Population Dynamics and Molecular Profiling of Microbial Ecosystems during Spontaneous Fermentation of *Carica Papaya* Leaves

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***Abstract*— The fermentation of *Carica Papaya* leaves in this research is spontaneous fermentation. There is none of studies has been doing research on the metabolic compounds in fermented Carica Papaya leaves. This has been a constraint to pursue further analysis on fermented Carica Papaya leaves. Other than that, there was also unknown microbial population.. In natural fermentation, the process is operated without the use of starter inoculum. In order to determine the microbial population dynamics, reliable methods for enumeration are required. Sample taken from the fermented *Carica Papaya* leaf may contain mixed population of bacteria. Therefore, selective media must be employed in which the culture medium used is depends on the target group, the product matrix and the taxonomic diversity of the bacterial background in the fermented *Carica Papaya* leaf. The chosen medium must allow the growth of the organisms of interest and restrain other microorganisms to encounter.**

***Keywords*— *Carica Papaya, Spontaneous fermentation, Microbial growth, DGGE*.**

I. INTRODUCTION

Fermentation is a popular method to preserve staple food, fruits, vegetables, herbs and other edible materials as well as improving their nutritional value and sensory quality [1, 2]. Some traditional fermented foods such as kimchi (originally Korea)[3] and saeurkraut [4] become international delicacy. Other traditional fermented foods derived from wheat, starch, milk, maize are known by their local names such as doklu (africa) [5], tape (Indonesia) [6], tempoyak (Malaysia) [7], kishk (Egypt). Despite long-time tradition of fermented foods spanning over millenia, their current production method is largely low-tech at household scale with the majority of production processes employ spontaneous fermentation using indigenous microorganisms inhabitating the raw materials. On the other hand, the use of starter culture evolved from repeated 'backslopping' processes to iterate the best-adapted strain [4]. For this reason, fermentation becomes a recommended food preservation method and nourishment programme for the population in impoverished areas [4]. Until recently, the health-promoting aspects of these fermented foods in terms of the presence of probiotics such as lactic acid bacteria (LAB) that is beneficial to cholesterol modulation, immune stimulation and toxin inactivation were revealed. In addition, the bacteriocin secretion of LAB inhibits the foodborne pathogenic growth and preventing diarrhea [6].

Carica papaya leaf (CPL) has wide range therapeutic properties such as antiinflammatory [8], antioxidant and immunomodulatory [9]. In the tropical region, the anti-dengue efficacy of CPL extract is supported by numerous clinical studies [10]. These therapeutic benefits are associated with its prominent polyphenolic compounds such as alkaloids, saponins, flavanoids, tannins and phenolic acids [11]. A few studies highlighted the ennhanced bioactivity of fermented plants such as mangosteen's α-mangostin as a result of fermentation by Colletotrichum gloeosporioides (EYL131) and Neosartorya spathulata (EYR042) fungi [12]. In addition, lactic acid fermentation of Myrus communis berries using L. plantarum and yeast extract was reportedly enhanced the antioxidant activity of the fruit with respect to DPPH scavenging activity, inhibition of linoleic acid peroxidation and increase of phenolic acids and flavonols content [13]. These findings may counteract with the problem of poor bioavailability of direct intake of dietary polyphenolic, although none implicated to CPL directly, which heavily relies on colonic microbiome to break down the complex polyphenols compounds in the digestive tract [14, 15].

In this study, spontaneous fermentation is used to enhance the bioavailability of CPL extract and possible enhancement of its sensory quality. Since spontaneous fermentation of CPL is unprecedented, the information of its biodiversity is imperative for process standardisation. Additional, the safety and health aspects of this novel product can be put to light from the presence of pathogens and probiotics respectively while information on dominant strain may be useful for the selection of starter culture for shorter fermentation time and better quality control in future. In the present study, the bacterial diversity of CPL fermentation was elucidated using polyphasic approach using both traditional plating method and culture-independent method. Denaturing gradient gel electrophoresis (DGGE) was used in the latter obtain a complete molecular fingerprinting of the bacterial species present in fermentation ecosystem which otherwise non-culturable on the traditional plating method.

II. METHODOLOGY

A. Carica Papaya fermentation

Carica Papaya leaves were collected from plantations in Banting, Selangor, Malaysia. The leaf was processed instantly after the harvest to maintain its original characteristics. This is to avoid the leaves from swollen and died thus the nutrient and phenolic compound in the leaves will be lost or decreased. The fresh leaf was washed to remove physical dirt. A kitchen blender was used to shred the leaves into smaller pieces. A 5 L benchtop reactor (INOFRS) was loaded with 10 %w/v of *C. Papaya* leaf and 10 %w/v unrefined sugar. Distilled water was added to bring 5 L

volume.

The fermentation was carried out in anaerobic condition for 90 days at room temperature and protected from direct sunlight. Broth samples were collected at day 0, 2, 4, 6, 8, 15, 30, 45, 60, 75 and 90 to be used for microbiological and molecular analyses.

A. Microbiological analysis

Enumeration of microbial populations

Broth samples were subjected to microbiological analysis for evaluation of microbial population dynamics during fermentation period. Accurately 0.1 ml of broth sample was homogenized in 0.9 ml of sterile saline-peptone water, 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), Dichloran Bengal Chloramphenicol (DRBC) agar for yeast and mould, Plate count agar (PCA) for aerobic mesophilic and total bacteria and MacConkey agar for coliforms and enterobacteria. Each agar solution was supplemented with L. cysteine of 0.05% w/v as reducing agent to improve anaerobic environment. All cultures were incubated. Cultures on PDA, DRBC, PCA and MRS agar were incubated at 30 ºC whereas MacConkey were incubated at 37ºC for 1-2 days in candle jar.

After incubation, three colonies from each media were randomly selected (approximately 150 isolates), purified by re-streaking twice, incubated in MRS and nutrient broths overnight and stored in -30 ºC in the same liquid media containing 30% glycerol until further analysis.

B. Chemical analysis

pH measurement

pH was measured by pH probe (Mettler-Toledo) as part of bioreactor outfit.

C. DNA extraction and purification

Broth sample was collected from each sampling time in triplicate and big debris deposition was formed. Extraction of DNA from cells of bacteria and yeast (grown in MRS, BHI and LB broth) followed the protocol in Wizard Genomic DNA Purification kit (Promega). DNA extract was used as template for PCR.

D. PCR amplification

PCR amplification of V3 region of 16S rDNA gene of each bacterial isolate was carried out using universal primer set of forward primer 27f (5'-AGAGTTTGATCMTGG CTCAG-3') and reverse primer 1492r (5'-TACGGYTACCTTGTTACGACTT-3'). Each PCR mixture of 50 µl total volume consisted of 5 µl of DNA template, 0.25 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 MgCl2 and 400 µM of each dNTPs and nuclease-free water. 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 purity of DNA fragments were analysed by running 2%(w/v) agarose gel with 1 × TAE buffer.

For fungal isolates, the 5.8S-Internally Transcribed Spacer (5.8S-ITS) rDNA region was amplified using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). Other PCR conditions were similar to bacterial isolates analysis.

E. 16S rRNA sequence analysis

The PCR products were submitted to Sanger sequencing by 1st BASE using the same primers used during PCR amplifications. The sequence identity was determined by a BLASTn search at NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) while the phylogenetic tree was constructed using MEGA software 7.0.26 ver.

F. Direct sampling and DNA extraction of broth sample

Fifty mililitre of broth samples were collected at day 2, 4, 8, 15, 33, 45, 60, 75 and 100 of the fermentation days into a 50 ml tube. The bulk DNA was isolated using bead beating method according to protocol by GenElute™ Soil DNA Isolation Kit (Sigma).

G. PCR-DGGE analysis

PCR amplification of V3 region of 16S rRNA gene was carried out using bacteria-specific primer set gc338f (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3') and 518r (5'-ATT ACC GCG GCT GCT GG-3') as described Muyzer et al [16] resulting ~200-250 bp DNA fragments. Forty GC nucleotide length (underlined) is 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 using a conventional thermocycler (Eppendorf Mastercycler) at ten cycles of denaturation at 95 °C for 1 min, followed by touchdown annealing temperature by 1 °C for 1 min from 65 °C to 55 °C during each successive cycle and elongation at 72 °C for 3 min. Additional twenty 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 [17]. PCR products were purified using FavorPrep PCR Purification Kit (FAVORGEN). About 5 µl of PCR products were analysed on 2%(w/v) agarose gel with 1 × TAE buffer using conventional gel electrophoresis chamber (Biorad), followed by staining with staining dye (SYBR®Safe) and quantified by 100 bp DNA ladder (SMOBIO DM2100 ExcelBand) under UV light in GelDoc system (Biorad).

The VS20WAVE-DGGE (Cleaver Scientific Ltd) was used for sequence specific separation of PCR products. Electrophoresis was performed in a 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 analysed using GelDoc for identification of significant DNA bands. Subsequently, DGGE bands were excised using sterile razor blade and the DNA of each band was eluted by overnight incubation at 4 °C in 50 µl of 0.1×TE buffer solution. Next, 5 µl of eluted DNA was reamplified using similar PCR conditions described earlier minus the GC-clamp of the forward primer..

The DNA fragments were purified prior to Sanger sequencing by 1st BASE using the same primer during reamplification. The sequence identity was determined by a BLASTn search at NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) while the phylogenetic tree was constructed using MEGA software 7.0.26 ver.

III. RESULTS AND DISCUSSION

A. Microbial growth of C. Papaya fermentation

Fig. 1 shows the growth of bacteria and yeasts during spontaneous fermentation of *C. Papaya* leaves for 90 days. It is characterized according to different types of agar media (PCA, PDA/DRBC, MRS, MC). PCA is a selective agar media for total bacteria growth whereas McConkey has a suitable environment for the growth of coliform and enterobacteria.[7] On the other hand, PDA and MRS are the most important media for the growth of yeasts and lactic acid bacteria respectively.

As its function, PCA agar media shows a consistent trend in increment starting from day 0 until day 4. The growth of total bacteria in PCA agar media (approximately 10^6-10^7 CFU/ml) was present at the commencement of fermentation and remained steadily until it dropped after 45 days of fermentation. However, there was a positive growth trend during day 90 of fermentation.

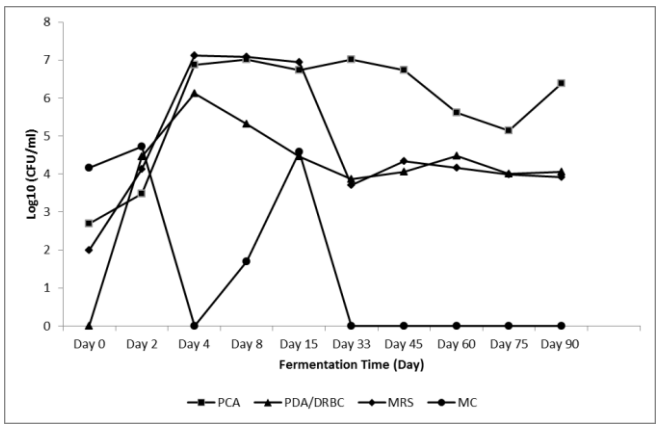

Fig.1 Growth of bacteria and yeasts during 90 days of fermentation

Enterobacteria were detected at the beginning of fermentation and grew from 10^4 CFU/ml to 10^5 CFU/ml. Differ with PCA, MRS and PDA/DRBC media, MC brings out the most growth from day 4 to day 15. It is shows that the growth of bacteria in McConkey agar had a sudden rise during day 8 compared to other media. The populations were facing a slump during day 4 and managed to surge to the highest population of 10^5 CFU/ml at day 15. There are no sign of enterobacteria growth at day 33 until the end of fermentation. This is good sign since enterobacteria is a type of pathogen that is unwanted in this fermentation.

No yeasts were detected at the commencement of fermentation and this can relate to the starting pH value of 5.87. Yeasts are more favourable to grow at acidic condition. From day 0 to 2, the growth of yeasts climbed to a population of 10^4 CFU/ml together with other selective agar media. There are fluctuations in growth pattern after the increasing and it is remained steadily until fermentation ends. On the other side, the growth of yeasts during 90 days of fermentation was equal to the trend of pH values.

LAB was first detected at day 0 (102 CFU/ml), at which they constantly grew to populations of 107 CFU/ml at day 4. This was the highest peak in LAB growth fermentation because by referring to Fig. 2, pH value starts to stable at day 4 and remained steady until the ends of fermentation. LAB was considered to be substantial in modulating the pH of the mixture. [2] During the first 4 days of fermentation, lactic acid was produced but more so when lactic acid bacteria grew. There was a decline in LAB growth from day 15 to 33 and this can relate by referring to Fig. 2, there was slightly increase in pH value.

B. Pure isolates of bacteria and yeasts

Fig. 3 shows that populations of different species of microorganisms present during 90 days of C. Papaya leaves fermentation. This phylogenetic tree comes with accession number for further usage in looking for information. Based on this figure, there are 6 genera of bacteria present in the ecosystem which are Lactobacillus, Bacillus, Lactococcus, Cronobacter, Enterobacter and Klebsiella. Bacillus, Lactococcus, Cronobacter and Klebsiella genera have only one type of strain, which are *Bacillus cereus*, *Lactococcus lactis*, *Cronobacter sakazakii* and *Klebsiella pneumoniae*.

On the other hand, there are two strain of lactobacillus; plantarum and brevis strain. These lactic acid bacteria make the most number of populations because they are living in their suitable ecosystem. During this time, lactic acid was produced and resulting to acidity condition according to Fig. 2. This environment was favourable for lactic acid bacteria growth. This is compatible with DGGE results in Fig. 4.

The existing of pathogens according to the phylogenetic tree can be assumed comes from day 0, 2, 8 and 15. The growth of bacteria in MC by that time displayed a tiny, round, white single colonies with a huge amount of colonies present (70-190 colonies counted). *Cronobacter sakazakii*, *Enterobacter tabaci* and *Enterobacter cloacae* were species that establish in the ecosystem but unfound after day 33 in Fig. 1.

In fermentation, the most common microorganisms present are LAB because of its exceptional metabolic characteristics. During fermentation, lactic acid bacteria, carbon dioxide, ethanol and hydrogen peroxide were being produced. The type of microorganisms present during the fermentation process might be affected by the ecology of where the C. Papaya leaves planted. This significant effect had a major impact on the processes and contributed to ensuring consistency of product and dependability of fermentation. Type of media used for the isolation also influences the growth of LAB.

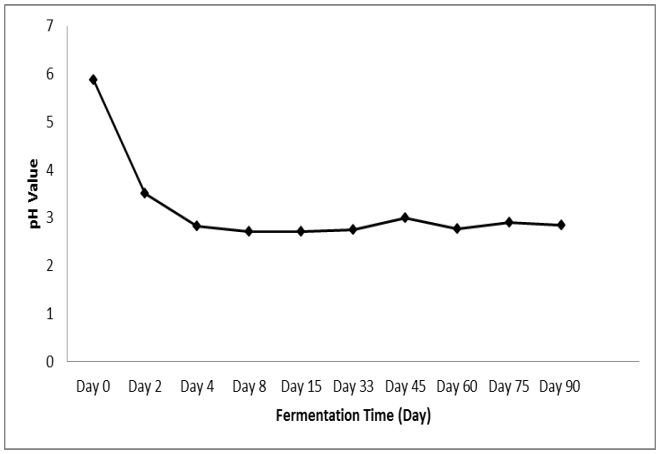

Fig. 2 pH Values during 90 days of fermentation

In this research, we examined the microbiological changes during the 90 days of fermentation to determine the microbial selection occurred during fermentation. Even though it is a non-aseptic fermentation because the process involved utilization of microflora that existed on the leaves, it is believe that the microflora of the leaves might not have differed significantly.

There are several studies reported that during fermentation, there were yeasts occurrence in association with LAB. It is observed in Fig. 3 and Fig. 4, there were numerous number of LAB and yeasts present during this 90 days of fermentation. *Lactobacillus plantarum* and *Pichia kudriavzevii* were the most obvious species that existed throughout the fermentation.

In this *C. Papaya* fermentation, there was no starter culture being used. Therefore, the fermentation process was naturally affected by the indigenous microflora. There is possibility that antibiotic-resistant organisms may be presented in the mixture

during the fermentation. LAB strains could act as a reservoir of antibiotic resistance genes and it is non-transferable to human antibiotic resistance gene. [7]

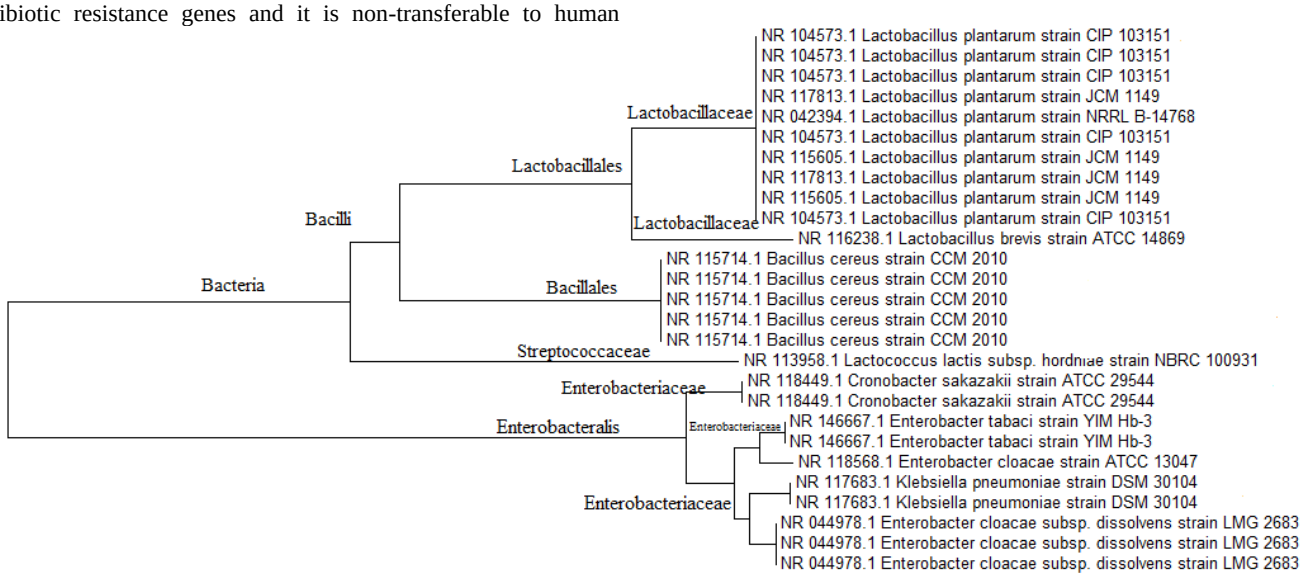

Fig. 3 Phylogenetic tree of pure isolation of bacteria

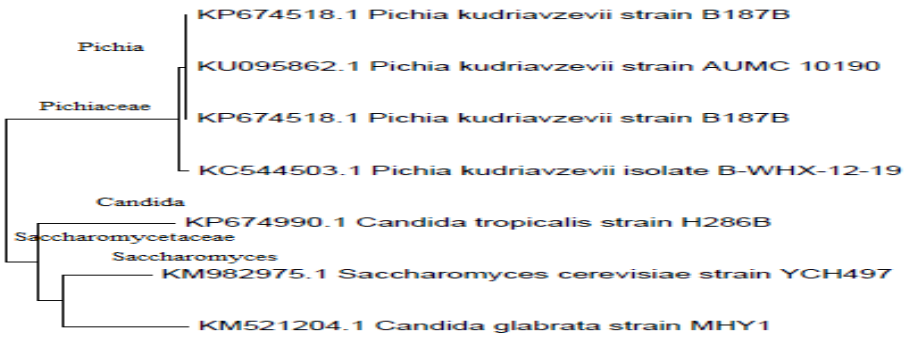

Fig. 4 Phylogenetic tree of pure isolation of yeasts

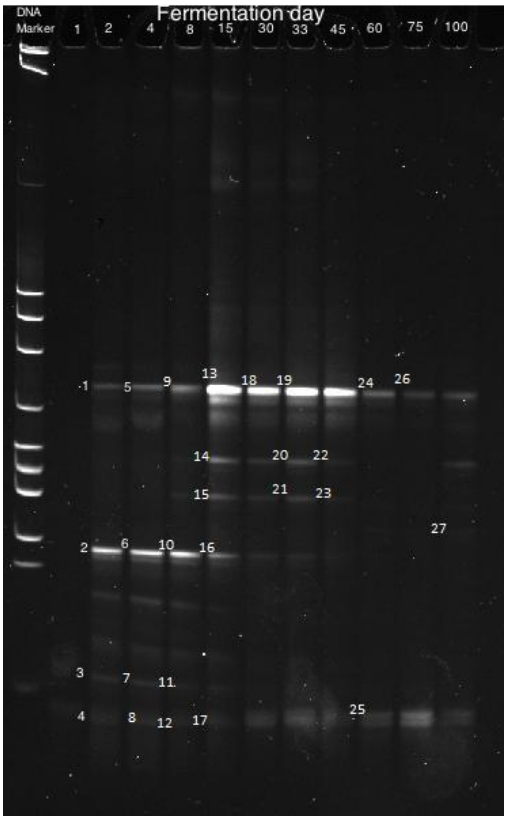

Fig. 5 Gel electrophoresis

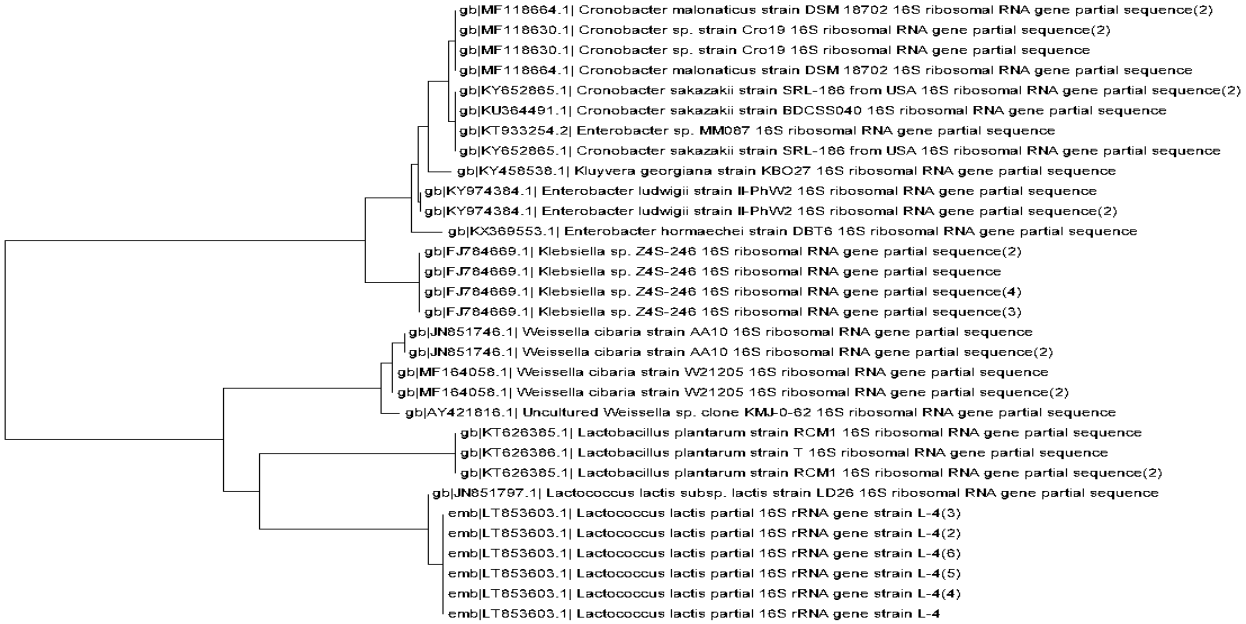

Fig. 6 Phylogenetic tree of DGGE analysis

Despite its high acidity, the consumption of naturally fermented product is a concern since the microbial safety is at questioned. Our results showed that pathogens such as *Salmonella spp*., and *Staph. aureus* are not presented in our fermentation. This indicates that the spontaneous fermentation of *C. Papaya* leaves does not support the growth of this kind of pathogens. In this research, rapid decline of pH value at day 0 to day 4 might proposed to acid adaptation of pathogens, thus resulting in slump growth of bacteria in McConkey agar media.

A. *PCR-DGGE*

Highlighted marker in Fig. 5 shows that LAB was coming out strongly as the dominant species mostly in day 15 until day 45. This can relate with pH values during that time which is around pH 3. This acidity condition comes out as the most favorable for the growth of lactic acid bacteria. This DGGE analysis identified the species in the fermentation and it was related with phylogenetic tree.

Based on Fig. 6, there are 7 genera displayed as species presented in the ecosystem. There were Cronobacter, Enterobacter, Klebsiella, Weisella, Lactobacillus, Lactococcus and Kluyvera genera.

Most of genera in this DGGE analysis have only one type of species. *Kluyvera georgiana, Klebsiella sp. Z4S-24, Weissella cibaria, lactobacillus plantarum* and *lactococcus lactis*.

For Cronobacter genus, 3 types of strain were available; *Cronobacter malonaticus, Cronobacter sp. Strain,* and *Cronobacter sakazakii*.

By comparing with Fig. 3, there were similarities of species in both of the phylogeny tree. Species identified in this analysis is much related with the phylogenetic tree in Fig. 3. Lactobacillus, bacillus, cronobacter, enterobacter, klebsiella and lactococcus can be seen existing in both figures.

IV. Conclusion

In conclusion, this study reports the microbial population dynamics in the spontaneous fermentation of Carica Papaya leaves. Based on the pH values, it can be predicted what types of microorganism would grow at certain period of time. pH value dropping indicates that the environment is in acidity condition which favorable for the growth of lactic acid bacteria. Based on the DGGE analysis and phylogenetic tree constructed, the validity of species present in the ecosystem can be confirmed because there were some similarities of genera existed such as lactobacillus, bacillus, cronobacter, enterobacter, klebsiella and lactococcus.

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