Beneficial effects of spontaneous fermentation in enhancing the phenolic contents, antioxidant activity and cultivation of lactic acid bacteria of *Carica papaya* leaf

M S So’aib1*, K H K Hamid2, J Salihon2, H L Tan2 and A. Hamid3

1Faculty of Chemical Engineering, Universiti Teknologi MARA, Cawangan Pulau Pinang, 13500 Permatang Pauh, Pulau Pinang, Malaysia
2Faculty of Chemical Engineering, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia

*Corresponding author’s email: sufian5129@uitm.edu.my

Abstract. Spontaneous fermentation using endogenous microorganisms is a reliable method for enhancing the bioactivity of medicinal plant. Here, the method was applied on *Carica papaya* leaf (CPL) for the first time to enhance the phenolic contents, antioxidant activity, and cultivation lactic acid bacteria. The antioxidant activity was estimated in terms of 2,2-diphenyl-1-picryl-hydrazyl DPPH scavenging activity. Meanwhile, the microorganism fingerprinting was carried out by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) method. Total phenolic content and antioxidant activity of fermented CPL (day 90) were 45.64±0.69 mg GAE/g dm and 467.38±4.09 mM TE/g dm, respectively, higher than 12.13±0.69 mg GAE/g dm and 275.46±3.09 mM TE/g dm, respectively of control, unfermented CPL. The cultivation of lactic acid bacteria (LAB) was marked by its viable cell counts between 10^3 to 10^7 colony forming unit (CFU)/mL on De Man, Rogosa and Sharpe (MRS) agar medium and the fingerprints of *Weisella*, *Lactobacillus* and *Lactococcus* species on PCR-DGGE throughout the fermentation period.

1. Introduction

*Carica papaya* Linn., also known as papaya or paw belongs to the family of *Caricaceae* and *Plantae* kingdom. It is originally from southern Mexico and now widely distributed in tropical region [1]. Various parts of *Carica papaya* are known to treat various illnesses and diseases. The fruits, roots, seeds and latex have been used in folk medicines to treat myriad diseases and sicknesses such as dyspepsia, urinary problems, external burns and infertility [2]. Therapeutic properties of *Carica papaya* leaf (CPL) such as analgesic [3], anti-tumor [1], immunomodulatory [4], anti-inflammatory [5], anti-hyperlipidemic, antifungal, antimalarial and anti-dengue fever [6], thrombocytotic [7], antioxidant [8] and anti-diabetic [9] have been proven by numerous in vitro and in vivo studies. These properties are contributed by the alkaloids (e.g. carpaine, pseudocarpaine, dehydrocarpaines I and II) flavonoids (e.g. quercetin 3-(2G-rhamnosylrutinoside), kaempferol 3-(2G-rhamnosylrutinoside), quercetin 3-rutinoside and kaempferol [10].

The limitation of dietary polyphenols, of which many of the bioactive compounds contained in CPL originate, is their limited digestion in digestive tract. Only small fraction (5-10%) of low molecular weight polyphenols are readily absorbed at the small intestine, whereas large molecular weight polyphenols rely on colonic microbiota for breakdown and eventually absorbed at the colon [11]. Previous study showed the beneficial effect in breaking down large polyphenols into low...
molecular weight phenolic acids, such as the application of *Enterococcus faecalis* and *Aspergillus oryze* strains during fermentation of papaya fruit which improved the immune system and modulated the gut microbiota of the consumers [12]. Fermentation using *Colletotrichum gloeosporioides* (EYL131) and *Neosartorya spathulata* (EYR042) fungi had been applied on mangosteen’s a-mangostin extract to enhance its bioactivity [13]. Numerous lactic acid fermentation using lactic acid bacteria (LAB) strains was reportedly enhancing the functionalities of several phenolic-rich plants such as of *Myrus communis* berries [14], cactus pear (*Opuntia ficus-indica* L.) [15] and *Echinacea* spp [16]. In addition, a fermentation technique known as spontaneous fermentation has been applied on spider flower (*Gynandropsis gynandra*) [17], leek [18], carrot juice [19], garlic [20] and cornelian cherry [21] which resulted enhanced bioactivities and cultivation of health-promoting microorganisms of the respective material.

In view of the benefit of fermentation in enhancing the functionality of fermented materials, spontaneous fermentation which employs indigenous microorganisms was chosen as the fermentation method of CPL [22-23]. Nevertheless, its impact of the spontaneous fermentation method on the total phenolic content and antioxidant activity of the CPL, as well as the microorganisms profile in the fermentation ecosystem were unknown, thus were investigated in this study.

## 2. Materials and methods

### 2.1. Fermentation

Fresh CPL was collected from a plantation in Banting, Selangor, Malaysia. It was washed to remove physical dirt and shredded into smaller pieces using kitchen blender. Later, it was loaded into 7 L benchtop fermenter (INOFRS) at 10 %w/v. Then, the distilled water was added to bring to 5 L working volume with 10 %w/v of unrefined sugar as initial substrate. The spontaneous fermentation was carried out in an anaerobic condition for 90 days at room temperature. Broth samples were collected at day 0, 2, 4, 6, 8, 15, 30, 30, 45, 60, 75 and 90 for phytochemical and microbiological analyses. The pH of broth sample of each sampling time was measured by pH probe (Mettler-Toledo).

### 2.2. Total phenolic content (TPC) and antioxidant activity

Fifteen millimetre (15 mL) of fermented CPL suspension were collected at day 90 of the fermentation. Solid debris was removed by centrifuging the suspension at 10,000 g for 20 min at 4°C. Then, the supernatant was dried using rotary evaporator at 30°C for 45 min to remove water content and later re-suspended with 80% of methanol (MeOH) at 1:1 (v/v) to yield methanolic extract (ME). Further drying by recentrifuging and nitrogen purging were implemented [14, 24].

TPC was estimated using Foulin Ciocalteu’s method [25]. Ten mg of gallic acid standard (Sigma-Aldrich) was dissolved in 100 mL of 50% MeOH (100 μg/mL) of stock solution and further diluted into 0, 6.25, 12.5, 25 and 50 μg/mL of working solutions. Then, the samples were incubated at room temperature for 5 min followed by the addition of 4 mL of 20% w/v Na₂CO₃ (Sigma-Aldrich). The absorbance value was measured at 765 nm using UV-vis spectrophotometer (Shimadzu) against blank (distilled water). Gallic acid standard curve was constructed by plotting absorbance of assay against known concentrations of gallic acid standard. Similarly, ME was dissolved in MeOH and its absorbance taken. All readings were done in triplicate and calculated as average±standard deviation.

Total phenolic content (TPC) of assay was expressed as mg gallic acid equivalent per ME dry mass (mg GAE/g dm) as shown in Equation (1) [24]:

\[
TPC = \frac{C \times V \times D}{W}
\]

where \(C\) is the concentration of gallic acid (mg/mL), \(V\) is volume of extract solution (mL) and \(W\) is mass of dried ME (g).

The antioxidant activity was estimated based on scavenging activity of fermented CPL extract against DPPH (Sigma-Aldrich) as free radical model. Standard calibration curve was prepared by
dissolving 10 mg of Trolox as antioxidant reference in 20 mL of MeOH and further diluted into 0 to 150 μg/mL (0 to 600 μM) of working solutions. For DPPH scavenging assay, 0.15 mL of Trolox assay was mixed with 2.85 ml of DPPH assay. The reaction of Trolox and DPPH was allowed for 24 hr of incubation under darkness followed by absorbance reading at 515 nm using UV-vis spectrophotometer. Similarly, ME was dissolved in MeOH and its absorbance measured. All readings were done in triplicate and calculated as average ± standard deviation. DPPH scavenging activity was expressed as μM Trolox equivalent (TE) per g of dried ME (mM TE/g dm) as shown in Equation (2) [24]:

\[ TE = \frac{C \cdot V \cdot D}{W} \]  

(2)

where \( C \) is the concentration of Trolox (mM), \( V \) is volume of extract solution (mL), \( D \) is dilution factor and \( W \) is mass of dried ME (g).

2.3. Microfloral population dynamics

Exactly 0.1 mL of broth collected at each sampling time 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. The Man Rogosa Sharpe agar (MRS) for lactic acid bacteria (LAB) and MacConkey agar for enterobacteria were used. All media were incubated at 30-37 °C for 1-2 days in candle jar.

2.4. PCR-denaturing gradient gel electrophoresis (DGGE) fingerprinting

Each broth sample was collected at day 0, 2, 4, 8, 15, 30, 33, 45, 60, 75 and 90. It was subjected to the total genomic DNA extraction using bead beating method according to protocol by GenElute™ Soil DNA Isolation Kit (Sigma). PCR amplification of V3 region of 16S rRNA gene of bacterial DNA was carried out 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 ~200-250 bp DNA fragments [26, 27]. Each reaction was carried out using a conventional thermocycler (Eppendorf Mastercycler) 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 VS20WAVE-DGGE (Cleaver Scientific Ltd) was used for sequence specific separation of PCR products DNA. 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 hr at constant temperature of 60 °C. After electrophoresis, the gel was stained and the selected DGGE bands were excised using sterile razor blade, eluted and reamplified.

2.5. DNA sequencing analysis

The PCR products of bacteria were submitted to Sanger sequencing service (1st BASE Laboratory, Selangor, Malaysia). The sequence identities were determined using BLASTn search from National Center for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [28].
3. Results and discussion

3.1. Total phenolic content and antioxidant activity

Total phenolic content of fermented CPL extract was 48.42±0.31 mg GAE/g dm, i.e. four-time higher than the control, unfermented extract, i.e. 12.13±0.39 mg GAE/g dm as shown in Figure 1(a). The TPC of fermented CPL was also higher compared to the unfermented CPL aqueous extract (28.61±0.33 mg GAE/g) and CPL methanol extract (15.03±0.39 mg GAE/g) reported by other researcher [25]. The enhanced TPC found in this study was in line with favourable effect of fermentation reported by other studies involving phenolic rich plant materials. Five-fold increase of TPC was reported on of fermented Myrtus communis [14] as well as fermented Echinacea spp. [16], albeit at a lesser degree in the latter.

Antioxidant activity is the hallmark of various health-promoting functionalities against cancer, obesity, cardiovascular disease and other chronic diseases. The antioxidant property of fermented CPL extract was measured in terms of its ability to scavenge DPPH radical which reduced the coloured DPPH to DPPH-H, the latter was stabilised in the absence of an antioxidant or hydrogen donor [15]. The DPPH scavenging activity of fermented CPL extract was higher than control, i.e. 467.38±4.09 mM TE/g dm and 275.46±3.09 dm, respectively as shown on Figure 1(b). This finding was in accordance to the benefit of fermentation on antioxidant activity as reported by other studies such as Myrtus communis [14], cactus cladodes (Opuntia ficus-indica L.) [15] and Echinacea spp [16]. Moreover, the DPPH scavenging activity of final fermented CPL extract was higher than CPL aqueous extract obtained i.e. 0.042 mM TE/g reported by other study [25]. Nonetheless, studies on spontaneously fermented spider flower (Gyanandropsis gynandra) [17] and leek [18] reported lower antioxidant capacity of spontaneously fermented than their unfermented extracts, respectively which was caused by the rise of certain flavonoids which inhibited the electron transfer mechanism of DPPH.

The increased of TPC, and correspondingly the antioxidant activity of the spontaneously fermented CPL may had been caused by the enzymatic breakdown of polymeric phenols into smaller, free phenols by wide-ranging enzyme portfolio of the microorganisms which involved during fermentation [14-16]. Thus, more free phenol molecules reacted with the reagents than their native compounds which were originally cryptic by strong chemical bonds such as glycosidic and ester bonds [14].

![Figure 1](image-url)  
**Figure 1.** Changes of a) total phenolic content and b) antioxidant activity between control CPL and fermented CPL.

3.2. Microbial population dynamics

CPL fermentation was characterized by steep growth of presumptive lactic acid bacteria (LAB) (on MRS medium) from $10^2$ CFU/mL at day 1 to $10^7$ CFU/mL at day 4 through day 15, as shown in
Figure 2(a). However, LAB population declined at day 30 and finally settled at $10^4$ CFU/mL throughout the remaining fermentation phase. The population dynamic of LAB was probably influenced by the conversion of substrates; sugar and phenolic compounds into various metabolites, as observed during spontaneous fermentation on other materials [29]. The presumptive enterobacteriaceae showed the highest growth of $10^5$ CFU/mL at day 2, then briefly disappeared at day 4, re-appeared at day 5 to $10^5$ CFU/mL before decreasing to undetectable level from day 33 till the end, as shown in Figure 2(b). Such dynamic was probably caused by the synthesis of inhibitive compounds by the more prevalent LAB against enterobacteriaceae, as usually occurred during other spontaneous fermentation processes [30]. The pH level rapidly dropped from pH 6.0 at the onset of the fermentation to pH 3.0 at day 4 as shown in Figure 2, which coincided with the rapid growth of presumptive LAB population. The pH value persisted at pH 3.0 throughout the remaining of CPL fermentation which possibly caused by the accumulation of lactic by the prevalent LAB [31].

**Figure 2.** Microbial population dynamics (solid line) of sample cultivated on a) MRS (LAB) and b) MacConkey (Enterobacteriaceae). pH is represented by dashed line.

### 3.3. Microorganisms fingerprinting

PCR-DGGE is a reliable method to obtain a complete fingerprints microbial community in fermentation ecosystem by resolving the amplified total DNA of the microorganisms into sequence-specific bands. The sequencing of selected 27 bands of DGGE is shown in Figure 3. The nucleotide sequence similarity searches at NCBI BLAST yielded a total of eight genera and 16 species is shown in Table 1. Major bands such as band no. 2, 6, 10 and 16 were identified as *Lactococcus lactis* where its stronger band intensity between day 2 to 15 of fermentation suggested its higher population at the onset of fermentation. Additionally, other major bands belonging to *Weisella* sp. and *Weisella cibaria*, *L. lactis*, *L. lactis subsp. cremoris*, *L. lactis subsp. lactis* and *Lb. plantarum* suggested the prevalence of LAB throughout the fermentation process as was marked by their relatively stronger band intensity. Their prevalence was at peak between day 15 and 45 and apparently outcompeted the presence of undesirable enterobacteriaceae (band no. 14, 15, 20, 21, 22 and 23) in which the latter was not detected during this period, as previously shown in Figure 2(b). LAB maintained a relatively strong presence towards the end of fermentation as previously shown in Figure 2(a) as indicated by the presence of *Lb. fermentum* and *Lactobacillus* sp. at day 60 and 75, respectively.
Figure 3. DGGE fingerprints of V3 region of 16S rDNA gene of bacteria.

Table 1. Identities of selected DGGE bands.

| Band no. | Closest relative                        | "aSource" | "bSimilarity (%)" | Band no. | Closest relative                        | "aSource" | "bSimilarity (%)" |
|----------|-----------------------------------------|-----------|-------------------|----------|-----------------------------------------|-----------|-------------------|
| 1        | Lactic acid bacteria                    | Weissella sp. | AY421816         | 3        | Klebsiella sp.                         | FJ784669  | 88                |
| 5        | Weissella cibaria                       | JN851746  | 99                | 4        | Cronobacter malonicus                  | MF118664  | 99                |
| 9        | Weissella cibaria                       | MF164058  | 99                | 12       | Cronobacter sakazakii                  | KY652865  | 99                |
| 13       | Lactobacillus plantarum                 | KT626385  | 100               | 8        | Cronobacter sakazakii                  | MF118630  | 99                |
| 18       | Lactobacillus plantarum                 | AB973314  | 99                | 15, 23   | Cronobacter sakazakii                  | KU364491  | 99                |
| 19       | Lactobacillus plantarum                 | KT626386  | 100               | 17, 21, 27| Cronobacter sakazakii                  | KY652865  | 98                |
| 24       | Lactobacillus acid fermentation         | HE858541  | 94                | 7        | Enterobacter ludwigi                   | KY974384  | 99                |
| 26       | Lactobacillus sp.                       | LT678435  | 88                | 20       | Enterobacter sp.                       | KT933254  | 98                |
| 2, 10    | Lactococcus lactis                     | LT853603  | 98                | 22       | Enterobacter sp.                       | KT933254  | 99                |
| 6        | Lactococcus lactis subsp. cremonis      | CP003157  | 100               | 11       | Enterobacter hormaechei                | HQ322395  | 98                |
| 16       | Lactococcus lactis subsp. lactis        | JN851797  | 100               | 14       | Kluyvera georgiana                     | KY458538  | 94                |
|          | Enterobacteria                          |           |                   |          | Escherichia vulneris                   | KP318474  | 98                |
The prevalence of LAB was reported in some spontaneously fermented foods such as sauerkraut [32], kimchi [33], pickles [17], carrots [19], pineapple [34], tempoyak [28], doklu [35], cocoa bean [30] and leek [18]. LAB species such as Lactobacillus, Lactococcus, and Weisella, of which detected in this study were known as health-promoting probiotics which prevent diarrhea and colorectal cancer, stimulate immune system and hypcholesterolemia effect [36]. Their presence also conferred the microbiological safety of fermented products by inhibiting food pathogens [37]. Such effect could be attributed to the disappearance of enterobacteriaceae after 30 days of fermentation as previously shown in Figure 2(b).

4. Conclusions
For the first time, application of spontaneous fermentation has successfully benefited the CPL properties in terms of enhancing its phenolic contents and antioxidant activity. Indirectly, the chosen method has also cultivated diverse LAB species, such as Weisella, Lactobacillus and Lactococcus species which potentially deliver an additional probiotic functionality. The microbiological safety of the fermented CPL was conferred by the disappearance of enterobacteriaceae after 30 days, which also coincided with the growth of pathogen-inhibiting LAB.

Acknowledgment
The authors gratefully acknowledge the Ministry of Education Malaysia for funding the research through 600-IRMI/FRGS 5/3 (188/2019) grant.

References
[1] Otsuki N, Dang N H, Kumagai E, Kondo A, Iwata S and Morimoto C 2010 J. Ethnopharmacol. 127 760–767
[2] Kovendan K, Murugan K, Kumar A N, Vincent S and Shiou Hwang 2012 Parasitol Res 110 669–678
[3] Hasimun P, Suwendar and Ermasari G I 2014 Procedia Chem. 13 147–149
[4] Canini A, Alesiani D, D’Arcangelo G and Tagliatesta P. 2007 J. Food Compos. Anal. 20 584–590
[5] Patil T, Patil S, Patil and Patil S 2014 Int. J. Pharmacogn. Phytochem. 6(2) 260–265
[6] Ismail Z, Halim S Z, Abdullah N R, Afzan A, Rashid B A A 2014 Evidence-Based Compl. Alt. Med. 2014 1-10
[7] Subenthiran S, Choong T C, Cheong K C, Thayan R, Teck M B, Munirandy P K, Afzan A, Abdullah N R and Ismail Z 2013 Evidence-Based Compl. Alt. Med. 2013 1-7
[8] Nugroho A, Heryani H., Cho J S and Park H J 2017 Asian Pac. J. Trop. Biomed. 7(3) 208–213
[9] Gheith I and El-mahmoudy A 2018 Biosci. Rep. 38 1–11
[10] Afzan A, Abdullah N R, Halim S Z, Rashid B A, Raja Semail R H, Abdulllah N, Jantan I, Muhammad H and Ismail Z 2012 Molec. 17 4326-4342
[11] Cardona F, Andrés-Lacueva C, Tulipania S, Tinahones F J, Queipo-Ortuño M I 2013 J. Nutr. Biochem. 24 1415–1422
[12] Fujita Y Tsuno H and Nakayama J 2017 PLoS One 12, 1 p. 1–19
[13] Arunrattiyakorn P 2011 Phytochemistry 72, p. 730–734
[14] Curiel J A, Pinto D, Marzani B, Filannino P, Farris G A, Gobbetti M and Rizzello C G 2015 Microb. Cell Fact. 14(67) 1-10
[15] Filannino P, Cavoski I, Thlien N, Vincentini O., De Angelis M., Silano M., Gobbetti M and Di Cagni R 2016 PLoS One. 11(3): 1–22
[16] Rizzello C G, Coda R, Macias D S, Pinto D, Marzani B, Filannino P, Giuliani G, Paradiso V M, Di Cagni R, Gobbetti M Microb. Cell Fact. 12(1) 1–15
[17] Muhiadlin B J Sukor R Ismail N Ahmad S W Me N C and Meor Hussin A S 2018 J. Pure Appl. Microbiol. 12, 2 p. 497–504
[18] Wouters D, Bernaert N, Conjaerts W, Van Droogenbroeck B, De Loose M and De Vuyst L 2013 Food Microbiol. 33(2) 185–196
[19] Wuyts S, Van Beeck W, Oerlemans EFM, Wittouck S, Claes I J J, De Boeck I, Weckx S,
Lievens B, De Vuyst L and Lebeer S 2018 *Appl. Environ. Microbiol.* 84(12) 1–16

[20] Kimura S Tung Y Pan M Su N Jang Lai Y and Chen Cheng K 2016 *J. Food Drug Anal.* 25(1) 62–70

[21] Kawa-Rygielska J Adamenko K Kucharska A Z and Piórecki N 2018 *Molecules* 23(2) 1–16

[22] Capozzi V, Fragasso M, Romaniello R, Berbegal C, Russo P and Spano G 2017 *Fermentation* 3(4) 1–19

[23] Holzapfel W H 2002 *Int. J. Food Microbiol.* 75 197–212

[24] Madaan R, Kumar S, Bansal G and Sharma A 2011 *Indian J. Pharm. Sci.* 73(6) 666

[25] Vuong Q V, Hirun S, Roach P D, Bowyer M C, Phillips P A and Scarlett C J 2013 *J. Herb. Med.* 3(3) 104–111

[26] Muyzer G, Dewaal E C and Uitterlinden A G 1993 *Appl. Environ. Microbiol.* 59(3) 695–700

[27] El Shoabaky A and Montet D 2015 *Egypt. J. basic Appl. Sci.* 2 327–333

[28] https://blast.ncbi.nlm.nih.gov/Blast.cgi

[29] Chuah L, Shamila-syuahada A K, Tze M, Rosma A, Lin K and Rusul G 2016 *Food Microbiol.* 58 95–104

[30] Moreira I M V, Miguel M G C P, Duarte W F, Dias D R and Schwann R F 2013 *Food Res Int.* 54 9-17

[31] Ho V T T, Zhao J and Fleet, G 2014 *Int. J. Food Microbiol.* 174 72–87

[32] Touret T, Oliveira M and Semedo-Lemsaddek T 2018 *PLoS One* 13(9) 1-16

[33] Jung J Y, Lee S H and Jeon C O 2014 *Appl. Microbiol. Biotechnol.* 98(6) 2385–2393

[34] Chanprasartsuk O O, Prakitchaiwattana C, Sanguandeekul R and Fleet G H 2010 *Bioresour. Technol.* 101(19) 7500–7509

[35] Assoumoun-djeni N M C, Djeni N T, Messaoudi S and Lhomme E 2016 *Food Control* 62 397–404

[36] Nuraida L 2015 *Food Sci. Hum. Wellness* 4(2) 47–55

[37] Dalié D K D, Deschamps A M, Richard-Forget F 2010 *Food Control* 21 370-380