Microbial Biodiversity Profiling of Spontaneous Fermentation of *Garcinia Mangostana* Pericarps

Azreen Zainaldin, Mohamad Sufian So'aib,

Faculty of Chemical Engineering, Universiti Teknologi Mara

***Abstract*— The Spontaneous fermentation of *Garcinia Mangostana* pericarps carried out over 100 days of period in order to enumerate the population dynamic of fermented mangosteen pericarps. The fermentation was conducted anaerobically in 20L benchtop bioreactor. Then, the samples was collected on each sampling day at day 5, 10, 20, 30, 45, 60, 75 and 90. The collected samples were cultivated on four different type of media which are Mac Conkey (MC), Man Ragosa Agar (MRS), Plate Counting Agar (PCA) and Potato Dextrose Agar (PDA) in order to determine the presence of *Enterobacteriaceae*, Lactic Acid Bacteria, Total Bacteria and Yeast respectively. DNA of bacteria and yeast were extracted and followed by PCR amplification using universal primer set. The sequencing of 16S rDNA gene of bacterial isolates yielded 5 genera and 7 species which are *Enterococcus* (1 species), *Bacillus* (1 species, *Gluconobacter* (1 species), *Enterobacter* (2 species) and Azotobacter (1 species) while the sequencing result of 5.8S-ITS rDNA gene of random selected isolates on PDA media from all sampling days yielded only 2 genera with 5 species including *Hanseniaspora* (4 species) and *Candida* (1 species). An *Enterococcus faecalis* which is one of the common species of Lactic Acid Bacteria (LAB) was become major bacterial group that presence in random selected isolates. The LAB has an ability to improve the nutritional value of food by providing and acidic condition in fermented food. In this acidic condition shows that the pathogens could not survive started from day 10 until the end of fermentation. However, in this study the small amount of LAB population was identified due to the limited number of random selected isolates.**

***Keywords*— *Garcinia mangostana*, Spontaneous fermentation, Lactic Acid Bacteria**

I. INTRODUCTION

Garcinia mangostana L. or mangosteen is a tropical evergreen fruit tree that is from Clusiaceae family [1] .It is consist of white pulp in an inner part while the dark purple pericarp in an outer part of the fruit. The mangosteen is called as 'Queen of Tropical Fruit' because of their remarkably pleasant flavour [1]. This kind of fruit tree is commonly cultivated in Southeast Asia such as Philippines, Thailand, Indonesia and Malaysia. It is required about 10 or more years to become completely mature which is ranges from 6 to 25m in height [2]. In the past hundreds years, the different part of *G. mangostana* such as bark, roots and fruit hull have being used in Southeast Asia as a source of medicine. It is widely used to treat many kind of diseases because of their medicinal and health properties

Fermentation process is widely applied to the food from a long time ago. According to the researchers, they said that fermentation process will make the food and beverage being preserved by lengthening the shell-life of the food. They also mentioned that the spontaneous fermentation or natural fermentation was use traditionally to preserve the food [3]. This oldest technology is a process that involving biological activity of microorganism that suppressed the growth and survival of unwanted micro flora in the fermented food.

The study of many kind of indigenous fermented fruits has widely carried out through many country included cocoa [4], table olives [5], grape, tempoyak [6] and also apple [7]. The different kind of fermented food will have different microbial biodiversity including the fermentation of mangosteen pericarps. Therefore, the combination conventional microbiological method and molecular biology techniques which is called poly-phasic approach is necessary to figure out a better understanding of microbial diversity. There are few studies that proved that the fermentation of *G. mangostana* will increase it bioavailability. So that, the knowledge on the population dynamic of microorganism are necessary to details out the properties of the fermented food that may contain some microbes that produce catalase enzyme as a helper to protect the cells from oxidative damage by the species of reactive oxygen. The natural fermentation was carried out in this study. The aim of this research study were to identify the microorganisms during spontaneous fermentation of *Garcinia mangostana* pericarps based on capability of microorganism to produce catalase enzyme and also depends on its gram morphological characteristic. The population dynamic of microorganism was enumerated throughout the spontaneous fermentation in order to establish the molecular profiling of the microflora diversity.

II. METHODOLOGY

A. G. Mangostana fermentation

The *Garcinia mangostana* was bought from a local market to get the pericarps of the fruit. The five kilograms of pulps were removed and the pericarps were collected in a good condition. The pericarps are straightaway processed in order to maintain its original characteristic from changing. Then, the collected pericarps were washed by using tap water in order to remove any dirt that attached to the rind. The pericarps of *Garcinia mangostana* were cut into small pieces before it is added into the kitchen blender for further shredded. Ten percent (w/v) of *G. mangostana* pericarps was added into 20L bench top bioreactor. While distilled water was loaded to make up 20L and initial substrate of 10% w/v of sugar was added. An anaerobic fermentation was carried out for 90 days at room temperature, 24°C. There were no starter cultures or preservative added.

B. *Microbiological Analysis Sampling*

The samples were collected at initial day of fermentation process followed by day 5, 10, 20, 30, 45, 60, 75 and finally at day 90 of fermentation. 50ml of sample was collected and centrifuged for a few seconds.

C. *pH determination*

The pH was determined for each sample days. The pH was measured two times by using Seven Easy™ pH meter S20. Then average pH were calculated before plotting the result.

D. *Enumeration of microbial population*

Each of the samples that have been collected were diluted by using serial dilution method. The sterile peptone water was used as diluent. A 0.9ml of sterile peptone was homogenized with 0.1ml of sample. The suspension was diluted in series up to five or six dilution factors. Then, each of serial dilution samples were spread on the four different selective media. The hockey stick spread plate method was used before placed inside the candle jar. The candle were used to provide anaerobic conditions. Then, the cultured plates were placed inside the incubator about 24hours. All the PDA, PCA and MRS cultured media were placed at 30˚C while MC cultured medium was placed at 37˚C. After incubation, the yeast and mould were isolated from PDA, lactic acid bacteria (LAB) isolated from MRS agar, mesophilic aerobic isolated from PCA and *Enterobacteriaceae* were isolated

All the well-growing bacteria and yeast colonies from the selective media were observed. Then, the colony counting method was used to enumerate the microbial that had growth in different kind of media. It is conducted by using colony counter. From the cultivated media, three random colony from each different media was picked and further purified by re-striking on other new agars. The purified isolated cultured were stored in the liquid broth for overnight inside incubator shaker. Lastly, the 0.5ml of growth culture inside the broth was mixed together with 0.5 ml of 30% glycerol to preserve it. It is stored at -30°C in that medium for further subsequent molecular analysis.

E. *DNA extraction and purification*

The Promega DNA extraction kit was used to extract the total of the DNA from broth culture and yeast from pure culture by following the instruction that have being provided by manufactures.

The Gram-Positive and Gram-negative Bacteria's genomic DNA were isolated. 1ml of an overnight culture was added to 1.5ml of micro centrifuge tube then centrifuged with speed 13,000 to 16,000 for 2 minutes in order to remove supernatant. The gram positive bacteria was preceded while the gram negative bacteria proceed with the addition of nuclei lysis solution. The supernatant were removed and the cell were suspended in total volume. An extracted DNA were preserved at -30 °C.

F. *PCR Amplification*

There are two universal primers were involved in PCR amplification of V3 region of 16S rDNA gene that have being obtain from isolated bacterial. The universal primer use are forward primer 27f (5'-AGAGTTTGATCMTGG CTACAG-3') and backward primer 1492r (5'-TACGGYTCCTTGTTACGACTT-3'). A mixture of 50µL total volume is made of 5µL of DNA template, 0.25µM of each primer, 25µL of REDiant 2x mastermix (1st BASE) that consist of reaction buffer, 0.006 U/µL of Taq DNA polymerase, 3 mm MgCl2 and 400µM of each dNTPs and nucleas-free water.

The conventional thermo cycler was used for each reaction in which Eppendorf Mastercycler was used at 35 cycles of denaturing at 95˚C for 30s. It is followed by annealing temperature at 55.5˚C for 30s. Then elongate at 72˚C for 1.5min. The initial denaturation were carried out 5minutes at 95˚C while final extension take 10min at 72 ˚C. By running 2% (w/v) of agarose gel with 1X TAE buffer, the purity of DNA fragment can be analysed.

For the fungal isolation, 5.8S –Internally transcribe Spacer (5.8S-ITS) rDNA region was amplified using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). Then, Eppendorf Mastercycler in 35 cycles of denaturation was used for thermocycling. It is conuducted at 95˚C for 2min and followed by annealing the temperature at 56˚C in 2min. Then elongate to 72˚C for 2min. The initial denaturation were carried out 5minutes at 95˚C while final extension take 10min at 72 ˚C. The bacterial isolates procedures similar to the composition of PCR Mastermix and other analyses.

The Sanger sequencing service (1st BASE Laboratory, Selangor, Malaysia) identified the PCR products from both methods by using the same primers in the earlier PCR amplification. It was identified by using the BLASTn search from the National Center Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The MEGA-X software 7.0.26 version was used in constructing the phylogenetic tree

G. *Cluster Analysis of microbial profiles*

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 version.

III. RESULTS AND DISCUSSION

A. *Enumeration of microbial population*

The microbial population dynamic during the spontaneous fermentation of *Garcinia Mangostana* over 90 days of period were showed in the Fig. 1, Fig. 2, Fig. 3 and Fig. 4. It shows the changes of different microorganism in different media. Fig. 1 referred to the population growth on Plate Counting Agar. Since PCA media consist of complex nutritional requirement, it will represent the total or viable growth of bacteria from sample. It is categorized as non-selective media. The selective media of Man Rogosa Agar was used to obtain the population growth of lactic acid bacteria because of the media will provide luxuriant growth for *lactobacilli.* The Fig. 2 shows the population growth on these media over 100 days fermentation period. In this study, the Mac Conkey media also was used to enumerate the population dynamic of *enterobacteriaceae* that may presence in the samples while the Potato Dextrose Agar media was used to obtain the profiling of yeast growth. The Fig. 3 and Fig. 4 shows the population dynamic of *enterobacteriaceae* and yeast respectively

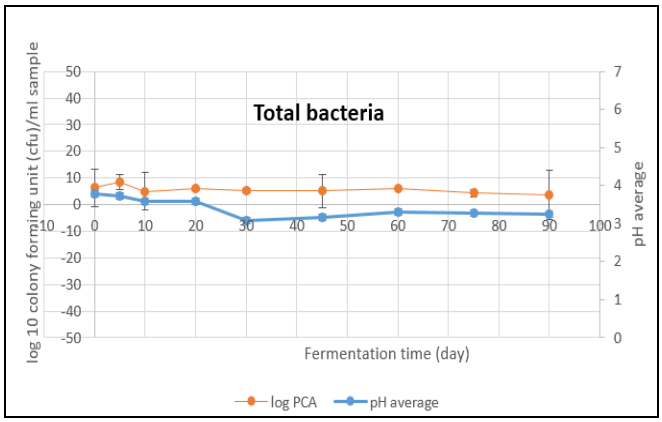

Fig. 1: Population Dynamic on PCA media

Fig. 1 shows the total bacteria counts at approximately 10^6 cfu/ml at an early stage of the Garcinia mangostana fermentation and reach about 10^8 cfu/ml during its peak on day 5. This kind of

population was maintain with little fluctuation until day 60 before it is decline until about 10^4 cfu/ml in between day 60 to day 90 which is at pH lower than pH 3.

The population dynamic in the Figure 2 have almost similar pattern with PCA media as it is start from approximately 10^6 cfu/ml at an early stage of the Garcinia mangostana fermentation and reach about 10^8 cfu/ml during its peak on day 5. The population dynamic suddenly decline at day 10 and maintain at a little changes until day 60. The lactic acid bacteria was reached its lowest population at day 75 with approximately 10 ^{4}cfu/ml of lactic acid bacteria

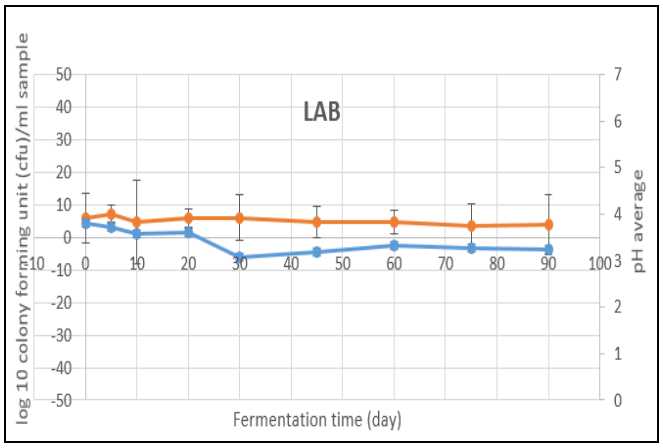

Fig. 2: Population Dynamic on MRS media

Yeast is a unicellular fungi that play vital roles in food biotechnology [8]. In this study, the population of yeast was reached its highest at same day with total bacteria and lactic acid bacteria at day 5. Figure 3 shows the population of yeast start decreasing from about 10^5 cfu/ml until 10^4 cfu/ml at day 60 until day 90 of *Garcinia mangostana* fermentation.

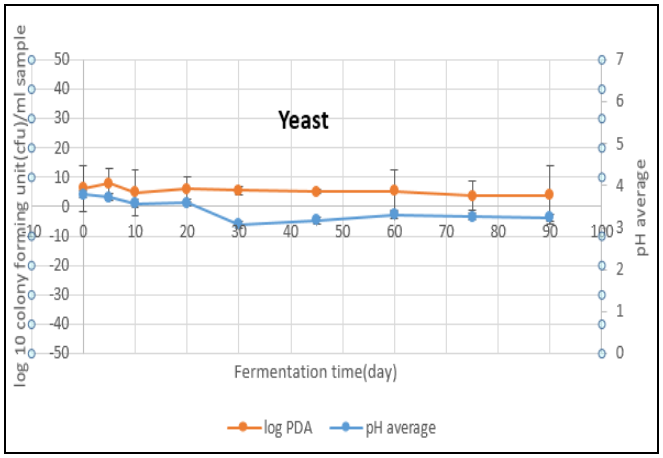

Fig. 3: Population Dynamic on PDA media

In contrast with the result from all three previous figure, this Fig. 4 shows slightly different pattern. It shows no growth of *enterobacteriaceae* at the beginning of the fermentation of *Garcinia mangostana* and followed by an abruptly increase of the population at day 5 with around 10^7 cfu/ml. Then it is suddenly disappeared for the next sampling day at day 10 as the pH was reached about pH 3.5 which is acidic condition. The enterobacteriaceae were totally absent until the end of the fermentation period.

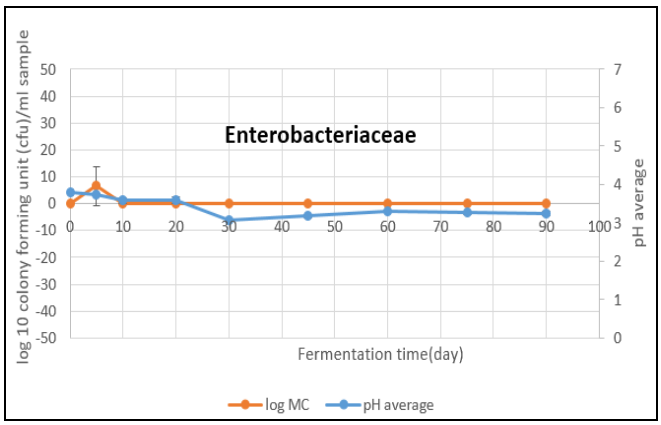

Fig. 4: Population Dynamic on MC media

B. *Cluster Analysis*

The 19 of bacteria and 19 of yeast were randomly selected isolates from PCA, MRS and PDA media. Both bacterial and yeast were subjected to Polymerase Chain Reaction (PCR), sequencing and homological comparison with online NCBI database. Some example of amplified DNA of pure isolates (PCR amplicons) were shown in Fig. 5. The sequencing of 16S rDNA gene of bacterial isolates yielded 5 genera and 7 species as shown in the Fig. 6; *Enterococcus* (1 species), *Bacillus* (1 species, *Gluconobacter* (1 species), *Enterobacter* (2 species) and Azotobacter (1 species) by using Mega7 software. Based on the Fig. 6, the *Enterococcus* species was become the major bacterial group that presence in random selected bacterial followed by other genera *Enterobacter*, *Bacillus*, *Gluconobacter* and *Azotobacter*. An *enterococcus* was categorized under *lactobacillales* that also known as lactic acid bacteria. The most common species of lactic acid bacteria (LAB) are *Lactobacillus*, *Pediacoccus*, *Weisella*, *Leuconostoc* and including *Enterococcus* [9]. Moreover, this kind of species are also widely presence in many fermented food and beverages such as *Lactobacillus brevis* from wine[10] and *Enterococcus faecalis* from *tempoyak* [9] and many more. However, due to the limited number of selected isolates leads to small amount of LAB diversity identified in this study. Interestingly, this lactic acid bacteria also have tendency to improve the nutritional value of food and control some type of cancer [11].

The sequencing result of 5.8S-ITS rDNA gene of random selected isolates on PDA media from all sampling days yielded only 2 genera with 5 species including *Hanseniaspora* (4 species) and *Candida* (1 species) were shown in the Fig. 7.

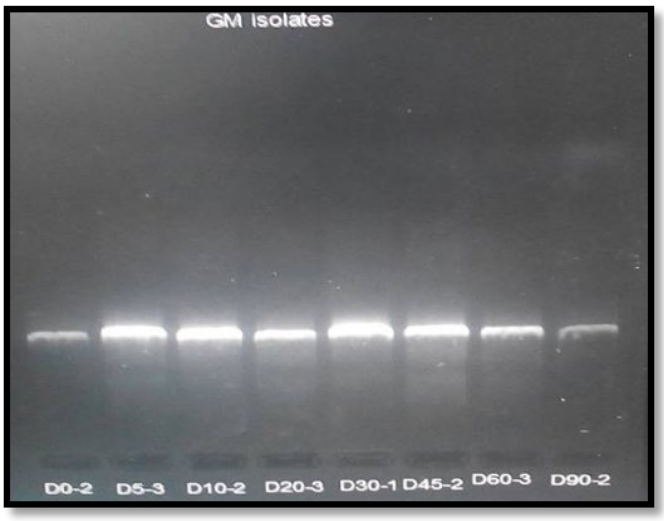

Figure 5: Amplified bacterial DNA at V3 region 16S rDNA genome

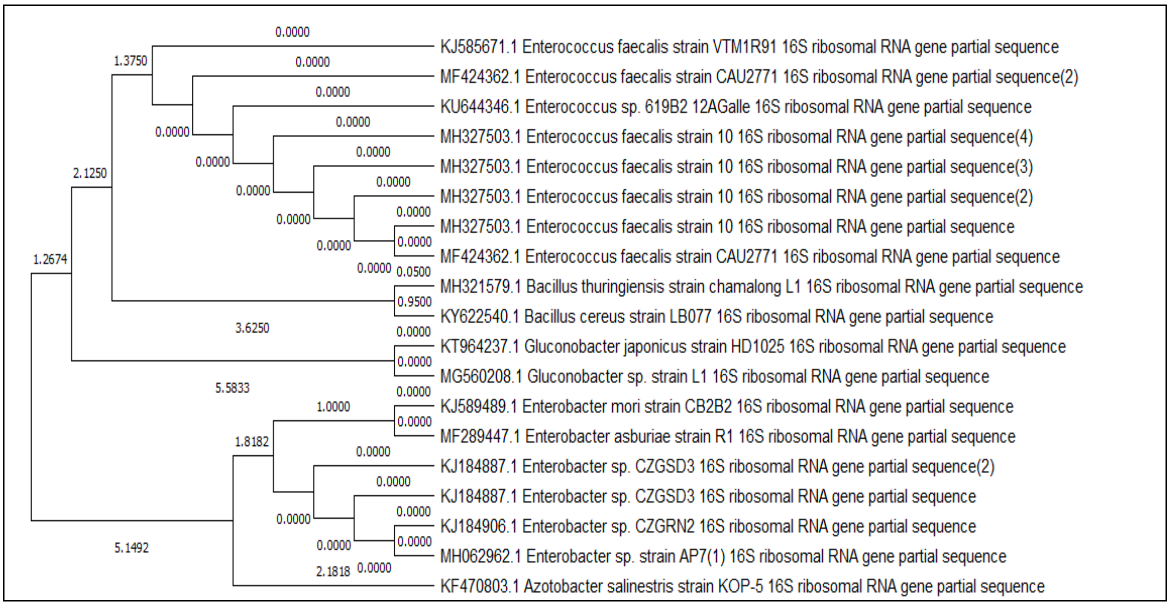

Fig. 6: Phylogenetic Tree of Bacteria

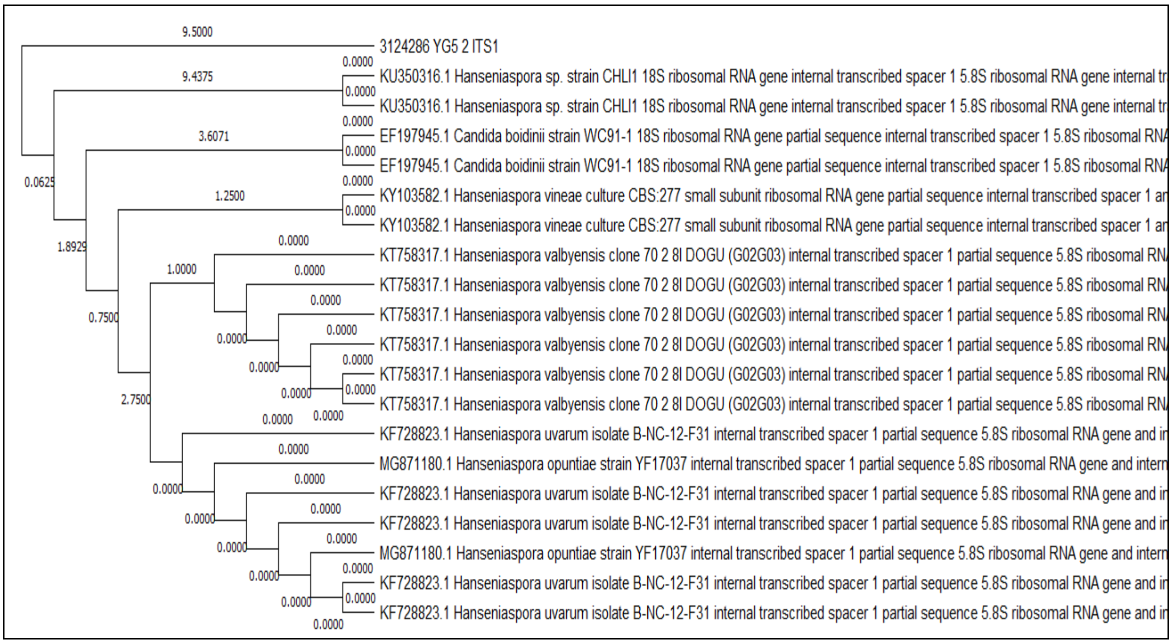

Fig. 7: Phylogenetic Tree of Yeast

From the NCBI results, the closest relative of the selected isolates was obtain by blasting the nucleotide sequence. The data obtain from NCBI database were tabulated in Table 1 for bacteria isolated and Table 2 for yeasts isolated. Table 2 shows the *Hanseniaspora uvarum and Hanseniaspora valbyensis* were the abundant yeast found. *H. uvarum* is the common species found in mature fruit that responsible fruit spoilage prevention due to its antagonist properties which is relevant to the industry [12].

Table 1: Sequencing results of selected DGGE bands in Fig. 7 and the rest of isolates bacteria.

Isolate no.	Closest relative	[a]Source	[b]Similarity (%)
D0-2	*Enterobacter* sp.	KJ 184919.1	96
D0-3	*Enterobacter* sp	KJ 184919.1	96
D5-1	*Enterobacter sp.*	KJ 184906.1	99
D5-2	*Gluonobacter sp.*	MG 560208.1	100
D5-3	*Enterobacter sp.*	MH 062962.1	99
D10-1	*Enterobacter sp.*	KJ 184887.1	99
D10-2	*Enterobacter sp.*	KJ 184887.1	99
D20-1	*Enterococcus faecalis*	MF 42432.1	94
D20-2	*Llliottia amnigena*	KR 045591.1	100
D20-3	*Bacillus cereus*	KY 622540.1	96
D30-1	*Enterococcus faecalis*	MH 327503.1	100
D30-2	*Enterococcus faecalis*	MH 327503.1	100
D30-3	*Enterococcus faecalis*	MH 327503.1	100
D45-1	*Enterococcus faecalis*	MH 327503.1	100

D45-2	*Azotobacter salinestris*	KF 470803.1	81
D45-3	*Enterococcus sp.*	KU 644346.1	99
D60-1	*Enterococcus faecalis*	MF 424362.1	100
D60-2	*Gluconobacter japonicus*	KT 964237.1	94
D75-1	*Enterococcus faecalis*	MH 327503.1	100
D75-3	*Bacillus thuringiensis*	KJ 589489.1	96
D90-1	*Enterococcus faecalis*	KJ 585671.1	98
D90-2	*Enterococcus faecalis*	KJ 585671.1	98

[a]NCBI accession number from http://blast.ncbi.nlm.nih.gov/Blast.cgi
[b]number of identical base/total length of DNA sequence

Table 2: Sequencing results of selected yeast

Isolate no.	Closest relative	[a]Source	[b]Similarity (%)
YGD0-1	*Hanseniaspora sp.*	MG 813538.1	100
YGDO-2	*Hanseniaspora uvarum*	MF 348240.1	100
YGD5-1	*Mycobacteroides abscessus*	CP 029074.1	94
YGD5-2	*Candida orthopsilosis*	MH 171915.1	87
YGD5-3	*Candida orthopsilosis*	MH 166846.1	99
YGD10-2	*Hanseniaspora uvarum*	KP 010406.1	91
YGD10-3	*Candida orthopsilosis*	MH 166846.1	100
YGD20-1	*Pichia kudriavzevii*	KP 675276.1	99
YGD20-2	*Hanseniaspora uvarum*	KF 728823.1	99
YGD20-3	*Hanseniaspora uvarum*	KF 728823.1	100
YGD30-1	*Hanseniaspora uvarum*	KF 728823.1	100
YGD45-1	*Candida boidinii*	EF 197945.1	100
YGD60-1	*Hanseniaspora uvarum*	KF 728823.1	100
YGD60-2	*Hanseniaspora opuntiae*	MG 871180.1	99
YGD60-3	*Hanseniaspora uvarum*	KF 728823.1	99
YGD75-2	*Hanseniaspora valbyensis*	KT 758317.1	99
YGD75-3	*Hanseniaspora valbyensis*	KT 758317.1	99
YGD90-1	*Hanseniaspora sp.*	KU 350316.1	85
YGD90-2	*Hanseniaspora vineae*	KY 103582.1	86

IV. CONCLUSION

In conclusion, this is the first research on the population dynamic involved in spontaneous fermentation of *Garcinia Mangostana* pericarps. The result shows that the *Enterococcus faecalis sp.* which is categorized in lactic acid bacteria was became a predominant microorganism that presence in the fermentation of *Garcinia Mangostana.* However, in this study the small amount of LAB population was identified due to the limited number of selected isolates .The LAB can be exploited as consortium for starter culture development since it have probiotic properties and have ability to improve the nutritional value of fermented food. This study also showed that the pathogen only survive from day 0 to day 5 and totally disappeared until the end of fermentation due to the acidic condition at lower than pH 3.5. Lastly, it is recommended to further research on the potential of lactic acid bacteria to become probiotic.

V. ACKNOWLEDGMENT

A very special thanks of gratitude to my research project supervisor, Sir Mohamad Sufian So'aib who gave me opportunity to do this great project and who also gave me ideas, guidance, patience, encouragement, feedback and endless support throughout the preparation of this research project. I would like to take this opportunity to thank to everyone who had assisted me and Universiti Teknologi Mara in the completion of this research project within the limited time.

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