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Molecular Characterization of Bacterial Isolates Contaminating from Dried Fruits

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Abstract

Producing and processing fruits are impacted by the global issue of fruit safety. Bacteria that might cause serious health issues can infect dried fruits. A total of 180 dried fruit samples including (raisins, dates, and apricots) were purchased from markets in Jeddah Governorate, Saudi Arabia. Morphological bacterial isolation of dried fruits was carried out by adding 5 g of each dried fruit sample to 5 ml of diluent (distilled water). A 0.1 ml volume of each dilution was used to make a series of pour plates. The plates were incubated at 30°C for 48 hr. In addition, the molecular identification analysis of bacterial isolates was confirmed by sequencing the 16S rRNA gene. The results revealed the isolation of 31 different species belonging to 3 genera of bacteria which are *Bacillus, Micrococcus*, and *Staphylococcus***.** The most dominant genus is *Bacillus sp.* (739 CFU) isolated from dried fruits. In conclusion, good manufacturing practices (GMPs) must be applied to maintain healthy dried fruits and reduce dried fruit contamination during and after harvest.

Keywords: Dried fruits, Bacteria, *Bacillus*, 16S rRNA

Introduction

Fruits and Vegetables, due to their high nutritional content, which helps prevent diseases like cancer, heart disease, and eye disease, are vital for maintaining a healthy body (Mostafidi *et al.*, 2020). Fruits are a great medium for microbial development even though they are a significant source of nutrients for humans (Mensah *et al.*, 2002; Sousa, 2008). Fruits are known to be carriers of both pathogenic and non-pathogenic microflora, and wrong handling can contaminate them at any point throughout their acquisition, including during growing and harvesting, processing, and distribution (Victor *et al.*, 2017). High levels of sugar, optimal water activity, harvesting techniques, and drying conditions are the primary causes of microbe contamination in dried fruits, rendering the fruits vulnerable to microbial infection (Trucksess & Scott, 2008; Ozer *et al.*, 2012). Thus, it is crucial to guarantee the safety of fruits and vegetables and to put the right policies in place to lower the possibility of microbial contamination (Olsen *et al.*, 2000; Beuchat, 2002). Contamination of dried fruits may indicate inadequate drying and storage conditions (Christensen & Kaufmann, 1965; Hedayati *et al.*, 2007). Sufficiently dried fruits are extremely low in moisture and include microbes such as fungi and bacteria. Previous reports on organic raisin samples have indicated the presence of bacterial isolates, including coliform bacteria like *E. coli*, in certain dried fruits (Torres, 2007). In this study, 180 dried fruits—both imported and locally produced—that were gathered from several Jeddah marketplaces had microorganisms identified from them. Both morphological and molecular techniques were used to identify the isolates.

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Materials and Methods

Collection of Dried Fruit Samples

A total of 180 local and imported dried fruit samples comprising Raisin, Date, and Apricot were collected under normal purchase conditions in January 2020 from different markets in Jeddah Governorate, Saudi Arabia. The samples were placed in sterile plastic bags and transferred to the laboratory for bacterial isolation.

Serial Dilution Method for Bacterial Isolation

From each of 20 samples of dried fruits (10 imported samples and 10 local samples), 5 g of each dried fruit sample (10 imported samples and 10 local samples) was suspended in 5 ml of sterilized distilled water to make serial dilution, and 100 ml of these dilutions were placed onto nutrient agar plates and incubated at 30°C for 48 hours (Aryal, 2021). To generate a pure culture and identify the isolates, a nutrient agar medium was utilized as a subculture medium.

Morphological and Molecular Identification of Bacteria Isolated from Three Different Types of Dried Fruits

Purification and Preservation of Isolated Bacteria

The isolated bacteria were streaked and sub-cultivated on nutrient agar plates, which were subsequently incubated at 30°C, to

produce pure cultures. After that, pure cultures were placed on slants made of the same medium and maintained at 4ºC. For long-term preservation, strains were maintained at -80ºC in 20% glycerol.

Identification of the Isolated Bacteria

Using morphological analysis, biochemical testing, and 16S rRNA, the isolated bacteria in this study were identified.

Morphological Characteristics

Gram Staining

Gram staining was used to identify the bacteria in cultures by distinguishing Gram-positive bacteria species from Gram-negative ones and determining the shape, size, and arrangement of the cells. The following reagents and equipment were used in this reaction: Crystal violet stain, Iodine, decolorizer (Acetone), Safranin, water, Light microscope, oil, slides, and loops.

Endospores Staining

Endospore staining was used to identify bacteria in specimens and cultures by distinguishing endospore-forming positive bacteria species from the endospore-forming negative ones. The following reagents and equipment were used in this reaction: Malachite green stain, Safranin, water, Light microscope, oil, slides, and loops.

Molecular Identification of Bacterial Isolates

Standard Buffers

1. Taq Master (Polymerase, dNTPs, and Buffer, 5X) for PCR amplification.
2. TAE running buffer (For nucleic acid horizontal and vertical electrophoresis).
3. DNA loading dye for electrophoresis.

Isolation of Genomic DNA

Bacterial isolates were characterized molecularly using the 16S rRNA gene sequencing method. The genomic DNA was obtained using a GeneJET Genomic DNA Purification kit according to the manufacturer's instructions. Cells were first cultured in nutrient broth medium, and then 10 ml of the culture (2×10^9 CFU/ ml) was harvested by centrifugation for 10 min. at 5000 × g. The pellet was suspended in 180 µL of bacteria lysis buffer and incubated for 30 min at 37°C. Then, 20 µL of proteinase K and 200 µL of lysis solution were added and vigorously mixed with a vortex or pipette to produce a homogeneous suspension. The sample was incubated for 30 minutes at 56°C in a water bath until the cells were lysed. 20 µL of RNase solution was added and mixed by using a vortex and incubated for 10 min at room temperature. After that, 400 µL of 50% ethanol was added and blended by pipetting or vortexing. In a collection tube, a GeneJET genomic DNA purification column was inserted, and the prepared lysate was transferred to it. After centrifuging the column for one minute at 6000 × g, the collection tube containing the flow-through solution was discarded. A new 2 ml collection tube was filled with the GeneJET genomic DNA purification column. After washing the DNA pellet with 500 µL of wash buffer I, it was centrifuged for 1 minute at 8000 × g. The flow-through was then discarded and returned to the collecting tube via the purification column. After that, the GeneJET genomic DNA purification column was filled with 500 µL of wash buffer II, and it was centrifuged for three minutes at its highest speed (≥12000 x g). Ultimately, the genomic DNA was extracted and placed in a sterile 1.5 ml microcentrifuge tube by centrifuging it for one minute at 8000 × g after incubating for two minutes at ambient temperature and adding 100 µL of elution Buffer to the middle of the GeneJET genomic DNA purification column membrane. The extracted DNA was kept at -20 °C and utilized right away in subsequent uses.

Polymerase Chain Reaction (PCR) Amplification

The isolates DNA was amplified using 16S rRNA universal primers: 27F (5′AGAGTTTGATCMTGGCTCAG3′) and 1492R (5′TACGGYTACCTTGTTACGACTT3′). the process was performed through a thermal Cycler. Each 50 µl reaction mixture contained 25 µL Mastermix (50 units/ml Taq polymerase, 400 µL moles DNTPs, 3 mM $MgCl_2$) 19 µL of ultrapure PCR water, 2 µL of each primer, and 2 µL of template DNA. The usual methods for amplification included an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for one minute, annealing at 58°C for one minute, first elongation at 72°C for two minutes, and final elongation at 72°C for five minutes. Ethidium bromide fluorescence validated the amplification process (Alrabiah *et al*., 2023; Ansari *et al*., 2023; Maiti *et al*., 2023 Petronis *et al*., 2023; Tirupathi *et al*., 2023).

DNA Visualization

After adding the PCR results to a 1.5% agarose gel, the experiment was performed at around 130 volts for 45 minutes. The electrophoresis gel (Horizontal gel electrophoresis, Cleaver Scientific, UK) was subjected to UV light visualization (gel DocImager, Biorad, USA) after being stained with ethidium bromide. To measure and identify PCR products, a 100 bp DNA ladder from Invitrogen USA was utilized as a marker. After that, the samples were shipped to the Macrogen Company in South Korea for sequencing and purification.

DNA Sequencing and BLAST Analysis

Sequencing was performed in Macrogen Company (South Korea). Low-quality bases were trimmed manually, and only the highest-quality bases were used. the DNA sequences of the fungi and bacterial isolates were used for BLAST analysis (blast.ncbi.nlm.nih.gov) using the default parameters, sequencing data were analyzed against the nucleotide collection database (Kiedrowicz *et al*., 2023; Kulkarni *et al*., 2023; Macrì *et al*., 2023; Marconcini *et al*., 2023; Shenoy *et al*., 2023).

Results and Discussion

Morphological and Biochemical Characteristics of Bacteria Isolated from Three Different Types of Imported and Local Dried Fruits

Bacteria were isolated from 60 (30 local and 30 imported) dried fruits **Table 1**. The samples were collected in January 2020 from different markets in Jeddah Governorate, Saudi Arabia. Serial dilution method was used for dried fruit samples and isolates were separately plated on nutrient agar medium, then incubated at 30°C for 48 hrs. The morphological characteristics of the bacterial isolates were observed on the nutrient agar the results of direct examination of each isolate using a light microscope based on morphology, gram staining, and endospores staining were presented in **Table 2**. 31 species belonging to 3 genera were isolated **Table 1**. These bacteria were identified to belong to the genera *Bacillus, Micrococcus,* and *Staphylococcus.* Total isolated bacteria were (753 CFU) from 60 dried fruit samples, the most dominant genera were *Bacillus* (739 CFU). The morphological appearance of the colony, such as colony color, shape, and surface characteristics, was documented. For fruits, by lowering their water content, they can be dried to enhance preservation by preventing microbial development and enzymatic reactions. The goods' high acidity, low moisture level, sugar content, and drying procedure will make them resistant to microbial deterioration. Food quality is decreased by microbial deterioration, endangering consumer health and resulting in large economic losses for the producing nations (Richard, 2007; Gonçalves *et al.*, 2019). When dried fruits come into contact with vegetative pathogens or their toxins—mostly derived from animal products—foodborne illness results. These are the main bacteria that affect food safety and cause illnesses in people all around the world when contaminated food is consumed. Bacteria are responsible for two-thirds of the world's high prevalence of foodborne illnesses in impoverished nations (Abebe *et al*., 2020). Thus, spoilage is seen as a serious issue for the global economy, agriculture, and public health. As a result, spoiled fruit poisoning poses a risk to human health as well as financial losses, particularly to exporting nations. The majority of dried fruits have thousands of microorganisms per gram of fruit, most of which are found on the outside surfaces. Improperly loaded and unclean drying trays can lead to a large increase in the number of germs growing throughout the drying process. Most dry fruits often deteriorate while being handled, transported, and stored (Frazier & Westhoff, 1988). The goal of the current investigation was to determine the kind and quantity of bacteria that are infecting dried fruits. The findings showed that 31 distinct species of bacteria from the genera Bacillus, Micrococcus, and Staphylococcus had been isolated. Bacillus sp. (739 CFU), the most prevalent species, was isolated from dried fruits (raisin, date, and apricot) that were imported and obtained from several marketplaces in the Jeddah Governorate, Saudi Arabia. It was discovered by Olsen *et al.* (2000), that pathogenic bacteria and their poisons exist. In this investigation, bacteria including Salmonella, Clostridium, E. coli, and Staphylococcus were isolated and identified. Victor *et al.* (2017), state that a range of microorganisms, including bacteria and fungi that are known to seriously harm fruit production, may have an impact on dried fruits. Spoilage is any change in food quality that makes it unpalatable or even poisonous. Unsanitary circumstances and surroundings might lead to the presence of pathogenic bacteria in fruits and vegetables that have been home-dried. As an example, slicing fruits and vegetables thinly with bare hands in the open might potentially contaminate the final goods with microorganisms. To increase the items' exposure to possible contamination, the pieces are also sun-dried for a few days outside (Victor *et al*., 2017). *Bacillus, Micrococcus,* and *Staphylococcus* were the main bacteria found in the dried fruits used in this experiment. For imported dried fruits, the total counts of bacteria were 242 CFU, but for local dried fruits, they were 511 CFU. Fruit safety is a major concern as, even with ideal farming, storage, and processing techniques, microbial contamination is believed to be an inevitable and unexpected problem (Quintela, 2020). Furthermore, it is important to adopt suitable food safety training, such as Hazard Analysis and Critical Control Points (HACCP), Good Manufacturing Practices (GMP), and Good Hygienic Practices (GHP), since these may significantly reduce pathogenic dangers in foods (Blumenthal *et al*., 2000; Ijabadeniyi & Buys, 2010).

Table 1. Diversity and count (CFU) of bacteria isolated from dried fruits at three different dried fruits (raisin, date, apricot) by Serial dilution method on NA medium at 30°C.

Isolate No.	Dried fruits / bacterial species	Raisin		Date		Apricot		Total Count (CFU)
		Imported Raisin (Yemeni)	Local Raisin (Saudi)	Imported Date (Egyptian)	Local Date (Saudi)	Imported Apricot (Turkish)	Local Apricot (Saudi)	
	Bacillus							**739**
YR7	*Bacillus aryabhattai strain ZXG-17*	5	-	-	-	-	-	5
YR9	*Bacillus atrophaeus*	2	-	-	-	-	-	2
YR4	*Bacillus cereus strain CASA51-1*	46	-	-	-	-	-	46
TA2	*Bacillus cereus strain S1-02*	-	-	-	-	62	-	62
YR6	*Bacillus clausii strain H39*	8	-	-	-	-	-	8
SD4	*Bacillus clausii strain KSM-K16*	-	-	-	1	-	-	1
ED14	*Bacillus haikouensis strain Q2As*	-	-	2	-	-	-	2

TA1	*Bacillus huizhouensis strain WJB119*	-	-	-	-	7	-	7
SR1	*Bacillus invictae strain MER_48*	-	3	-	-	-	-	3
SD6	*Bacillus megaterium strain 2-a*	-	-	-	1	-	-	1
SD8	*Bacillus methylotrophicus strain IARI-B-11*	-	-	-	1	-	-	1
TA9	*Bacillus niabensis strain ZJY-790*	-	-	-	-	7	-	7
SA3	*Bacillus pumilus strain PPL-SSC8*	-	-	-	-	-	2	2
ED2	*Bacillus* sp.	-	-	4	-	-	-	4
YR10	*Bacillus* sp. *hb114*	1	-	-	-	-	-	1
TA3	*Bacillus* sp. *HYC-1-3*	-	-	-	-	13	-	13
YR2	*Bacillus* sp. *KP071r*	37	-	-	-	-	-	37
SR4	*Bacillus* sp. *strain BAB-6021*	-	73	-	-	-	-	73
SD3	*Bacillus* sp. *strain BAB-6035*	-	-	-	325	-	-	325
YR8	*Bacillus* sp. *strain EFB(1B)B16*	5	-	-	-	-	-	5
ED13	*Bacillus* sp. *strain FJAT-21930*	-	-	4	-	-	-	4
ED15	*Bacillus* sp. *strain FJAT-22452*	-	-	1	-	-	-	1
TA7	*Bacillus* sp. *strain nenu_DS-R05*	-	-	-	-	22	-	22
SR2	*Bacillus* sp. *S205*	-	10	-	-	-	-	10
SR3	*Bacillus* sp. *X21(2015)*	-	30	-	-	-	-	30
ED12	*Bacillus* sp. *210_10*	-	-	2	-	-	-	2
SD7	*Bacillus* sp. *3LF 6TD*	-	-	-	1	-	-	1
SA4	*Bacillus subtilis strain STA2B*	-	-	-	57	-	4	61
SA1	*Bacillus subtilis subsp. inaquosorum strain St09*	-	-	-	-	-	3	3
	Micrococcus							**12**
ED5	*Micrococcus* sp. MT-L-S2	-	-	12	-	-	-	12
	Staphylococcus							**2**
ED9	*Staphylococcus caprae* partial	-	-	2	-	-	-	2
	Total count in all samples							**753**

(-) = Not detected

Table 2. Morphological characterization of bacterial strains bacteria isolated from dried fruits at three different dried fruits (raisin, date, apricot).

Isolate No.	Bacterial species	Morphological characteristics of bacterial isolates					
		Color	Form	Surface	Shape	Gram stain	Endospores stain
YR7	*Bacillus aryabhattai strain ZXG-17*	creamy	circular	smooth	rod	+ve	+ve
YR9	*Bacillus atrophaeus*	creamy	circular	smooth	rod	+ve	+ve
YR4	*Bacillus cereus strain CASA51-1*	creamy	irregular	smooth	rod	+ve	+ve
TA2	*Bacillus cereus strain S1-02*	creamy	circular	smooth	rod	+ve	+ve
YR6	*Bacillus clausii strain H39*	creamy	circular	smooth	rod	+ve	+ve
SD4	*Bacillus clausii strain KSM-K16*	creamy	circular	smooth	rod	+ve	+ve
ED14	*Bacillus haikouensis strain Q2aS*	orange	circular	smooth	rod	+ve	+ve
TA1	*Bacillus huizhouensis strain WJB119*	creamy	circular	smooth	rod	+ve	+ve
SR1	*Bacillus invictae strain MER_48*	creamy	circular	smooth	rod	+ve	+ve
SD6	*Bacillus megaterium strain 2-a*	creamy	circular	wrinkled	rod	+ve	+ve
SD8	*Bacillus methylotrophicus strain IARI-B-11*	creamy	circular	smooth	rod	+ve	+ve
TA9	*Bacillus niabensis strain ZJY-790*	creamy	circular	smooth	rod	+ve	+ve
SA3	*Bacillus pumilus strain PPL-SSC8*	creamy	irregular	wrinkled	rod	+ve	+ve
ED2	*Bacillus* sp.	creamy	circular	smooth	rod	+ve	+ve
YR10	*Bacillus* sp. *hb114*	creamy	circular	smooth	rod	+ve	+ve
TA3	*Bacillus* sp. *HYC-1-3*	creamy	circular	smooth	rod	+ve	+ve

YR2	*Bacillus* sp. *KP071r*	creamy	circular	smooth	rod	+ve	+ve
SR4	*Bacillus* sp. *strain BAB-6021*	creamy	circular	wrinkled	rod	+ve	+ve
SD3	*Bacillus* sp. *strain BAB-6035*	creamy	circular	smooth	rod	+ve	+ve
YR8	*Bacillus* sp. *strain EFB(1B)B16*	creamy	circular	wrinkled	rod	+ve	+ve
ED13	*Bacillus* sp. *strain FJAT-21930*	orange	circular	smooth	rod	+ve	+ve
ED15	*Bacillus* sp. *strain FJAT-22452*	orange	circular	smooth	rod	+ve	+ve
TA7	*Bacillus* sp. *strain nenu_DS-R05*	creamy	circular	smooth	rod	+ve	+ve
SR2	*Bacillus* sp. *S205*	creamy	circular	smooth	rod	+ve	+ve
SR3	*Bacillus* sp. *X21(2015)*	creamy	circular	wrinkled	rod	+ve	+ve
ED12	*Bacillus* sp. *210_10*	creamy	circular	smooth	rod	+ve	+ve
SD7	*Bacillus* sp. *3LF 6TD*	creamy	circular	smooth	rod	+ve	+ve
SA4	*Bacillus subtilis strain STA2B*	creamy	circular	wrinkled	rod	+ve	+ve
SA1	*Bacillus subtilis subsp. inaquosorum strain St09*	creamy	circular	wrinkled	rod	+ve	+ve
ED5	*Micrococcus* sp.*MT-L-S2*	yellowish	circular	smooth	coccus	+ve	-ve
ED9	*Staphylococcus caprae*	creamy	circular	smooth	coccus	+ve	-ve

(+ve): Indicates positive result with purple and green staining
(-ve): Indicates negative result with red staining

Molecular Identification of Isolated Bacteria

The DNA was extracted from 31 bacterial isolates and PCR amplification of the 16S rDNA gene was carried out using universal bacterial primers. The 31 isolates strong bands on agarose gel appeared as PCR products with predicted sizes ranging from 1500 bp are shown in **Figure 1**. Sequencing data from bacterial strains were aligned with sequences from closely related strains found in GenBank. Sequences from closely related strains found in GenBank were aligned with sequencing data from bacterial strains. Molecular identification of 31 bacterial strains to species level was based on the similarity level and comparison with sequences of the known species already published in NCBI databases. **Figure 2** shows Phylogenetic trees based on sequences of rDNA of the bacterial strains recovered in the current investigation that were aligned with closely related sequences obtained from the GenBank (Alrabiah *et al*., 2023; Ansari *et al*., 2023; Maiti *et al*., 2023; Petronis *et al*., 2023; Tirupathi *et al*., 2023).

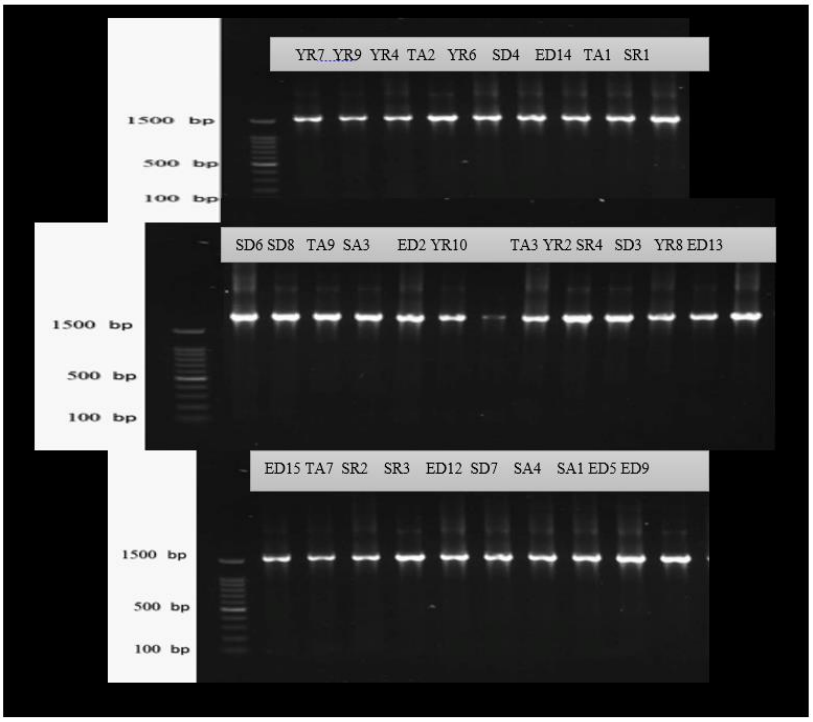

Figure 1. Gel electrophoresis of PCR product from 31 Bacterial strains. All data shown were obtained with 1492R primer. The range of DNA molecular size was 1500 bp compared to M (100 bp marker on the left side of the image).

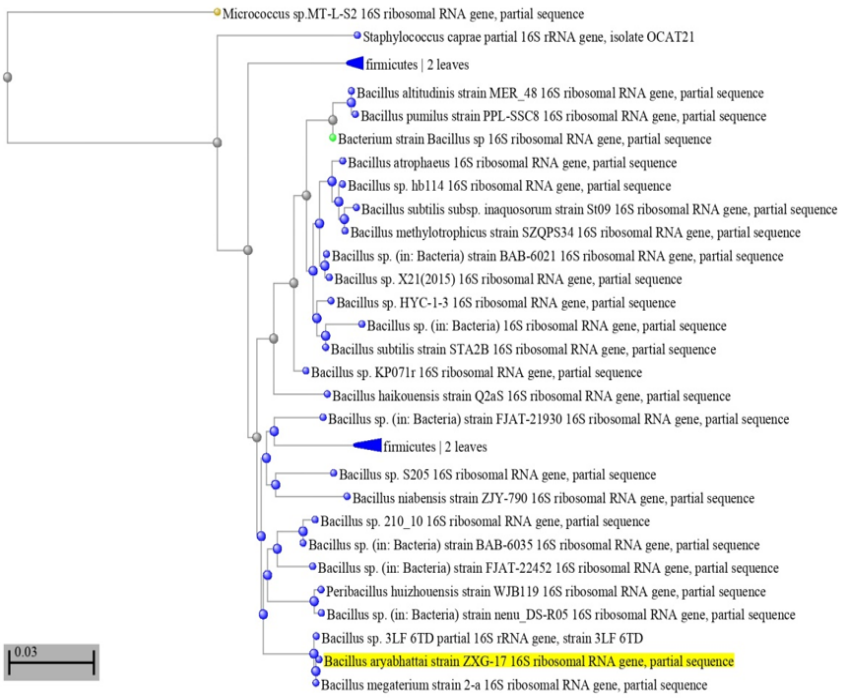

Figure 2. The Phylogenetic tree based on sequences of rDNA of the bacterial strains isolated in the present study aligned with closely related sequences accessed from the GenBank.

Conclusion

Bacterial contamination and development are common in dried fruits. The microbiological quality of imported and local dried fruits in Saudi Arabia was studied in this study, which resulted in the discovery of various potentially toxigenic bacteria such as *Bacillus* and others. The presence of potentially pathogenic organisms on dried fruits poses a public health risk to consumers. To minimize contamination of dried fruits that are hazardous to human health, thorough microbiological hygiene procedures must be implemented at all phases, including harvest, handling, transportation, storage, and drying.

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