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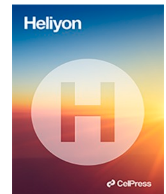

The indigenous microbial diversity involved in the spontaneous fermentation of red dragon fruit (*Hylocereus polyrhizus*) identified by means of molecular tools

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ABSTRACT

Red dragon fruit (RDF) is well-known for its high nutritional content, especially the red pigment betacyanins that possess high antioxidant activity. Natural fermentation is an ancient yet outstanding technique that relies on the autochthonous microbiota from fruits and vegetables surfaces to preserve and improve the nutritional values and quality of the food product. The present study was to evaluate and identify the indigenous microbial community (bacteria and fungi) that are involved in the natural fermentation of RDF. Results revealed a total of twenty bacterial pure cultures and nine fungal pure cultures were successfully isolated from fermented red dragon fruit drink (FRDFD). For the first time, the PCR amplification of 16S rRNA and ITS regions and sequence analysis suggested nine genera of bacteria and three genera of fungi (*Aureobasidium pullulans*, *Clavispora opuntiae*, and *Talaromyces aurantiacus*) present in the FRDFD. Four dominant (≥10 % isolates) bacteria species identified from FRDFD were *Klebsiella pneumonia*, *Brevibacillus parabrevis*, *Bacillus tequilensis* and *Bacillus subtilis*. The carbohydrate fermentation test showed that all the indigenous microbes identified were able to serve as useful starter culture by fermenting sucrose and glucose, thereby producing acid to lower the pH of FRDFD to around pH 4 for better betacyanins stability. The present study provides a more comprehensive understanding of the indigenous microbial community that serves as the starter culture in the fermentation of RDF. Besides, this study provides a useful guide for future research to be conducted on studying the rare bacterial strains (such as *B. tequilensis*) identified from the FRDFD for their potential bioactivities and applications in medical treatment and functional foods industries.

1. Introduction

Over the past decades, the development of foods that promote health and well-being is one of the key research priorities of the food industry. This has led to increased production and consumption of foods containing ingredients that may modulate various

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physiological functions; something beyond nutritional needs, which are recognised as functional foods [1]. One of the emerging functional food products introduced in recent years is fermented fruit/vegetable juice like fermented red dragon fruit drink (FRDFD) [2]. Fermentation is an ancient but outstanding technique to transform edible raw food materials into new food products for better preservation and improvement of food quality [3]. Usually, fermented juices are made by putting in the fruits and/or vegetables (single or mixed), with or without the addition of starter culture and other value-adding ingredients in an enclosed container to allow anaerobic fermentation to happen for weeks or months [4]. In this context, spontaneous fermentation usually relies on the abundant sources of autochthonous microbiota from the fruits/vegetables surfaces, or to some extent from the utensils used and the exclusive uncontrollable environmental conditions during the fermentation process [5]. The diverse microbiota, which includes coliforms, lactic acid bacteria (LAB), yeasts and moulds may play an important role during the spontaneous fermentation process [6]. These indigenous microbes are vital in improving the sensory quality and providing some additional values and functionalities to the product [4,5].

In 2012, a study had successfully identified several bioactive compounds (campesterol, stigmasterol, B-sitosterol, betanin and isobetanin) in fermented red dragon fruit drink (FRDFD) with betanin as the prominent bioactive compounds (with high antioxidant activity) present at a concentration of 14.23 g/L [2]. Following this, it has been proven that optimised fermentation was not only able to significantly increase the betacyanins concentration (ten-fold higher) but also capable of improving the stability of betacyanins (<20 % of loss after 8 weeks storage at 4 °C) in the red dragon fruits (RDF) [7,8]. As FRDFD is a product of natural fermentation, information on the different types of microorganisms involved in the fermentation process is important as those microorganisms are involved directly in the physical and chemical changes that occur during the fermentation process. To date, only a study conducted in 2012 has identified the LAB that present in FRDFD were either *Enterococcus faecalis* or *Enterococcus durans* [9]. However, fermented products often possess heterogeneous nature due to the variations in microbial diversity and properties as well as the fermentation parameters [5]. This infers that there are limited published studies on FRDFD, especially in the aspect of detailed evaluations of the diversity of the indigenous microbial community that takes part in the fermentation of RDF. The microbiota profile of FRDFD is crucial not only in revealing the food safety information of FRDFD but also could form the basis for the improvement of product quality and consistency. Therefore, the aim of this study was to isolate and identify the bacteria and fungi microbiota from FRDFD using molecular approaches. This study will provide a better understanding of the microbial community (specifically bacteria and fungi) involved in the fermentation of RDF, which served as a foundation for the development of suitable starter cultures for FRDFD. In addition, the FRDFD can be a valuable source of novel strains [10]. Hence, the investigations on the FRDFD microbial community could also help in discovering the potential microbes that can be isolated, cultivated and applied in commercial products to provide useful health benefits and/or medical functions.

2. Materials and methods

2.1. Raw materials

Fresh red dragon fruits (RDF) (*Hylocereus polyrhizus*) with an average weight of 0.4–0.6 kg were purchased from a local market (Aeon). The selected fruits were all at commercial maturity level; without damage on the skin and absent of insect. Brown raw cane sugar (BRCS) were purchased from local market.

2.2. Chemicals

Chemicals used in this study such as plate count nutrient agar, standard method broth, potato dextrose agar, potato dextrose broth and phenol red broth base are all obtained from HiMedia (India). Meanwhile, crystal violet solution, gram iodine solution, ethanol solution (95 % v/v), safranin and methylene blue solution were purchased from Sigma (St. Louis, MO, USA). The chemicals for molecular analyses including GF-1 bacterial DNA extraction kit, GF-1 plant DNA extraction kit, 2X Taq Master Mix reaction buffer, nuclease-free water and GF-1 PCR Clean-up Kit are all from Vivantis Technologies (Malaysia).

2.3. Preparation of fermented red dragon fruit drink (FRDFD)

RDF was rinsed with tap water to remove dirt and residues followed by air dried. Then, the skin of RDF was peeled while the flesh was cut into slices with each having an average thickness of 5 mm. The surface of the table was sterilised and wiped with 70 % ethanol before conducting the work to prevent microbial contamination. Stainless steel fermentation tanks with 2-L volume were autoclaved while other utensils were cleaned and rinsed with hot water and air-dried prior usage. Fermentation of RDF was carried out in 2-L stainless steel fermentation tank according to previously published protocols with slight modifications [2]. The RDF slices were arranged layer by layer alternately with 20 % sugar (w/w) in the fermentation tank in a sterile condition, with the air conditioning turned off and carried out beside flame lighted by Bunsen burner to prevent any specks of dust and particles that could contaminate the fermentation process. Lastly, fermentation tanks were closed tightly and stored for seven days in a clean cabinet at 25 °C. The FRDFD from the tank was strained and filtered through a sterilised sieve bag, pasteurised at 75 °C for 15 s and stored in individual amber bottles at 4 °C until further analysis.

2.4. Identification of microbes in FRDFD

2.4.1. Isolation of bacteria

The FRDFD was serially diluted (10^{-1}, 10^{-2} and 10^{-3}) and plated on plate count nutrient agar and incubated at 37 °C for 2 days. Isolates were sub-cultured on plate count agar at 37 °C until a pure culture was obtained. A single colony of pure culture was then transferred into standard method broth and incubated overnight at 37 °C [9]. The identification and selection of representative bacterial colonies was based on the difference in morphologies (shape, size and colour) [11–13].

2.4.2. Isolation of fungi

The FRDFD was serially diluted (10^{-1}, 10^{-2} and 10^{-3}) and plated on potato dextrose agar and incubated at 25 °C for 5 days. Isolates were sub-cultured on potato dextrose agar at 25 °C until a pure culture was obtained. A single colony of pure culture was then transferred into potato dextrose broth and incubated overnight at 37 °C [9]. The identification and selection of representative fungal colonies was based on the difference in morphologies (shape, size and colour) [11–13].

2.4.3. Gram stain (bacteria isolates) and simple stain (fungal isolates)

Gram stain was carried out on bacteria isolates. First, a loop of water was first placed on a microscope slide and an appropriate amount of culture from a single colony on the agar plate was mixed well with the water in a circular motion on the microscope slide. Then, the microscope slide was let air dry in the laminar flow and pass through the flame quickly a few times to fix the culture. After that, a few drops of crystal violet solution were added to the specimen and allowed to stand for 1 min. The solution was washed away with distilled water and gram iodine solution was added onto the microscope slide and stand for 1 min. Subsequently, gram iodine solution was washed away with ethanol solution (95 % v/v) and safranin was added onto the microscope slide. Lastly, safranin solution was washed away with distilled water and the microscope slide was blotted dry with filter paper before observed under a light microscope [14]. The morphology and Gram stain results of isolated bacteria were recorded.

The simple stain was carried out on fungal isolates. First, a fungal suspension was placed on a glass microscope slide and attached by gentle heating. Then, a few drops of methylene blue solution were added to the specimen and allowed to stand for 1 min. After that, methylene blue solution was washed away with distilled water and the microscope slide was blotted dry with filter paper before observed under a light microscope [15]. The morphology of isolated fungal was recorded.

2.4.4. Genomic DNA extraction

A total of 2 mL of cell suspension was centrifuged at 10,000 rpm for 3 min in a microcentrifuge. The genomic DNA of the isolates was extracted by using GF-1 bacterial DNA extraction kit for bacteria and GF-1 plant DNA extraction kit for fungus. Manufacturer's instructions were strictly followed to extract DNA from bacterial and fungal isolates. The extracted DNA was stored at −20 °C for further use [16].

2.4.5. Amplification of 16S rRNA and ITS regions using polymerase chain reaction (PCR)

Amplification of the genomic 16S rRNA region was performed in 50 μL reaction containing 25 μL of 2X Taq Master Mix reaction buffer (Taq DNA Polymerase, 0.4 mM dNTPs, and 3.0 mM $MgCl_2$), 2 μL each of 10 μM universal forward primer 27F and universal reverse primer 1492R, 2 μL of genomic DNA, and 19 μL of nuclease-free water. PCR was carried out in a Mastercycler thermal cycler (Eppendorf, Hamburg, Germany) with the following conditions: initial denaturation at 94 °C for 1 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min and final extension at 72 °C for 5 min [9,17].

Amplification of the internal transcribed spacer (ITS) regions was performed in 50 μL reaction as described above, using 2 μL each of 10 μM ITS1 forward primer and ITS4 reverse primer, with sequence shown in Table 1, 2 μL of genomic DNA, and 19 μL of nuclease-free water. PCR was carried out in a Mastercycler thermal cycler (Eppendorf, Hamburg, Germany) with the following conditions: initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 94 °C for 2 min, annealing at 56 °C for 2 min, extension at 72 °C for 2 min and final extension at 72 °C for 10 min [18].

The PCR products were analysed on a 1.0 % (w/v) agarose gel in 1X TAE buffer at 90 V for 30 min and purified using the GF-1 PCR Clean-up Kit. The purified PCR products were sent to First Base Laboratories for DNA sequencing (two directional) using the respective primer sets for 16S rRNA genes and ITS regions mentioned above [9,17].

2.4.6. Sequence analysis

The 16S rRNA and ITS sequences obtained from bacterial and fungal isolates were compared with the highest homology sequences

Table 1
Primers used for amplification.

Primer	Sequence (5′-3′)	T_m^a (°C)	GC Content (%)	References
27F	AGAGTTTGATCMTGGCTCAG	54.6	47.5	Pyary et al. (2016)
1492R	TACGGYTACCTTGTTACGACTT	54.6	43.2	Pyary et al. (2016)
ITS1	TCCGTAGGTGAACCTGCGG	60.6	63.2	Ciardo et al. (2006)
ITS4	TCCTCCGCTTATTGATATGC	54.7	45.0	Ciardo et al. (2006)

T_m^a, Melting temperature.

in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) from National Center for Biotechnology Information (NCBI, USA) using the BLASTN search [9].

2.4.7. Carbohydrate fermentation test

A cost-effective carbohydrate fermentation test was conducted using a 96-well (8 × 12 wells) autoclavable polypropylene microtitre plate. 300 μL of sterilised phenol red broth base added with 2 % of glucose or sucrose was added to each well. Each well was labelled and inoculated with bacterial or fungi culture while the un-inoculated well was served as control. The microtitre plate was covered with a lid and incubated at 37 °C for 2 days and at room temperature for 5 days for the growth of bacteria and fungi, respectively. Development of yellow colour was considered as a positive result [19].

3. Results and discussion

3.1. Isolation and identification of bacteria

In the present study, a total of twenty pure cultures of bacteria were isolated from FRDFD. Total genomic DNA was extracted from the microbial cultures and was subjected to molecular analysis. Approximately 1200 bp amplicon was obtained from bacterial isolates using 16S rRNA primers 27F and 1492R as shown in Fig. 1.

The nucleotide analysis identified the bacterial isolates to be *Klebsiella pneumonia*, *Brevibacillus parabrevis*, *Bacillus tequilensis*, *Bacillus subtilis*, *Bacillus licheniformis*, *Kocuria rhizophila*, *Lactococcus lactis*, *Leuconostoc pseudomesenteroides*, *Serratia liquefaciens*, *Enterobacter cloacae*, *Klebsiella oxytoca* and *Kosakonia sacchari*, as shown in Table 2.

Previously, a study carried out in 2012 has identified *Enterococcus faecalis* and *Enterococcus durans* to be responsible for the fermentation of RDF [9]. The disagreement of results with the present study could be caused by differences in the fermentation condition and processing techniques used, such as fermentation duration as well as the amount of sugar added during the preparation of FRDFD. Besides, there are multiple factors that could affect the populations and community structure of microorganisms present in any one type of fruit, including fruit cultivar, geographical location, fruit developmental stage, harvesting season, storage condition as well as nutritional compositions [6,20,21].

At the similarity level of 97 %, *K. pneumonia* was found to be the predominant bacteria (25.0 %) responsible for the fermentation of red dragon fruits in the present study. This is possible as several studies showed that the presence of *K. pneumonia* was common in fruit juice and fresh products, indicating that this bacterium is highly associated with fruits and vegetables due to its wide ecological habitats which included surface water, sewage, soils, and plants [22–24]. The past studies further confirmed that *K. pneumonia* can be frequently found in many types of raw vegetables and fruits such as cucumber, lettuce, pear, strawberry and grape [25,26]. Although it is well-known that *K. pneumonia* is an opportunistic pathogen that primarily attacks immune-compromised individuals and causes pneumonia, it was also often reported to have participated in the fermentation of many food products like soy sauces, tempeh and rice-based fermented beverage [11,25,27–29]. As the previous studies showed that *K. pneumonia* was able to synthesise vitamin B_{12} in the fermentation of tempeh, this indicated that *K. pneumonia* may possess some similar useful characteristics and functions in the fermentation process of RDF, such as possibly improving the nutritional values of the FRDFD by enhancing the vitamin B12 content [30]. Importantly to mention, the optimum growth temperature for *K. pneumonia* is between 35 °C and 37 °C and will be killed or inactivated by temperatures above 50 °C [31,32]. Therefore, heat treatment using pasteurisation (75 °C) in the present study is effective in preventing the growth of this pathogenic microorganism in FRDFD while inhibiting its rapid cross-contamination feature with other foods [33].

In this study, two bacteria that are considered rare to be found from fruits were isolated and identified (at the identity level of 97 %) from the FRDFD for the first time, namely *Brevibacillus parabrevis* and *Bacillus tequilensis* at 15.0 % and 10.0 % respectively. *B. parabrevis* is a short, small rod bacterium that was reported to have the common features of *Bacillus brevis* [34,35]. Recently, this species was also found in a fermented food condiment called "soumbala" which was made from African locust bean seed [36]. As a previous study has proven *B. parabrevis* for possessing positive plant growth-promoting characteristics by protecting the plant from metals uptake and

Table 2
Prediction of bacteria isolated from FRDFD based on 16S rRNA gene sequencing and their % identity based on NCBI Blast results.

Bacterial isolates	% identity	Number of isolates (%)	Gram stain
Klebsiella pneumonia	97	5 (25.0)	Negative
Brevibacillus parabrevis	97	3 (15.0)	Positive
Bacillus tequilensis	97	2 (10.0)	Positive
Bacillus subtilis	97	2 (10.0)	Positive
Bacillus licheniformis	96	1 (5.0)	Positive
Kocuria rhizophila	99	1 (5.0)	Positive
Lactococcus lactis	98	1 (5.0)	Positive
Leuconostoc pseudomesenteroides	96	1 (5.0)	Positive
Serratia liquefaciens	95	1 (5.0)	Negative
Enterobacter cloacae	94	1 (5.0)	Negative
Klebsiella oxytoca	91	1 (5.0)	Negative
Kosakonia sacchari	91	1 (5.0)	Negative

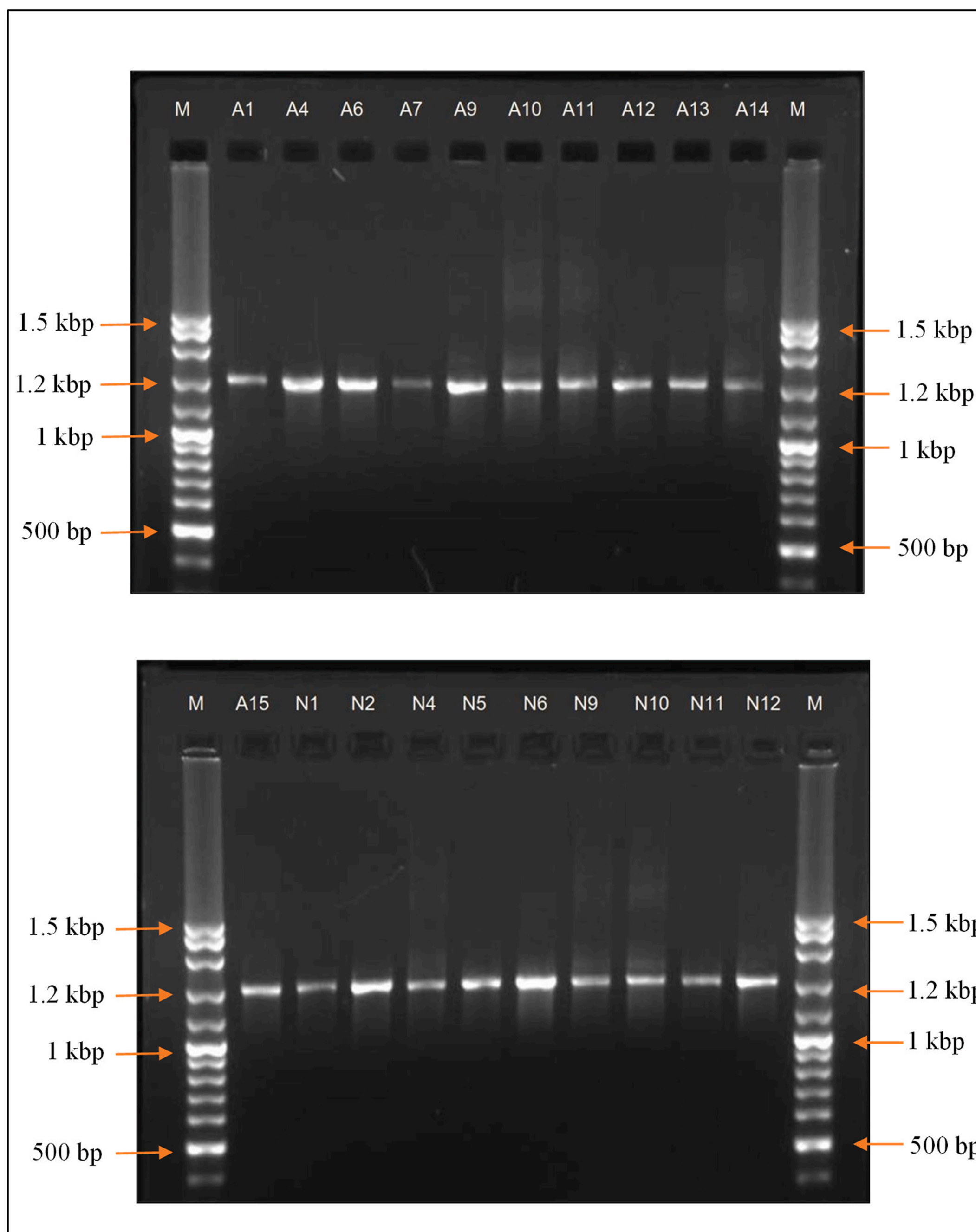

Fig. 1. Agarose gel analysis of bacterial amplicons using 16S rRNA primers. Lane M contained 0.5 μg of VC 100 bp PLUS DNA Ladder pre-mixed with loading dye. Lane A1 – N12 contained 2 μL of DNA amplification product generated with 2× Taq Master Mix for each isolate.

accumulating more nutrients for the plant, this indicated that *B. parabrevis* may assist in enhancing the RDF growth as well [37]. Besides that, this bacteria is a potential candidate that can be isolated from FRDFD to be cultured and studied for its possible application in the biodegradation of low-density polyethylene (LDPE), which is a type of polymers that is still difficult to be degraded safely nowadays [38].

In the previous study, *B. tequilensis* (a type of soil bacterium) was found in the fermented intestine of *Holothuria scabra* and has the ability to produce fibrinolytic protease with thrombolysis activity that is vital in the treatment of cardiovascular diseases [39]. Besides, the exopolysaccharides (EPS) produced by the *B. tequilensis* isolated from Thai milk kefir may also have a wide range of potential bioactivities including antioxidant, anti-inflammation, antibacterial and anticancer [40]. In a recent study, the *B. tequilensis* isolated from prolonged fermented soybean food was also found to be able to produce food-grade protease that is important in many food-grade

processes [41]. As the present study results (for the first time) showed that *B. tequilensis* was one of the dominant microbes found from FRDFD, the FRDFD can therefore serve as a good source for isolation of this bacteria to be cultured and studied on its potential bioactivities for future applications in medical treatment, functional foods and whitening cosmetics industries [40].

Similar to *B. tequilensis*, *B. subtilis* and *B. licheniformis* are usually present in soil and other natural environments to serve as plant growth-promoting rhizobacteria [42,43]. Besides that, many researches have shown that both species are also playing important roles in the food fermentation. For examples, *B. subtilis* was previously found in traditional rice-based fermented food and fermented soybean while *B. licheniformis* produced protease as food additive and showed probiotic potential in traditional fermented beverage [44–46]. Both species are also excellent GRAS (generally regarded as safe) candidates that can be isolated from FRDFD and cultured for large-scale production of commercially important chemicals and enzymes [47,48].

In addition, some of the bacterial isolates shown in Table 2 are common in food fermentation, such as *K. rhizophila* in dairy and meat fermentation; *L. lactis* and *L. pseudomesenteroides* in fruits and vegetables (like beetroot) and dairy products fermentation [10,12]. Recently, the fermented fruits and vegetables like FRDFD in the present study are important research targets due to many novel EPS produced by the LAB like *L. lactis* and *L. pseudomesenteroides* are found to associate with various outstanding biological properties [49].

In 2022, *S. liquefaciens* was isolated and proved to be the opportunistic pathogen that caused the rot disease of apple fruit [50]. Meanwhile, a previous study has isolated and discovered that *E. cloacae* and *K. oxytoca* were the bacteria responsible for attacking dragon fruit plants [51]. Different from these pathogenic bacteria, *K. sacchari* was found to be responsible for nitrogen fixation in relation to sugarcane plants [52]. This indicated that these microorganisms are common endophytes associated with fruits and plants, hence justifying their presence in the fermentation of RDF [51].

3.2. Isolation and identification of fungi

There was a total of nine pure cultures of fungi isolated from FRDFD in this study. The selection of representative fungi colonies was based on the difference in shape, size and colour [13]. Total genomic DNA was extracted from the microbial cultures and subjected to molecular analysis. ITS primer sets ITS1 and ITS4 generated approximately 350–600 bp amplicons from fungal isolates as shown in Fig. 2. The fragments generated in the present study were within the expected size of 350–800 bp long, as shown in a previous published study [53]. The nucleotide analysis identified the fungal isolates to be *Aureobasidium pullulans*, *Clavispora opuntiae*, and *Talaromyces aurantiacus* as shown in Table 3. The morphologies of the three identified fungal isolates were shown in Figure S1, S2 and S3, respectively.

Table 3 showed that *A. pullulans* appeared to be the prevailing fungal isolates (55.6 %) identified from FRDFD at a similarity level of 100 %. A literature search found that *A. pullulans* was associated with the stem and fruits spot of RDF in China [54]. The findings indicated that the same plant disease might have occurred in Malaysia's RDF plantation. Besides, *A. pullulans* was commonly found in the natural fermentation of fruit products such as pineapple juice and masau fruits [6,55]. A study published in 2020 revealed the capability of *A. pullulans* in modulating the fermentation kinetics and affecting the composition of fermented grape juice via the production of enzymes and extracellular polymers [56]. In recent years, the ability to produce pullulan (poly-α-1,6-maltotriose) for application in the food industry, coatings and wrappings potential has made *A. pullulans* to be one of the industrially important microorganisms. As the prevalent fungi identified from FRDFD, *A. pullulans* can be isolated to study for its function in producing metabolites that can be utilised as medical supplements or food ingredients/additives in the future [57].

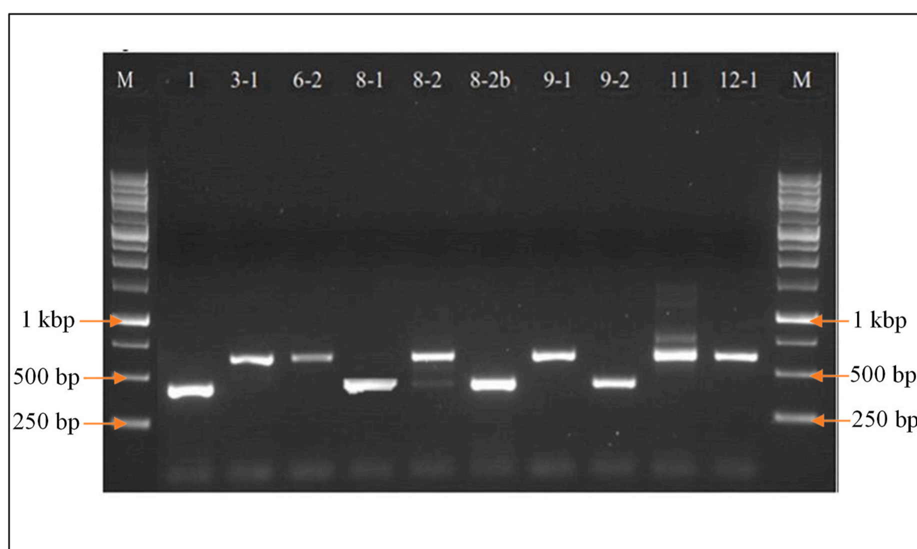

Fig. 2. Agarose gel analysis of fungi amplicons using ITS rRNA primers. Lane M contained 0.5 μg of VC 1 kb DNA Ladder pre-mixed with loading dye. Lane 1–12-1 contained 2 μL of DNA amplification product generated with 2× Taq Master Mix for each isolate.

Table 3
Prediction of fungal isolated from RDFD based on ITS gene sequencing and their % identity based on NCBI Blast results.

Fungal isolates	% identity	Number of isolates (%)
Aureobasidium pullulans	100	5 (55.6)
Clavispora opuntiae	99	3 (33.3)
Talaromyces aurantiacus	99	1 (11.1)

Apart from this, *C. opuntiae* was likely to occur in decaying cactus fruit as well as somatic tissue [58]. Meanwhile, previous studies have reported the isolation of *T. aurantiacus* from the soil in different countries [59,60]. Therefore, the occurrence of these two fungi in FRDFD was in expectation. *C. opuntiae* is a potential candidate that can be isolated and cultured for the production of ethanol in continuous fermentation from D-xylose, which is a major lignocellulosic crop residues and wood by-products [61]. On the other hand, it is also worth isolating and studying the *T. aurantiacus* from FRDFD for its potential bioactivities as a recent study has discovered the ability of this fungi (isolated from *Huperzia serrata*) to produce asterric acid derivatives that can function as acetylcholinesterase (AChE) inhibitor to treat Alzheimer's disease [62].

3.3. Spontaneous fermentation of red dragon fruit

At present, FRDFD was prepared by spontaneous fermentation relying on the indigenous microbes of the raw materials. Yeast and lactic acid bacteria are implicated in the fermentation of a wide variety of traditional food and beverage fermentations [63]. Results showed that the number of bacteria isolated (twenty pure cultures) was higher than the number of fungi (nine pure cultures), which suggested that bacteria have a stronger influence on the fermentation of red dragon fruit. It is well-known that the higher betacyanins content (which could be achieved by fermentation) could enhance the betacyanins stability [8,64]. Previously, it has been proven that the FRDFD contained high ethanol content (2.41 %) which indicated the high activity of the microbes such as the microbial protease activity could aid in the biosynthesis of betacyanins, through the breakdown of protein present in the RDF into free amino acids like tyrosine [8,65,66]. Moreover, the microbes implicated in the fermentation could also assist in hydrolysing the bounded phenolics complexes using proteolytic enzymes into the soluble free phenols, thereby releasing the bounded form betacyanins to increase the betacyanins content [8,65]. Besides that, the microbial consumption on the brown sugar during the fermentation process could cause the lowering of the total soluble solids (TSS) content and subsequently enhance the release of water-soluble betacyanins into the FRDFD through reverse osmosis process [8,67]. Nevertheless, a simple yet effective carbohydrate fermentation test showed that all the microbes identified in the present study were tested positive for sucrose and glucose fermentation. This indicates acid production as the end product of fermentation and explained the drop in pH from 5.06 in RDF juice to 3.64 in FRDFD. This further evidenced that fermentation serves as a way to lower the pH naturally to around pH 4 which is great for betacyanins stability [68]. Although some of the microorganisms identified from FRDFD possess a threat of foodborne pathogens, pasteurisation will be carried out before the consumption of this drink. The past study by Foong et al. (2012) and present studies confirmed that there was no presence of microorganisms in FRDFD after pasteurisation. Hence, the FRDFD in the present study falls under the satisfactory level of microbiological standards for ready-to-eat food, which is less than 10^4 CFU/mL of sample [69].

4. Conclusion

The present study successfully isolated and identified twenty pure cultures of bacteria (comprising nine genera) and nine pure cultures of fungi (consisting of three genera) from FRDFD using molecular approaches. All microorganisms identified from FRDFD have common ecological habitats such as soil and plants. This was likely to explain the occurrence of these microorganisms during the natural fermentation of RDF. The present results provided a more comprehensive understanding of the indigenous microbial community that participated in the fermentation of RDF. Yet, further studies can be aimed at the physicochemical and microbiological changes of the RDF throughout the fermentation process. Further investigations can be carried out on the metabolites produced by the identified microorganisms and their ability to produce and improve the content of beneficial compounds in the FRDFD. Among all the microbial isolates, *Bacillus tequilensis* was considered rare to be found from the natural fermentation of fruits and vegetables. The present study is the first to report *B. tequilensis* as one of the dominant microbes in the FRDFD. Hence, it is also recommended that future study can be conducted on isolating *B. tequilensis* from FRDFD to study their potential bioactivities in producing useful metabolites that can be applied in different aspects, particularly in the medical treatment and functional foods industries.

Data availability statement

The data associated with the present study has not been deposited into a publicly available repository. The data associated with the present study is included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Teck Wei Lim: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Formal analysis, Data

curation. **Kah Yee Choo:** Writing – original draft, Resources, Methodology, Formal analysis, Data curation. **Renee Lay Hong Lim:** Supervision, Conceptualization. **Liew Phing Pui:** Supervision, Conceptualization. **Chin Ping Tan:** Supervision, Conceptualization. **Chun Wai Ho:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21940.

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