Biodiversity analysis of spontaneously fermented *Garcinia mangostana* pericarp: From laboratory scale to pilot scale

M S So’aib1*, J Salihon2 and K H Ku Hamid2

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

*sufian5129@salam.uitm.edu.my

Abstract. Biodiversity analysis was carried out on *Garcinia mangostana* pericarp (GMP) which underwent spontaneous fermentation. Population dynamic was carried out using the plating method while genotyping employed gene sequencing of 16S rDNA (bacteria) and 5.8S-Internally Transcribed Spacer (5.8S-ITS) rDNA (yeasts). Throughout 90-day fermentation in 5-L fermenter (laboratory scale) ecosystem, the prevalence of lactic acid bacteria (LAB) and yeast were indicated by their viable cell counts at $10^3$ to $10^4$ colony forming unit per ml (CFU/mL) on MRS medium and $10^3$ to $10^7$ CFU/mL on PDA medium respectively, while the prevalence of LAB and yeasts in 50-L fermenter (pilot scale) ecosystem were marked by $10^3$ to $10^7$ CFU/mL and $10^2$ to $10^5$ CFU/mL on the respective medium. Complete inhibition of enterobacteriaceae population conferred a microbiological safety of the fermented GMP. Genotyping of isolates revealed Firmicutes and Proteobacteria in 5-L fermenter ecosystem, while Firmicutes and Proteobacteria were isolated from 50-L fermenter ecosystem. *Lactobacillus plantarum* and *Enterococcus faecalis* were amongst probiotic species isolated from 5-L and 50-L ecosystems respectively, whereas *Saccharomyces* yeasts were ubiquitous in both ecosystems.

1. Introduction

The bioavailability of native xanthones of the mangosteen pericarp in human metabolisms, such as its abundant α and γ-mangostins content is particularly low [1][2]. Such limitation was demonstrated by rapid conjugation of flavonoids and other polyphenols into for phase II metabolites in the form of glucuronide/sulfate conjugates rather than direct absorption of free xanthones by the first-pass metabolism at the intestine's enterocyte. This caused the polyphenols to take a longer route via enterohepatic recirculation before finally absorbed into the systemic system [2]. Additionally, the majority of undigested free xanthones in the upper gut have to rely on the colonic microbiome for conversion into more bioavailable and bioactive metabolites [3]. Realizing the important role of the colonic microbiome in the digestion of polyphenols, fermentation was used to enhance their bioavailability, such as α-mangostin fermentation using *Colletotrichum gloeosporioides* and *Neosartorya spathulata* fungal species [4] and the fermentation of whole mangosteen fruit by *Saccharomyces boulardii* starter culture [5]. Various fermentation techniques were also used on other polyphenol-rich plants such as radish (*Raphanus sativus* L.) [6], leek (*Allium ampeloprasum var.*...
porrum) [7], garlic (Allium sativum L.) [8], spider flower (Gynandropsis gynandra) [9] and cornelian cherry (Cornus mas L.) [10], resulting an enhanced bioactivities of the respective material.

To our knowledge, spontaneous fermentation has never been applied on mangosteen pericarp. The chosen process demonstrated the centuries-old spontaneous fermentation tradition which allows the complex ‘wild strain’ to colonize the materials and performs diverse metabolic functions and products in which available starter culture maybe in deficient as a result of different species, geography, cultivars and the farming practice of the mangosteen pericarp used [11].

In view of the benefit of the fermentation process, biodiversity analysis of the spontaneously fermented mangosteen pericarp was carried out in a laboratory (5-L) and pilot scale (50-L) capacities.

2. Materials and methods
2.1 G. Mangostana pericarp (GMP) fermentation
GMP was separated from the pulp of fresh mangosteen fruits obtained from a local market in Shah Alam, Malaysia. Unrefined sugar (10 %w/v) was added as substrate and purified water was added to make up 5-L and 50-L volumes of respective fermenters. The fermentation in each fermenter was carried out anaerobically for 90 days at room temperature. Sample collection was done at 0, 5, 10, 20, 30, 45, 60, 75 and 90 day of fermentation from each fermenter for analysis.

2.2 Enumeration of microorganism population.
At each sampling time, 0.1 ml of collected broth sample was homogenized in 0.9 ml (1:10 dilution) sterile peptone water, then serially diluted into appropriate dilution factors and cultivated onto several selective media in duplicates; plate count agar (PCA) for total bacteria, Man Rogosa Sharpe (MRS) agar for LAB, MacConkey (MC) agar for Enterobacteriacae and Potato-dextrose agar (PDA) for yeast. The cultivated plates were incubated anaerobically in candle jars at 30 °C for 1 to 2 days. After incubation, the viable microbial colonies were counted and expressed in terms of colony forming units (CFU) per millilitre of broth sample (CFU/mL). Three well-forming colonies from each medium were randomly selected, purified, enriched by overnight cultivation in selective liquid media and finally stored at -30 °C in 1.5 mL tubes containing 25% glycerol until further analysis.

2.3 DNA extraction and purification
The DNA extraction and purification of pure cultures were carried out using rapid DNA extraction kit (Wizard® Genomic DNA Purification Kit, Promega USA) according to the manufacturer's protocol.

2.4 PCR amplification
PCR amplification of V3 region of 16S rDNA gene of purified DNA extract was carried out using a pair of universal primers consisting of forwarding primer 27f (5'-AGAGTTTGTTCCTGTTACGACTT-3') and reverse primer 1492r (5'-TACGGYTACCTTGTTACGACTT-3'). Each reaction was carried out using a conventional thermocycler (Eppendorf Mastercycler) at 35 cycles of denaturation at 95 °C for 30 s, followed by annealing temperature at 55.5 °C for 30 s and elongation at 72 °C for 1.5 min. The initial denaturation and final extension were carried out at 95 °C for 5 min and 72 °C for 10 min respectively.

The 5.8S-Internally Transcribed Spacer (5.8S-ITS) rDNA region of fungal isolate DNA was amplified using ITS1 (5'-TCCGTAAGGTGAACCTGCGG-3') and ITS4 (5'-TCCCTCCGCTTATTGATAG-3'). Thermocycling program was set for 35 cycles of denaturation at 95 °C for 2 min followed by annealing temperature at 56 °C for 2 min and elongation at 72 °C for 2 min. The initial denaturation and final extension were set at 95 °C for 5 min and 72 °C for 10 min respectively.
3. Results and discussion

3.1 Microbial population dynamics
The microbial population dynamics of different microorganisms reveals the prevalence of LAB (Figure 1(b)) and inhibition of enterobacteriaceae (Figure 1(c)) for both 5-L and 50-L ecosystems. The presumptive LAB population in 5-L fermenter rose from absence at the fermentation onset (day 0) to a relatively steady population of $10^3$ to $10^4$ CFU/ml after the first week of fermentation, while it displayed a strong presence in 50-L fermenter since the start of fermentation and achieved a higher population ($10^6$-$10^7$ CFU/mL) at day 90 of fermentation. The complete inhibition of presumptive enterobacteriaceae was observed in both 5-L and 50-L fermenters, albeit it took 45 days in the 5-L fermenter ecosystem. Interestingly, the yeast population, as shown in Figure 1(d) displayed almost a similar pattern in both 5-L and 50-L fermenters ecosystem where it showed strong presence since the start of the fermentation and settled at $10^3$ to $10^4$ CFU/mL at day 90 of fermentation. The population dynamics of LAB was similarly observed during spontaneous fermentation of leek [7], sauerkraut [12], cocoa bean [13] and carrot juice [14], whereas the prevalence of yeast population throughout fermentation was in accordance to spontaneous fermentation of cocoa bean [13].

![Figure 1](image_url). Microbial population dynamics of sample cultivated on the following media (a) PCA (total bacteria) (b) MRS (LAB) (c) MacConkey (enterobacteriaceae), and (d) PDA (yeast).

3.2 Genotyping of bacteria and yeasts
Sequencing the 16 rDNA gene of bacterial isolates originating from 5-L fermenter (Table 1A) belonged to two main groups; Firmicutes and Proteobacteria. Firmicutes consisted of two genera: Lactobacillus and Bacillus, whereas Proteobacteriaceae consisted of four genera: Klebsiella, Komagataeibacter, Mangrovibacter, and Enterobacter. Proteobacteria were frequently isolated during the first half of the fermentation period which is between day 0 to 45, whereas Firmicutes were frequently isolated during the remaining days (between day 45 to 90).
Table 1. Closest relatives (NCBI database) of the isolated bacteria and yeasts in spontaneously fermented Garcinia mangostana pericarp simple originated from 5-L fermenter (A) and 50-L fermenter (B). Strain no.: the designated identity of isolate after isolation; bp: number of sequenced base pairs; Similarity (%): percentage of identical base pairs (NCBI database) to sequenced base pairs.

| Organism                        | Strain no. | Plating medium | bp  | Acc. no. | Similarity (%) | Day of fermentation |
|---------------------------------|------------|----------------|-----|----------|----------------|---------------------|
| **Day of fermentation**         |            |                |     |          |                |                     |
| **0**                           |            |                |     |          |                |                     |
| **5**                           |            |                |     |          |                |                     |
| **10**                          |            |                |     |          |                |                     |
| **20**                          |            |                |     |          |                |                     |
| **30**                          |            |                |     |          |                |                     |
| **45**                          |            |                |     |          |                |                     |
| **60**                          |            |                |     |          |                |                     |
| **75**                          |            |                |     |          |                |                     |
| **90**                          |            |                |     |          |                |                     |
| **Klebsiella pneumoniae**        | 0C1        | MC             | 1429| KC876640 | 97             | x                   |
| **Komagataeibacter saccharivorans** | 75M1   | MRS            | 1423| LN886705 | 95             | x                   |
| **Lactobacillus plantarum**     | 6M1        | MRS            | 1028| KC836663 | 97             | x                   |
| **Lactobacillus varicola**      | 0C3        | MC             | 1432| KJ638976 | 97             | x                   |
| **Bacillus thuringiensis**      | 75A1       | PCA            | 1466| KM280648 | 97             | x                   |
| **Bacillus cereus**             | 60M2       | MRS            | 229 | MG737475 | 97             | x                   |
| **Bacillus sp.**                | 75A2       | PCA            | 1392| MG645164 | 97             | x                   |
| **Enterobacter asburiae**       | 30C3       | MC             | 1418| KU714599 | 96             | x                   |
| **Enterobacter sp.**            | 48C3       | MC             | 1389| KJ638989 | 98             | x                   |
| **Enterobacter asburiae**       | 30C3       | MC             | 1413| KT287073 | 99             | x                   |
| **Mangrovibacter plantisponsor**| 21A1       | PCA            | 1393| KT025846 | 95             | x                   |
| **Enterobacter sp.**            | 48C1       | MC             | 1372| JN899572 | 98             | x                   |


### Table 1. (Continued)

| A | Organism       | Strain no. | Plating medium | bp   | Acc. no.     | Similarity (%) | Day of fermentation |
|---|----------------|------------|----------------|------|--------------|-----------------|---------------------|
|   |                |            |                |      |              |                  |                     |
| Pichia kudriavzeii | 0D2 | PDA | 493 | HQ122937 | 97 | x  |
|   | 0D2 | PDA | 493 | KY104575 | 99 | x  |
|   | 8D2 | PDA | 492 | KP878240 | 97 | x  |
|   | 8D2 | PDA | 490 | JX174414 | 99 | x  |
|   | 21D1 | PDA | 426 | KJ535099 | 100 | x  |
|   | 21D1 | PDA | 429 | EU315761 | 99 | x  |
| Yeasts | Saccharomyces sp. | 30D3 | PDA | 758 | FM199954 | 98 | x |
|   | 30D3 | PDA | 758 | FM199954 | 98 | x |
|   | 48D2 | PDA | 561 | MH988779 | 100 | x  |
|   | 48D2 | PDA | 554 | MF623892 | 100 | x  |
|   | 75D2 | PDA | 556 | KP675260 | 100 | x  |

### Table 1. (Continued)

| B | Organism       | Strain no. | Plating medium | bp   | Acc. no.     | Similarity (%) | Day of fermentation |
|---|----------------|------------|----------------|------|--------------|-----------------|---------------------|
|   |                |            |                |      |              |                  |                     |
| Bacteria | Enterobacter sp. | 75D2 | PDA | 550 | KY104222 | 99 | x  |
|   | 0D3 | MC | 1214 | GQ871449 | 96 | x  |
|   | 5D1 | MC | 1461 | KM253008 | 94 | x  |
|   | 10D2 | MC | 1434 | LC014955 | 94 | x  |
|   | 20D2 | MC | 1208 | LC014951 | 93 | x  |
|   | 45D2 | MC | 1300 | HM461159 | 79 | x  |
|   | 60D3 | MC | 1233 | MG754444 | 77 | x  |
|   | 90D1 | MC | 1207 | MH790145 | 88 | x  |
|   | Gluconobacter frateurii | 5D2 | PCA | 1409 | AB682110 | 97 | x  |

Other formatted elements can be copied and pasted accordingly.
Table 1. (Continued)

| B    | Organism                     | Strain no. | Plating medium | bp  | Acc. no.   | Similarity (%) | Day of fermentation |
|------|-----------------------------|------------|----------------|-----|------------|-----------------|---------------------|
|      |                             |            |                |     |            |                 | 0  | 5  | 10 | 20 | 30 | 45 | 60 | 75 | 90 |
| **Bacteria** |                           |            |                |     |            |                 |     |     |     |     |     |     |     |     |     |
|      | Gluconobacter sp            | 60D2       | PCA            | 1431| LC081223   | 98              | x  |     |     |     |     |     |     |     |     |
|      |                             | 20D1       | MRS            | 1286| MF369837   | 88              | x  |     |     |     |     |     |     |     |     |
|      |                             | 30D1       | MRS            | 1399| MF369842   | 97              | x  |     |     |     |     |     |     |     |     |
|      |                              | 45D3       | MRS            | 1437| MF369840   | 97              | x  |     |     |     |     |     |     |     |     |
|      | Enterococcus faecalis       | 60D1       | MRS            | 1232| MH385347   | 92              | x  |     |     |     |     |     |     |     |     |
|      |                             | 75D1       | MRS            | 1457| MG736061   | 96              |     |     |     |     |     |     |     |     | x  |
|      |                              | 90D2       | MRS            | 1234| MG694636   | 94              |     |     |     |     |     |     |     |     |     |
|      | Bacillus sp.                | 20D3       | MRS            | 1231| LN869534   | 94              |     |     |     |     |     |     |     |     |     |
|      | Bacillus cereus             | 75D3       | MC             | 1467| KY750690   | 97              |     |     |     |     |     |     |     |     | x  |
|      | Staphylococcus sp.          | 75D2       | MC             | 1224| JX082300   | 87              |     |     |     |     |     |     |     |     |     |
| **Yeasts** |                           |            |                |     |            |                 |     |     |     |     |     |     |     |     |     |
|      | Hanseniaspora opuntiae      | 0D2        | PDA            | 729 | KT029780   | 88              |     |     |     |     | x  |     |     |     |     |
|      |                             | 20D2       | PDA            | 728 | FM199951   | 99              |     |     |     |     | x  |     |     |     |     |
|      |                             | 30D2       | PDA            | 732 | KC544485   | 99              |     |     |     |     | x  |     |     |     |     |
|      |                              | 60D1       | PDA            | 728 | KC544485   | 99              |     |     |     |     | x  |     |     |     |     |
|      | Hanseniaspora valbyensis    | 75D1       | PDA            | 732 | MK044019   | 98              |     |     |     |     | x  |     |     |     |     |
|      | Hanseniaspora uvarum        | 10D2       | PDA            | 732 | KP010406   | 92              |     |     |     |     | x  |     |     |     |     |
|      | Hanseniaspora vineae        | 90D2       | PDA            | 722 | KY103582   | 77              |     |     |     |     | x  |     |     |     |     |
|      | Pichia kudriavzeii          | 20D1       | PDA            | 930 | KP675276   | 99              |     |     |     |     | x  |     |     |     |     |
|      | Uncultured Candida          | 0D3        | PDA            | 304 | JX159547   | 90              |     |     |     |     | x  |     |     |     |     |
|      | Candida                     | 5D2        | PDA            | 359 | KM014597   | 87              |     |     |     |     | x  |     |     |     |     |
|      | orthopsilosis               | 10D3       | PDA            | 492 | LC389314   | 99              |     |     |     |     | x  |     |     |     |     |
|      | Candida boidinii            | 45D2       | PDA            | 680 | EF197945   | 99              |     |     |     |     | x  |     |     |     |     |
Genotyping of bacterial isolates from 50-L fermenter (Table 1B) revealed two main groups; Firmicutes and Acetobacteria. Firmicutes were represented by four genera: Enterococcus, Bacillus, Staphylococcus, and Enterobacter, whereas Acetobacteriaceae was only represented by Gluconobacter.

The presence of Lactobacillus plantarum shown in Table 1A presented a desirable probiotic aspect of 5-L fermenter ecosystem, though it was not found in the 50-L fermenter. Nevertheless, Gluconobacter frateurii, an acetic acid bacteria, was isolated in the latter. Generally, Lactobacillus is ubiquitous in many spontaneously fermented plant ecosystem such as kimchi [15], leek (Belgian fermented vegetable) [7] and fermented carrot juice [14]. Interestingly, the Enterococcus faecalis, which is shown in Table 1B were frequently isolated from the 50-L fermenter. This species has been used as a starter culture for spontaneous fermentation of papaya fruit; Japan (SAIDO-PS501: PS-501) as highly nutritious nutraceutical product which was proven for its antioxidant [16] and immunity-inducing effects [17].

Sequencing of 5.8S-ITS rDNA gene of the selected yeast isolates revealed a diverse yeast strain from 5-L fermenter (Table 1A) ecosystem which belonged to Saccharomycetaceae (Pichia, Saccharomyces sp.), Saccharomycodaceae (Hanseniapora) and Debaryomycesaceae (Meyerozyma). Pichia kudriavzevii was frequently isolated from day 0 to 20 of the fermentation, while Hanseniaspora opuntiae (day 30 and 45) and Meyerozyma caribbica at day 75. The suspected main fermenting organism Saccharomyces was isolated at day 20 and 90. Yeasts isolated from 50-L fermenter (Table 1B) belonged to Saccharomycetales (Candida), Saccharomycetaceae (Pichia, Saccharomyces sp.) and Saccharomycodaceae (Hanseniapora). Hanseniapora strains were frequently isolated throughout the entire fermentation period while Candida strains were isolated between days 0 to 45.

The presence of Saccharomycetaceae and Saccharomycodaceae in both 5-L and 50-L ecosystems shown in Table 1A and Table 1B highlighted the ubiquitously of the species in many spontaneous fermentation processes. They presented the hallmark of alcoholic fermentation, as Candida bolitinii was also discovered from spontaneously fermented grape must [18]. The presence of Saccharomycyes cerevisiae, along with non-Saccharomyces yeasts such as Hanseniaspora, Pichia, Candida, Clavispora, Rhodotorula, Saccharomyopsis, Torulaspora, Metschnikowia, Issatchenka, and Geotrichum were also common in the spontaneously fermented palm, pineapple and orange juices, apple cider and mangosteen paste [19], whereas Candida spp. and Saccharomyces spp. were among the prevalent yeast species in spontaneously fermented kimchi [20].

4. Conclusion
Spontaneous fermentation of GMP highlighted the prevalence of LAB and yeast populations. LAB may confer a probiotic element of the fermented GMP, while the discovery of a few microorganisms from genotyping analysis such as Lactobacillus plantarum and Enterococcus faecalis elucidated their use as a potential starter culture. Several saccharomycetaceae strains may also serve as potential starter culture which can contribute to sensory quality of the fermented GMP. On the other hand, the inhibition of enterobacteriaeae was an important indicator of the microbiological safety of the fermented GMP.

5. Acknowledgement
The authors gratefully acknowledge the Universiti Teknologi MARA Cawangan Pulau Pinang for funding this research through LESTARI grant (600-IRMI/DANA KCM 5/3/LESTARI (194/2017)).

References
[1] Gutierrez-Orozco F Chitchumroonchokchai C Lesinski G B Suksamrarn Sand Failla M L 2013 α-Mangostin: Anti-Inflammatory Activity and Metabolism by Human Cells J Agric Food Chem. 24, 61 p. 3891–3900.
[2] Li L Han A R Kinghorn A D Frye R F, Derendorf H and Butterweck V 2013 Pharmacokinetic properties of pure xanthones in comparison to a mangosteen fruit extract in rats Planta Med. 79, 8 p. 646–653.
[3] Chitchumroonchokchai C, Riedl K M, Suksumrarn S, Clinton S K, Kinghorn A D and Failla M L 2012 Xanthones in mangosteen juice are absorbed and partially conjugated by healthy adults J. Nutr. 142, p. 675–680.

[4] Arunrattiyakorn P 2011 Microbial metabolism of a-mangostin isolated from Garcinia mangostana L. Phytochemistry 72, p. 730–734.

[5] Mantovani M 2010 US Patent Mangosteen extract 1(19).

[6] Pappa S, Papadelli M, Paramithiotis S, Daferera D, MG Polissiou and EH Drosinos 2018 Effect of herb addition on spontaneous fermentation of radish (Raphanus sativus L.) roots in brine and the fate of L. monocytogenes and E. coli J. Med. Plant Stud. 6, p. 32–39.

[7] Wouters D, Bernaert N, Conjaerts W, Van Droogenbroeck B, De Loose M and De Vuyst L 2013 Species diversity, community dynamics, and metabolite kinetics of spontaneous leek fermentations Food Microbiol. 33, p. 185–196.

[8] Kimura S, Tung Y, Pan M, Su N, Jang L, and Chen Cheng K 2016 Black garlic: A critical review of its production, bioactivity, and application J. Food Drug Anal. 25, p. 62–70.

[9] Muhialdin B, J Sukor R, Ismail N, Ahmad S W, Me N, C and Meor Hussin A S 2018 The Effects of Fermentation Process on the Chemical Composition and Biological Activity of Spider Flower (Gynandropsis gynandra) J. Pure Appl. Microbiol. 12, p. 497–504.

[10] Kawa-Rygelska J, Adamenko K, Kucharska A Z and Piórecki N 2018 Bioactive compounds in cornelian cherry vinegars Molecules 23, p. 1–16.

[11] Capozzi V, Fragasso M, Romaniello R, Berbegal C, Russo P and Spano G 2017 Spontaneous food fermentations and potential risks for human health Fermentation 3, p. 1–19.

[12] Touret T, Oliveira M and Semedo-Lemsaddek T 2018 Putative probiotic lactic acid bacteria isolated from sauerkraut fermentations PLoS One 13, p. 1–16.

[13] Ho V T, T Zhao J and Fleet G 2014 Yeasts are essential for cocoa bean fermentation Int. J. Food Microbiol. 174, p. 72–87.

[14] Wuyts S, Van Beeck W, Oerlemans EFM, Wittouck S, Claes IJJ, De Boeck I, Weckx S, Lievens B and De Vuyst L 2018 Carrot juice fermentations as man-made microbial ecosystems dominated by lactic acid bacteria Appl. Environ. Microbiol. 84, p. 1–16.

[15] Jung J Y, Lee S H and Jeon C O 2014 Kimchi microflora: History, current status, and perspectives for industrial kimchi production Appl. Microbiol. Biotechnol. 98, 6 p. 2385–2393.

[16] Noda Y, Murakami S, Mankura M and Mori A 2008 Inhibitory effect of fermented papaya preparation on hydroxyl radical generation from methylguanidine J. Clin. Biochem. Nutr. 43, p. 185–190.

[17] Fujita Y, Tsuno H and Nakayama J 2017 Fermented papaya preparation restores age-related reductions in peripheral blood mononuclear cell cytolytic activity in tube-fed patients PLoS One 12, p. 1–19.

[18] Utlée A, Wacker A, Kunz D, Löwenstein R and König H 2013 Microbial succession in spontaneously fermented grape must before, during and after stuck fermentation South African J. Enol. Vitic. 34, p. 68–78.

[19] Chanprasartsuk O O, Prakitchaiwattana C, Sanguandeekul R and Fleet G H 2010 Autochthonous yeasts associated with mature pineapple fruits, freshly crushed juice and their ferments; and the chemical changes during natural fermentation Bioresour. Technol. 101, p. 7500–7509.

[20] C. Ho-Won Kim K H, Nam Y D, Roh S W, Kim M S, Jeon C O, Oh H M, Bae J W 2008 Analysis of yeast and archael population dynamics in kimchi using denaturing gradient gel electrophoresis Int. J. Food Microbiol. 126, p. 159–166.