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Population dynamics and biodiversity during spontaneous fermentation of *Garcinia mangostana* pericarps

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Abstract

This study investigates the population dynamics and microbial diversity during spontaneous fermentation of *Garcinia mangostana* pericarp (GMP). The spontaneous fermentation was carried out anaerobically for 100 days. Population dynamics was determined by enumerating pure isolates on different selective media. The microorganism's identities were determined from the amplified 16S rDNA and 5.8S-ITS rDNA genes of bacteria and yeasts respectively. LAB and yeast were prevalent during fermentation at 10^3–10^4 cfu/ml and 10^3–10^7 cfu/ml, respectively. Around eight bacterial genera such as *Klebsiella, Enterobactor, Mangrovibactor, Escherichia, Cronobactor, Komagataeibacter, Lactobacillus* and *Bacillus* whereas five yeast genera *Meyerozyma, Saccharomycete, Hanseniaaspora, Mycobacterium* and *Pichia* were detected. *Lactobacillus brevis* and *Lactobacillus plantarum* were amongst LAB species identified. Lactic acid was suspected to promote LAB growth and total inhibition of pathogens after 21 days. The results highlighted potential starter culture consortium for future product development and conferred the microbiological safety of spontaneously fermented GMP product.

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1.0 Introduction

Fermentation is a common technique to preserve staple food, fruits, vegetables, herbs, and other edible materials as well as improving their nutritional value and sensory quality (Swain et al., 2014; Ho et al., 2015). The excellent attributes of some traditional fermented foods such as *kimchi* (Korea) (Chang et al., 2008) and *sauerkraut* (Holzapfel, 2002) earned them as international delicacy. In various regions of the globe, traditionally fermented foods are derived from wheat, starch, milk, maize, and known by their local names such as *doklu* (maize-Africa) (Assohoun-Djeini et al., 2016), *tape* (tapioca-Indonesia) (Lilis, 2015), *tempoyak* (Malaysian durian) (Chuah et al., 2016), and *kishk* (milk-Egypt) (Holzapfel, 2002). Despite their millennial manufacturing tradition, the standard production method is largely a low-tech, a household scale by applying spontaneous fermentation. In fact, the starter culture used on tape (ragi), dairies (*Lactobacillus*) (Gutierrez-Orozco & Failla 2013), and soy sauce (*Aspergillus oryzae*) (Peres et al., 2000) were originally evolved from repeated 'back slopping' of multiple spontaneous fermentation batches which iterated the best-adapted strain (Holzapfel, 2000). For this reason, fermentation becomes the most convenient food preservation method for the undernourished and impoverished population (Holzapfel, 2000). 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 (Lilis, 2015). The safety and shelf stability of the fermented products were attributed to the bacteriocin secretion of LAB which inhibited the growth of foodborne pathogens which otherwise become potential cause of food poisoning such as diarrhoea (Lilis, 2015).

Fermentation has been applied on some medicinal plants to overcome the low bioavailability of their native polyphenolic compounds. This was demonstrated by fermentation of Myrus communis berries (Curiel et al., 2015), cactus pear (*Opuntia ficus-indica* L.) (Filannino et al., 2016), and *Echinacea spp* (Rizzello et al., 2013) using LAB such as *L. plantarum, L. brevis, and L. rossiae* as starter culture. The fermented extract of these plants displayed enhanced bioactivities in terms of higher antioxidant activity

upon various *in vitro* and *ex vivo* bio-assays which can be attributed to the release of free phenolic acids and flavanols because of esterase activity during fermentation. The low bioavailability is also relevant to mangosteen (*Garcinia mangostana*) which possesses myriad therapeutic properties such as anticancer (Yu et al., 2009), anti-inflammatory (Chen et al., 2008), antiviral (Pedraza-Chaverri, 2008), and antioxidant (Yu et al. 2007). Substantial attention has been dedicated to its prominent xanthone compounds (α-mangostin, β-mangostin, and γ-mangostin) of its pericarp in these studies. The fermentation of mangosteen was demonstrated by α-mangostin extract fermentation by *Colletotrichum gloeosporioides* (EYL131) and *Neosartorya spathulata* (EYR042) fungi which aimed to enhance the α-mangostin bioactivity (Arunrattiyakorn et al., 2011). Another application of fungi (*Saccharomyces boulardii*) as starter culture was reported by Mantovani (2010) who invented an encapsulated mixture of fermented whole mangosteen fruit suspension and conventional mangosteen extract which reportedly had 11–16 times higher bioavailability than conventional mangosteen extract. Biotree PLT—a local company, used fermented extract of koji fermentation by *Aspergillus oryzae* as substrate for its subsequent fermentation of mangosteen pericarp. However, the bioavailability and bioactivity of the fermented product were neither reported (Biotree, 2016).

To date, limited study on spontaneous fermentation of GMP known to exist. Nevertheless, this fermentation technique may improve the palatability, nutrition, and therapeutic functionality of GMP as demonstrated by traditional fermented foods and various medicinal plants which used fermentation as their manufacturing technique. In this study, population dynamics and the microorganism identities during spontaneous fermentation of GMP were revealed. The results will be useful to assert the biological safety and probiotic content of fermented GMP as well as potential starter consortium for process improvement in future

2.0 Methodology

2.1 GMP fermentation and sampling

Mangosteen fruits were purchased from a local market in Shah Alam, Malaysia. After removing the pulp and washing the pericarp, it was shredded into smaller pieces and used as feedstock. The feedstock was loaded into 5 L benchtop bioreactor (INFORS) at 10% (w/v). The distilled water was added to make up 5 L and sugar was added at 10% (w/v) as initial substrate. The fermentation was carried out anaerobically for 90 days at room temperature. Sample collection was carried out at day 0, 2, 6, 8, 21, 30, 48, 60, 75, 90, and 100 of the fermentation. The pH reading of each sampling day was taken.

2.2 Microbiological analysis: Enumeration of microbial population

Each fermented *G. Mangostana* pericarp broth sample was properly mixed to ensure homogenisation of the microbes present in the fermented product. About 0.1 ml of collected sample was pipetted aseptically into 0.9 ml (1:10 dilution) of sterile peptone solution. The mixture was spread-inoculated on several selective agar media: Potato dextrose agar (PDA), Man-Rogosa Sharpe (MRS) agar, plate count agar (PCA), and MacConkey agar. All inoculated plates were incubated anaerobically in candle jar at 30 °C for 1–2 days (for PDA, MRS, and PCA) and 37 °C for 1–2 days (for MacConkey). After incubation, the viable single colony was enumerated and expressed in terms of colony forming units per millilitre (cfu/ml) of broth sample. Later, three colonies from each medium of each sampling time were randomly selected, enriched in liquid broth and stored at −33 °C in 50% glycerol solution until further analysis.

2.3 DNA extraction and purification

A tool kit for rapid DNA extraction from pure culture was used according to the protocol described by the manufacturer; Wizard® Genomic DNA Purification Kit (Promega Corporation, USA) for Isolating Genomic DNA from Yeast and Isolating Genomic DNA from Gram Positive and Gram Negative Bacteria.

2.4 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 $MgCl_2$, 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 was 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.

2.5 Sequencing 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.

3.0 Results and discussion

3.1 Population dynamics of microorganisms

The population dynamics during spontaneous fermentation of GMP is shown in Fig. 1. The LAB prevalence (on MRS agar) since the early phase into the final phase of fermentation was based on its population range of 10^3 to 10^4 cfu/ml. Yeast population showed a sharp spike in the first week of fermentation, and then rapidly declined and finally settled at 10^3 to 10^4 cfu/ml. Interestingly, the growth of enterobacteria on MacConkey agar stopped at day 48 onwards.

Simultaneous increase of yeasts and LAB could be due to a symbiotic relationship between them in which LAB provide an acidic environment which enables the proliferation of yeasts. The yeasts in turn provide growth factors like amino acids and vitamins for the growth of the LAB (Muyanja et al., 2003). Yeasts and LAB were recognised as the principal microorganisms in most of traditional fermented foods, cereals for the production of indigenous Nigerian foods (Nwachukwu et al., 2010), *kimchi* (Park et al., 2009), and *tempoyak* (Wardani et al., 2010). The composition of the microbial populations of both the traditional food and fermented *Garcinia mangostana* pericarp has consistently shown the presence of both LAB and yeasts during the different steps of the production process. The association of yeasts and LAB could also result in the production of metabolites which could impact on the flavour and taste of fermented products (Hansen & Hansen, 1996).

The LAB presence in fermented product is desirable to provide the probiotic effects and inhibiting the pathogen's growth. The inhibition of pathogen's growth could be attributed to the low pH caused by the proliferation of LAB and competitive factor which the dominance bacteria like LAB and yeast uptake all the nutrient (Forsythe, 2011). The key preservative effect of LAB is through the production of lactic acid (Schnürer and Magnusson, 2005) which decreases the internal pH of bacterial cells, thus denaturing their proteins and causing a loss in cell viability. This action causes inhibition to the growth of food spoilage organisms as well as pathogens which can cause food poisoning and disease (Ananou et al., 2007).

The change in pH during fermentation time is also Fig. 1. The pH value was initially high, around 3.9 and decreasing with time with a stationary value around 3.3. Such trend hinted accumulation of lactic acid fermentation which was correlated with the growth of LAB mentioned earlier. In turn, lactic acid played as inhibitor to pathogenic growth, thus explaining the absence of pathogen's population after day 48. This observation is highly desirable because the absence of pathogens confers the product's safe consumption (Kunene et al., 2000).

3.2 BLASTn analysis

Prior to sequencing analysis, amplified DNA extract of pure isolates were screened by electrophoresis on agarose gel to check their purity. Pure DNA appeared as single band under UV light illumination as shown in Fig. 2. The identity of genomic DNA based on its sequence homology in NCBI database. Later, phylogenetic tree as shown in Fig. 3 was constructed to show genetic relatedness between different species.

The pure culture from PCA agar (total plate count) showed a result of DNA sequence of *Klebsiella* species for early sampling day from day 0 to day 8 with similarities of 98 to 96%. On day 21, *Mangrovibacter plantisponsor* was identified based on similarities of 98 to 96%. For day 48 to 90, bacteria from *Bacillus* species was found with similarities of 98 to 97%.

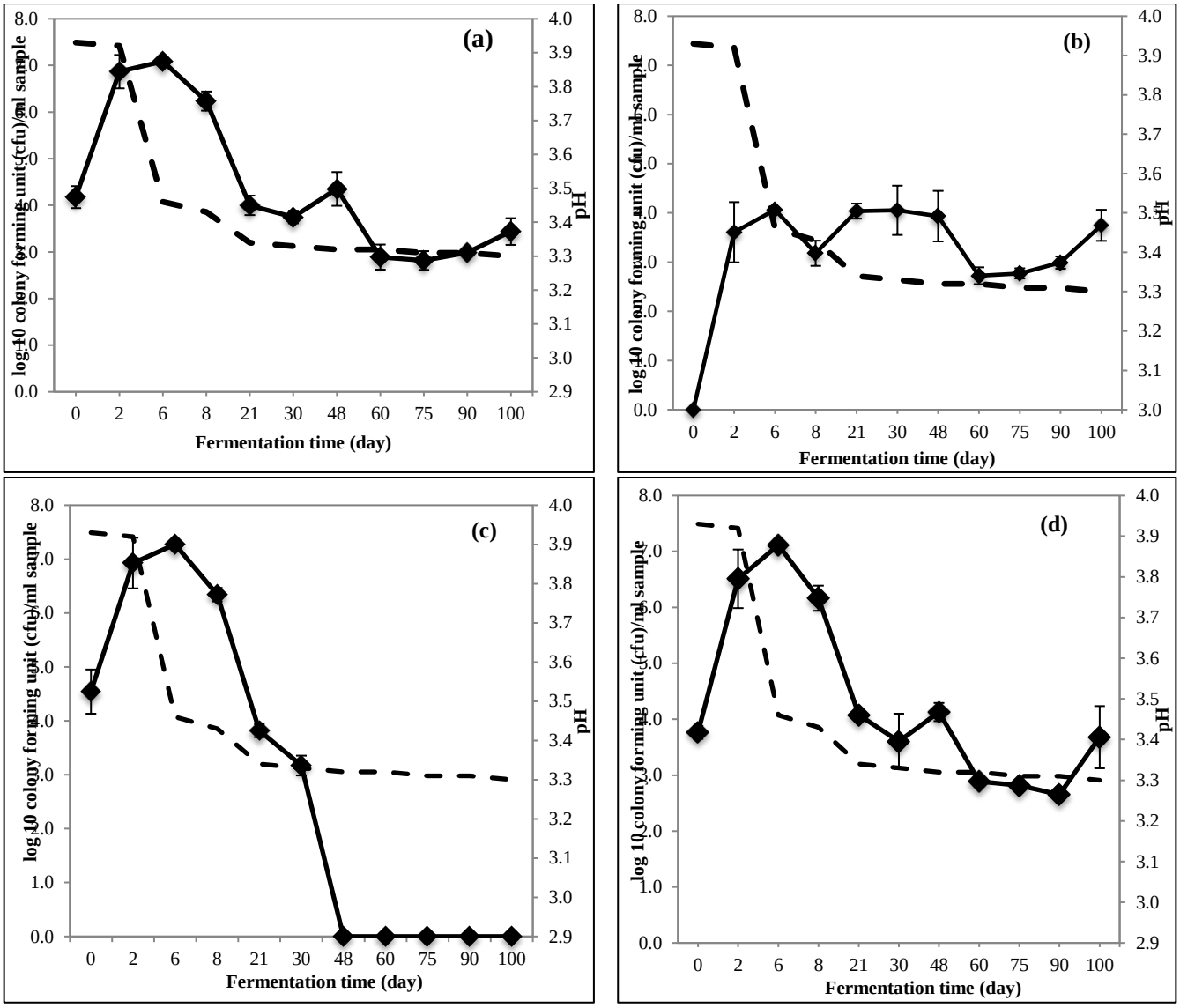

Fig. 1: Population dynamics of bacteria and yeast on different selective media a) PCA b) MRS c) MacConkey d) PDA.

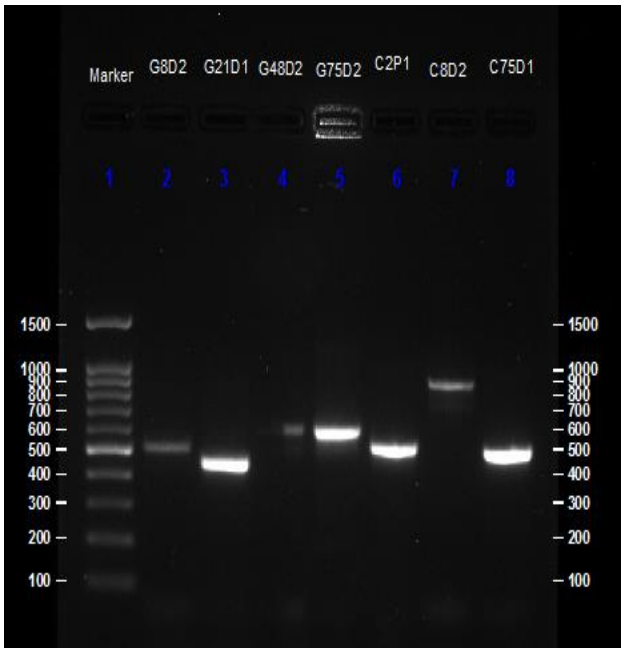

Fig. 2: DNA bands of pure isolates on agarose gel.

It was shown that throughout fermentation day, population dynamics of different bacteria species indicated their different degree of survival in the ecosystem. For example, the pathogenic bacteria were present during early stage before being substituted by the *Bacillus* species at the end.

Meanwhile, for the selective media sample (PDA agar) of fungal isolates, the blast analysis showed that four genera; *Pichia kudriavzevii*, *Saccharomycete sp.*, *Hanseniaspora lachancei*, and *Meyerozyma caribbica* were present in the ecosystem with high similarities over 99 to 100% with NCBI database. For the selective media MRS for lactic acid bacteria (LAB), the similarities obtained were between 88 to 97 %. The overall BLASTn result identified species from *Lactobacillus plantarum, Mycobacterium sp., Komagataeibacter saccharivorans, Lactobacillus brevis*, and *Bacillus cereus*; all belong to LAB species.

The presence of *Bacillus* genus is desirable in this fermentation for its role in enhancing the bio-availability of the compound. As for the MacConkey agar, *Klebsiella variicola, Enterobacter sp., Enterobacter asburiae, Escherichia coli,* and *Cronobacter sakazakii* were species identified from BLASTn analysis.

These bacteria were considered pathogenic and harmful to health. Throughout the fermentation, these bacteria were very likely inhibited by the low pH. However, they were viable on MacConkey medium which provided ideal enough growth condition (which otherwise inhibited in original fermentation ecosystem)

3.3 Phylogenetic tree construction

The alignment of sequence retrieved from the Gene Bank database available at the NCBI website (http://www.ncbi.nlm.nih.gov/) and construction of neighbour joining tree analysis using Clustal W (1.6) were performed using mega 7.0.26 software. Phylogenetic trees contain a lot of information about the inferred evolutionary relationships between a set of species. The clustered taxa in the phylogenetic tree are descendent from a common ancestor. The phylogenetic tree displays evolutionary relationship between different species of microorganisms as shown in Fig. 3.

The evaluator lineages can be determined from the horizontal line generated from the phylogenetic tree which changing over time; the shorter the horizontal branch the amount of change is less. Meanwhile, the vertical lines connected to the horizontal lines represent how long they are present. Based on phylogenetic tree of Fig. 3, the phylum of proteobacteria had the highest number of species among the selected pure isolates i.e. 13 species. Meanwhile the bacteria species of *Komagataeibacter saccharivorans* was the outgroup in proteobacteria. It was possible that the genomic profile of the out-group differed significantly from other bacterial species.

There were eight bacteria genera were identified; *Klebsiella, Enterobactor, Mangrovibactor, Escherichia, Cronobactor, Komagataeibacter, Lactobacillus,* and *Bacillus*. Previous studies have attributed the fermentation of *tempoyak* to different LAB species and these studies concluded that *Lb. plantarum* was the predominant organism responsible for the fermentation (Leisner et al., 2001; Yuliani & Dixon 2011; Chuah et al., 2016). In this study, the *Lb. plantarum* was detected at mid-fermentation time. This species commonly found on fermented vegetable and may have been the predominant species during fermentation. The existence of large species of *Bacillus cereus* was unfavourable due to its pathogenic characteristic. However, since this spontaneous fermentation employed indigenous microflora rather than starter culture, the complete profile of the microorganisms presents during fermentation well characterized. From the phylogenetic tree, the only LAB species that found is *Lb. plantarum* and *Lb. brevis*. This is due to poor sequence result and few samplings were damaged during the experiment. As for the fungi kingdom, four genera of fungi were found which were *Hanseniaspora, Pichia, Saccharamycetes* and *Meyerozyma.* The existence of fungi group was concurrent with the LAB where the yeasts provided growth factors like amino acids and vitamins for the growth of the LAB.

3.4 Pure isolates

Commonly, the spontaneous fermentation is associated with lactic acid fermentation. The rapid decline in pH and increase in lactic, acetic and propionic acids content can be attributed to LAB dominance. Microbiological studies on fermented products of Western Europe, Africa and Asia which were derived from various raw materials such as milks, meats, cereals, fruits and vegetables highlighted the predominance of LAB which were believed to benefit sensory quality, digestibility, detoxification, microbiological safety, health-promoting and shelf stability of the spontaneous fermentation products (Caplice & Fitzgerald 1999). Identification of LAB in the fermentation ecosystem yielded two prominent LAB species which were *Lb. plantarum* and *Lb. brevis*. Both species play an important role in nutritious and safety aspects of fermented foods.

Various studies have reported on the role of *Lb. plantarum* and *Lb. brevis* in the fermented products such as cucumber, *sauerkraut*, *tempoyak* and *kimchi* (Chuah et al., 2016, Breidt, 2004, Jung et al., 2014). The LAB presence during GMP fermentation was expected since it employed similar spontaneous fermentation technique with the foregoing examples.

As reported by Breidt (2004), the dominance of heterofermentative *Leu. mesenteroides* during the early stages of vegetable fermentation produced a significant amount of acetic acid in addition to lactic acid, as well as carbon dioxide which rapidly lower the pH and

creating an anaerobic condition which favoured the growth of more acid tolerant homofermentative *lactobacilli* such as *Lb. plantarum*. In this study, *Lb. plantarum* was found during the early stage of fermentation and *Lb. brevis* was found later at the end of fermentation.

Since GMP fermentation exploited indigenous microflora rather than pure culture (starter culture), the complete microorganism profile which responsible during fermentation was yet to be highlighted. Antibiotic-resistant organisms may be presented in fermented GMP. Previous study of fermentation of *tempoyak* (Chuah et al., 2016), the *Lb. plantarum* isolated from *tempoyak* were resistant to multiple antibiotics of various classes. This study was in agreement with the study reported by Nawaz et al. (2011) who also reported on multi drug resistant of *Lb. plantarum* isolated from fermented foods. The LAB could serve as a reservoir of antibiotic resistance genes with the potential to be transferred to humans, animals and pathogenic microbes via the food chain though many LAB strains are not pathogenic (Ashraf & Shah 2011). Numerous studies have reported on a wide range of antibiotic resistance detected in *lactobacilli* as being intrinsic, hence, safety issue is not of the worry (Ashraf & Shah, 2011).

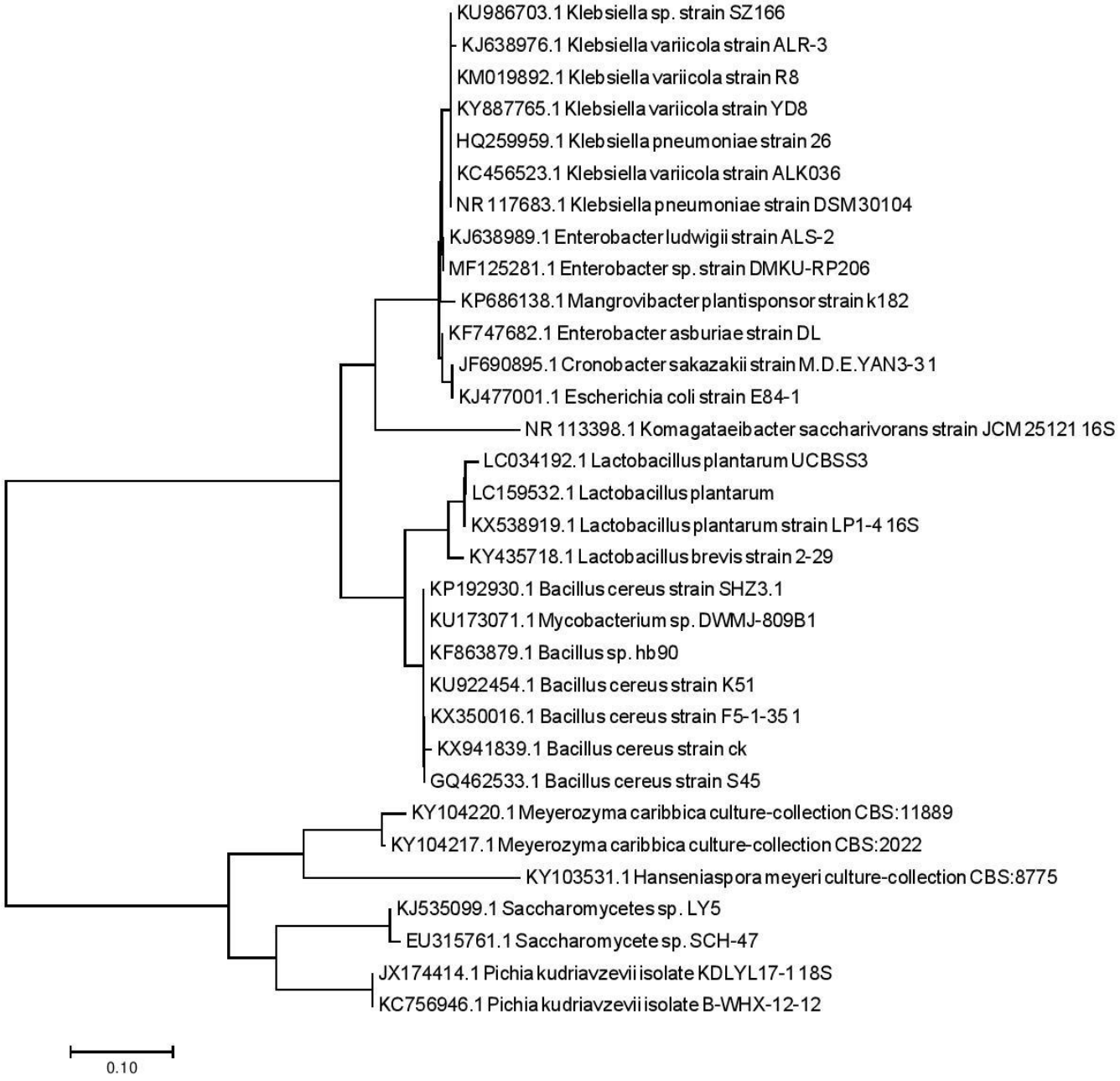

Fig. 3: Phylogenetic of microbial species relationships among 32 pure isolates identified from their 16S rRNA gene sequences. The tree was constructed using the NJ method.

4.0 Conclusion

In conclusion, the study shed light on the diverse LAB, other bacterial species and yeast during the course of the fermentation. The increase in yeasts and LAB population could be due to a symbiotic relationship between the two where LAB provided an acidic environment which favoured the proliferation of yeasts. Nevertheless, this study showed that the pathogens survive up to 21 days if there was contamination at any stage of fermentation. The diverse indigenous LAB microflora provided a prospective consortium for product development in future. Even though this study does not anticipate the selection of starter culture for GMP fermentation, further research on investigating the potential probiotic properties of LAB microflora is necessary.

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