structure of DNA fragment. Each PCR mixture of 50 µl volume consisted of 5 µl of DNA template, 0.5 µM of each primer, 25 µl of REDiant 2× mastermix (1st BASE) which comprised of reaction buffer, 0.06 U/µl of Taq DNA polymerase, 3 mM $MgCl_2$ and 400 µM of each dNTPs and nuclease-free water. Each reaction was carried out according to Chanprasartsuk *et al.* [23] using a thermocycler (Applied Biosystems Veriti) at 10 cycles of

denaturation at 95 °C for 1 min, followed by touchdown annealing temperature by 1 °C from 65 °C to 55 °C after each successive cycle for 1 min and elongation at 72 °C for 3 min. Additional 20 cycles were carried out at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min. Initial denaturation and final extension were carried out at 95 °C for 1 min and 72 °C for 10 min respectively. PCR products were purified using FavorPrep PCR Purification Kit (FAVORGEN). About 5 µl of PCR products were analysed on 2%(w/v) agarose gel in 1 × TAE buffer.

The amplification of D1/D2 region of 26S rDNA gene of yeast DNA was carried out in two-step nested PCR, according to Mills *et al.* [24]. The first PCR used a set of universal yeast primer, forward NL1 and reverse NL4 (5'-GGTCCGTGTTTCAAGACGG-3') resulting approximately 600 bp amplicons. The reactions were carried out at denaturation at 95 °C for 1 min, annealing at 52 °C for 45 s and extension at 72 °C for 1 min for 30 cycles. Initial denaturation and final extension were carried out at 95 °C for 5 min and 72 °C for 7 min respectively. The amplicons was later purified using FavorPrep PCR Purification Kit (FAVORGEN) prior to be used as DNA template for reamplification using a set of GC-clamp (underlined) NL1 forward primer (5'-GCGGGCCGCGCGACCGCCGGGACGCGCGAGCCGGCGGCGGGCCATATCAATAAGCGGAGGAAAAG-3') and reverse primer LS2 (ATTAAACAACTCGACTC) resulting approximately 200-250 bp amplicons. The reactions were carried out according to Bae [25, 26] at denaturation at 95 °C for 1 min, annealing at 52 °C for 2 min and extension at 72 °C for 2 min for 30 cycles. Initial denaturation and final extension were carried out at 95 °C for 5 min and 72 °C for 7 min respectively. The PCR mixing conditions and PCR product purification were similar to bacterial analysis.

The VS20WAVE-DGGE (Cleaver Scientific Ltd) was used for sequence specific separation of PCR products of both bacteria and yeast samples. Electrophoresis was performed using 1.0 mm thick 8%(w/v) polyacrylamide gel (acrylamide-bisacrylamide [37.5:1]) containing denaturing gradient of 30 to 60% of urea and formamide (100% corresponds to 7 M urea and 40%(w/v) formamide), increasing in the direction of the electrophoretic run. Electrophoresis was performed at 130 V for 4 h at constant temperature of 60 °C. After electrophoresis, the gel was stained with SYBR®Safe staining dye for 30 min and the resulting DNA bands were analysed using a gel documentation system (Bio-Rad's Gel Doc XR+). Subsequently, selected DGGE bands were excised using sterile razor blade and the DNA of each band was eluted in 20-50 µl of 0.1×TE buffer solution and overnight incubation at 4 °C [25]. Next, for both bacterial and yeast DNA, 5 µl of eluted DNA was re-amplified using similar PCR conditions described earlier using same primer sets minus the GC-clamp of the respective forward primer.

2.7 DNA Sequencing Analysis

The PCR products from both methods were submitted to Sanger sequencing service (1st BASE Laboratory, Selangor, Malaysia) using the same primers of earlier PCR amplifications. The sequence identities were determined using BLASTn search from National Center for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.0 RESULTS AND DISCUSSION

3.1 TPC and Antioxidant Activity

Figures 1 (A) and (B) illustrates the changes of TPC and DPPH scavenging capacity between unfermented and fermented CPL extracts. The final extract (from day 90 of fermentation) exhibits higher value in both TPC and DPPH scavenging capacity i.e. 31.14 mg GAE g^{-1} dm and 405.8 mM TE g^{-1} dm of TPC and DPPH scavenging activity respectively in comparison to the fresh extract (day 0) i.e. 5.71 mg GAE g^{-1} dm and 130.5 mM TE g^{-1} dm respectively.

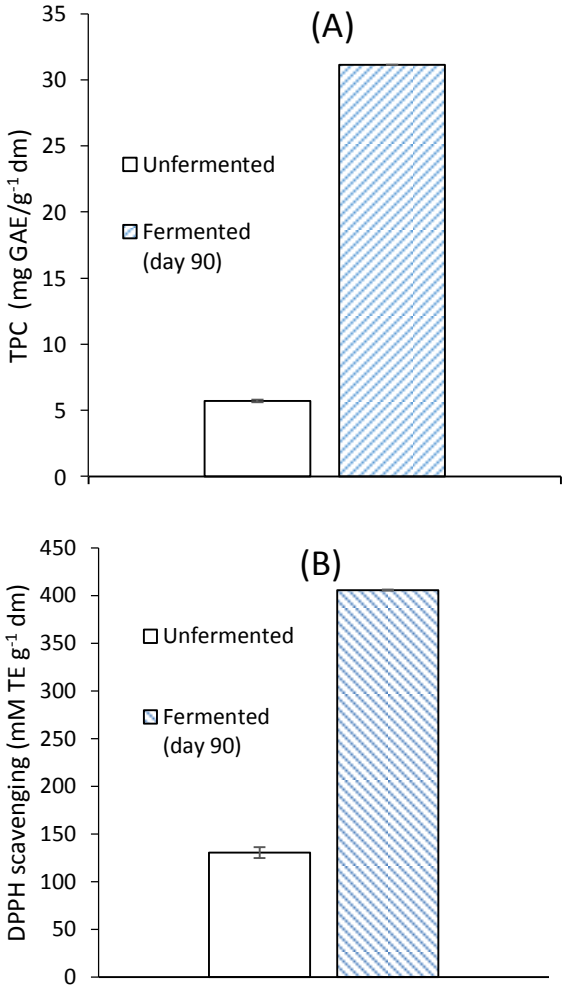


Figure 1 Changes of total phenolic content (A) and antioxidant activity (B) along fermentation time

The favourable effect of spontaneously fermented CPL was in accordance to several lactic acid fermentation of other plants, e.g. five-time higher total phenolics of fermented *Myrtus communis* berries [9] and *Echinacea spp*. [27] extracts, as compared to their respective unfermented extracts. The fermented materials also demonstrated better antioxidant and antibacterial functionalities in those studies. The benefit of fermentation was also demonstrated by fermented Cactus Cladodes (*Opuntia ficus-indica L.*) in terms of enhanced antioxidant and immune-modulation properties [10], while spontaneously fermented Cornelian cherry (*C. mas.* L.) exhibited high TPC and antioxidant activity [17]. Through enzymatic action, the role of microorganisms such as *Lb. plantarum* (to be discussed in later section) during fermentation may have caused the breakdown of glycosidic and ester bonds of polymeric phenols into free monomers, hence boosting the phenolic contents and antioxidant capacity [9]. This view was coherent with the predominance of phenolic acids and other flavonoids in spontaneously fermented Cornelian cherry as a result of metabolic activities during fermentation [17] and also shared by fermented papaya for its improved bioactivity [7]. However, contradictory outcomes of antioxidant capacity were reported on spontaneously fermented leek and spider flower (*Gyanandropsis gynandra*), where lower antioxidant capacity of the fermented parts as compared to their initial materials was blamed on the rise of certain flavonoids which negatively impacting the electron transfer mechanism of DPPH, respectively [13, 14].

3.2 Microbial Population Dynamic

Microbial population dynamics of presumptive LAB, yeasts and *Enterobacteriaceae* during fermentation is depicted in Figure 2. Presumptive number of LAB (to be identified in later section) on MRS grew exponentially since the initial phase of fermentation to reach maximum (10^9 CFU/mL) at day 5, then fluctuating (between 10^4 to 10^6 CFU/mL) towards the end of the fermentation. Presumptive yeast in DRBC was present throughout fermentation time, where it reached maximum (10^8 CFU/mL) at day 30, then fluctuating between 10^4 to 10^6 CFU/mL towards the end of fermentation. Presumptive *Enterobacteriaceae*, harboring potential foodborne pathogens were only detected during first week of fermentation and disappeared afterwards.

The initial pH was initially 9.0, then rapidly dropped to 6.0 at day 2. The rapid acidification could be correlated with exponential growth of presumptive LAB observed earlier due to the accumulation of lactic acid [28]. Afterwards, the pH drop continued, until it reached the final value of pH 3.0 after 90 day.

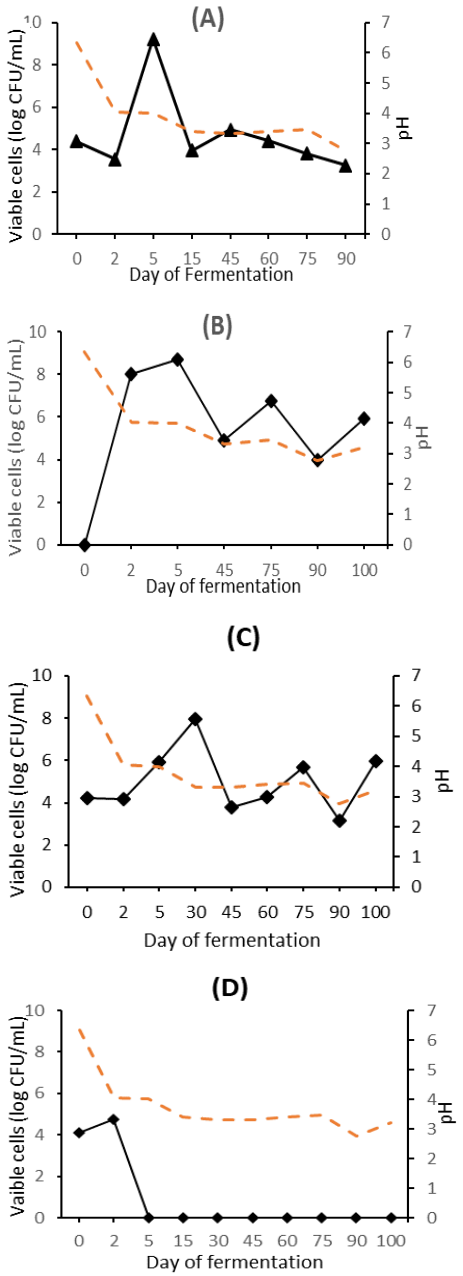


Figure 2 Variation of viable cell counts (solid line) on various selective medium; PCA (A), MRS (B), DRBC (C) and MacConkey (D) selective media along fermentation time. pH (dashed line)

The rapid growth of presumptive LAB was also observed during spontaneous fermentation of leek [29], sauerkraut [11], cocoa bean [30], and carrot juice [15]. LAB also demonstrated steady population throughout the fermentation time in those cases. It confers benefits to the fermented product in many ways; suppressing the growth of spoilage, thus prolonging the product's shelf-life and improving the product's flavor. On the other hand, the population dynamic of undesirable *Enterobacteriaceae*, where they were only observable at initial phase of fermentation agreed well with the spontaneous

fermentation of other vegetables (carrot juice, caper berry, leek, sauerkraut, eggplant and cucumber) [31, 32].

3.3 PCR-DGGE Fingerprinting

PCR-DGGE is a culture-independent method which can provide a better representation of microorganism fingerprints of a fermentation than traditional plating method. Eight distinct DGGE bands, shown in Figure 3 which represents the microbial community throughout fermentation age, were selected for 16S rDNA sequencing and later compared with NCBI genbank. All selected bands displayed 99-100% similarity with NCBI database where they all belonged to a single *Lactobacillus* genus and *Lb. plantarum* species as presented in Table 1.

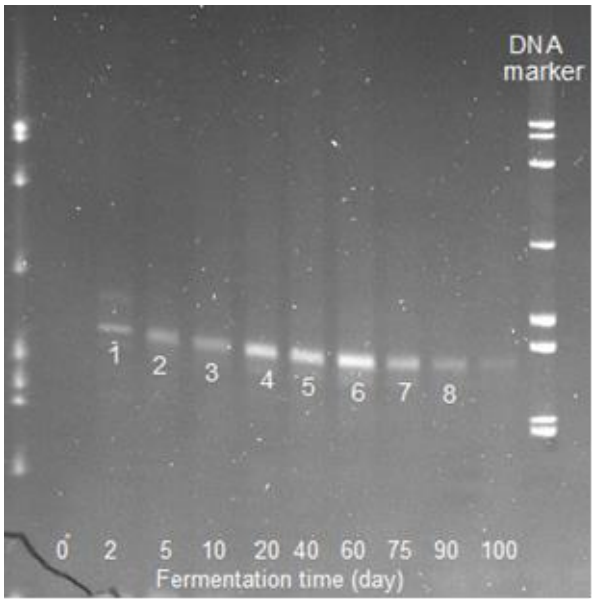


Figure 3 DGGE fingerprints of V3 region of 16S rDNA gene of bacteria

Table 1 Sequencing results of selected DGGE bands in Figure 3

Band no.	Closest relative	[a]Source	[b]Similarity (%)
1	*Lb. plantarum*	CP017374	99
2	*Lb. plantarum*	KT626385	99
3	*Lb. plantarum*	KT626385	100
4	*Lb. plantarum*	KT626385	100
5	*Lb. plantarum*	KT626385	100
6	*Lb. plantarum*	KT626385	100
7	*Lb. plantarum*	KT626385	100
8	*Lb. plantarum*	KT626385	100

[a]NCBI accession no. from https://blast.ncbi.nlm.nih.gov/Blast.cgi
[b]number of identical base/total length of DNA sequence

Lactic acid bacteria (LAB) is the hallmark of a nutritious and functional fermented food [33]. Among diverse group of LAB, *Lb. plantarum* species, such as the one detected in fermented CPL, characterizes "generally recognized as safe" (GRAS) and qualified presumption of safety (QPS) status of fermented food products [34]. Implicitly, the prevalence of *Lb. plantarum* sufficiently confers the safety of fermented CPL. In addition to its role in the safety of the fermented product, LAB or *Lb. plantarum* in particular, act as probiotics which prevent infection in gastrointestinal tract (by inhibiting pathogen adhesion on the intestinal lines), possess immunomodulation, antioxidant, antimicrobial, antifungal and antimutagenic functionalities [35] as well as treating various chronic diseases such as cardiovascular disease, diabetes, cancer, Alzheimer and Parkinson [34]. The presence of *Lb. plantarum* in the fermented CPL was in agreement with *sauerkraut* (fermented cabbage) which was produced by spontaneous fermentation method, although the latter reported a more diverse LAB presence such as the ones belonging to *Weisella* and *Leuconostoc* genera [11]. Such diverse microflora was also ubiquitous in *kimchi* (Korean traditional fermented cabbage) [36], leek [29] and spontaneously fermented carrot juice [15]. Diverse species of LAB (*Lb. fariminis, Lb. fermentum, Lb. namurensis, Lb. paralimentarius* and *Lb. plantarum*) was also reported during spontaneous fermentation of Indonesian *sayur asin* (fermented mustard), where *Lb. plantarum* was the most dominant [26]. The lack of LAB diversity in the fermented CPL is a subject of future investigation, particularly the absence of salt addition during its fermentation, since it was a key step during spontaneous fermentation of other aforementioned products.

The prevalent of yeast population throughout CPL fermentation was in accordance to spontaneous fermentation of cocoa bean [30] and pineapple juice [23], while differed from leek fermentation [29] and carrot juice [15] where the growth of yeast was only observable at initial fermentation. This discrepancy was caused by different fermentation methods between the two; cocoa bean and pineapple juice were single-stage where the fermented materials were mixed with natural inoculum since the first day, whereas the fermentation of leek was preceded by brine fermentation of fermentation. Furthermore, competitive interaction between bacteria and yeast could inhibit the growth of either component [37].

The selected DGGE bands of yeast, shown in Figure 4 revealed four genera belonging to five species; *Zygosaccharomyces rouxii, Saccharomyces cerivisiae, Candida glabrata, Aspergillus oryzae* and *Aspergillus flavus* as presented in Table 2. Generally, yeast influences the sensory quality and flavour of the fermented food by producing vast array of aromatic, volatile metabolites such as higher alcohols, organic acids, esters, aldehydes, ketones, etc. [30]. For example, *Saccharomyces cerevisiae* was instrumental to chocolate flavor produced from spontaneously fermented cocoa bean [30]. It is also

common that *Saccharomyces cerevisiae,* along with non-*Saccharomyces* yeasts such as *Hanseniaspora, Pichia, Candida, Clavispora, Rhodotorula, Saccharomycopsis, Torulaspora, Metschnikowia, Issatchenkia and Geotrichum* present in the spontaneously fermented palm, pineapple and orange juices, apple cider and mangosteen paste [23]. *Saccharomyces cerevisiae* and *Z. rouxii*, where both were present during spontaneous fermentation of CPL, produced higher alcohols during fermentation of soy sauce [38]. *Candida spp.* and *Saccharomyces spp.* were among the prevalent yeast species in spontaneously fermented *kimchi* [25] while *Aspergillus oryzae* was instrumental part of koji fermentation for good quality soy sauce [38].

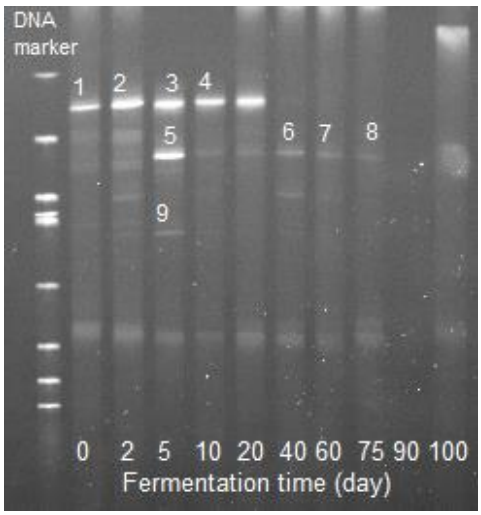


Figure 4 DGGE fingerprints of D1/D2 region of 26S rDNA gene of yeast

Table 1 Sequencing results yeast species from selected DGGE bands in Figure 4

Band no.	Identity	[a]Source	[b]Similarity (%)
1	*Zygosaccharomyces rouxii*	LT631808	97
2	*Zygosaccharomyces rouxii*	LT631808	99
3	*Saccharomyces cerevisiae*	KX428530	100
4	*Saccharomyces cerevisiae*	KY400198	100
5	*Candida glabrata*	KF880794	97
6	*Aspergillus oryzae*	MH997652	97
7	*Aspergillus oryzae*	MH997653	97
8	*Aspergillus flavus*	MH997655	97
9	*Candida glabrata*	KM103010	99

[a]NCBI accession no. from https://blast.ncbi.nlm.nih.gov/Blast.cgi
[b]number of identical base/total length of DNA sequence

4.0 CONCLUSION

Spontaneous fermentation has successfully enhanced the TPC and antioxidant capacity of the CPL. The process also cultivated *Lb. plantarum*, a prominent LAB which may deliver probiotic functionality. The safety of fermented CPL was conferred by the disappearance of *Enterobacteriaceae*, which only present briefly at the initial phase of fermentation as shown by microbial population dynamic results, and later undetected by PCR-DGGE fingerprinting. Fermented CPL may potentially has better sensory quality due to the presence of diverse yeast community which frequently present in many successful fermented foods to overcome the unpleasant, bitter taste of conventional CPL products. Further works are necessary to identify the phenolic and sugar metabolites, organic acids, alcohols etc. to elucidate their impacts on the functionality and sensory of the fermented CPL.

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