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Article

Phenotype and Molecular Characterization of Fungal Isolation in Local Markets

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Abstract: The study included the isolation of fungi from different food samples of 38 local markets in Al-Karkh and Al-Rusafa areas in Baghdad. The samples included (shawarma samples , fried chicken samples , hamburger samples , pizza samples , lahm bi-ajeen (meat pie) samples , bulgur wheat and cucumber salad samples (tabbouleh) , cucumber-yogurt salad samples , mixed green salad samples , potato chip samples , popcorn samples , finger potato food samples cake samples The results of the study showed six species: Pencillium spp, Aspergillus spp, Saccharomyces spp, Rhizopus spp, Mucor spp, and Altenaria spp. Aspergillus spp was the most frequently occurring species with a percentage of 34.2%. Pencillium spp was the second most frequently occurring species in the samples with a percentage of 26.3%. Rhizopus spp was the third most frequently occurring species with a percentage of 23.1%. Saccharomyces spp was the fourth most frequently occurring species with a percentage of 13.1%. Mucor spp was the fifth most frequently occurring species with a percentage of 5.2%. The genus Altenaria spp was the least common fungus, at 2.6%. They were diagnosed morphologically and microscopically based on reliable taxonomic sources as well as molecular diagnosis of the samples.

Keywords: Foodborne fungi, Ready-to-eat foods, Molecular identification, PCR, ITS region, Aspergillus spp

Introduction

The fungi are heterotrophs, which means they cannot produce their own food, because they do not have the pigment chlorophyll [1]. They are eukaryotic. Thereby they acquire nutrients through the other organisms or saprophytic breakdown of organic substance. Those are the important properties that allow them to undertake a variety of tasks which have direct connections with human life [2]. The fungal challenge is one of the most important challenges at present because fungi are everywhere, in food, water, agricultural crops and animal products, which are all part of human food intake. Therefore, they pose a serious risk to human health due to their presence as a product of mycotoxins formed by some fungal species in the food chain [3].

Fungal contamination continues to be an important issue because of the food spoilage it brings about, which is mostly due to mycotoxins (secondary metabolites produced by a few genera of fungi including Aspergillus spp. and Penicillium spp.) [4]. The issue of the proliferation of toxigenic fungi in food products and the production of mycotoxins is of great concern in public health and consumer protection

[5]. The Food and Agriculture Organization (FAO) reports that storage molds produce mycotoxins that account for the loss of about 1,000 million metric tons of food products around the world annually [6]. In addition, mycotoxins have many negative effects on human health such as neurotoxic, nephrotoxic and hepatotoxic effects [7].

Aspergillus spp. and Penicillium spp. are known to produce mycotoxins in food during their preparation or storage [8], while genera like Fusarium spp., Aspergillus spp. and Penicillium spp. are predominantly present in agricultural crop fields [9]. Fungi and fungal toxins contaminated foods represent serious problems in many countries, especially in those where food preservation and storage conditions are poor [10]. Mycotoxins are metabolic by-products produced by fungi and these toxins contaminate food and feed commodities. Hazard of fungi is further worsened by the ability of fungi to produce mycotoxins in foods that are directly involved in human pathogenesis [11]. There is no doubt that around 300 fungal species are capable of causing disease from allergic reactions to life-threatening invasive infection [12].

Mycotoxins are regularly detected in food and chronically consumed (even in minimal quantities, which are already harmful to the human body) [13]. Thus, the aim of the current study was to isolate the fungal contaminants of the food products that are currently available in the local markets and to identify the fungi using the conventional phenotypic (morphological and microscopic) method and molecular diagnostic method.

Materials and Methods

Sample Collection:

A total of 38 random samples of various ready-to-eat (RTE) and fast-food items regularly or occasionally used by the consumers were collected from the local markets in Al-Rusafa and Al-Karkh districts in Baghdad. The samples collected included 4 shawarma, 4 fried chicken (Kentucky style), 6 French fries, 4 burgers, 4 appetizers (2 tabbouleh and 2 tzatziki/cacik), 4 pizzas, 4 meat pastries (lahmacun), 2 vegetable salads, 2 desserts/sweets, 2 potato chips and 2 popcorn samples.

Culture Media:

Potato Dextrose Agar (PDA):

39.9 g of PDA powder was dissolved in 1 L of distilled water to prepare the medium following the manufacturer's instructions. It was sterilized by autoclaving at 121 °C and 15 psi for 20 minutes. The antibiotic chloramphenicol was then added at 250 mg/L upon cooling, to inhibit bacteria growth. This medium was then transferred to sterile petri dishes and stored for future use.

Morphological and Microscopic Examination:

Potato Dextrose Agar (PDA) was used for the isolation of fungal species. For the solid foods (shawarma, burger, fried chicken, and French fries), 1 g was taken from each food sample and divided into 5 small pieces and placed onto a PDA plate, and three replicates were used for each sample. A swab was taken from the sample and streaked on the agar plate for an appetizer. The potato chips and the popcorn were ground in a ceramic mortar and 1 g of the ground sample was weighed and placed into a 25 mL glass vial filled with 9 mL sterile distilled water. The vial was shaken well and 1 mL of the supernatant was pipetted onto PDA plates (three replicates) allowing the particulate material to settle at the bottom of the vial. Colonies of fungi were isolated from all inoculated plates using the same procedure, sealing with Parafilm and incubating at 25 °C for 7 days [14].

The colonies were macro-morphologically studied with regard to their shape, colour and size and were identified based on their cultural features. A few colonies growing on PDA were removed with a sterile inoculating loop from the plate, transferred to a clean glass slide, stained with a drop of Lactophenol Cotton Blue, a few drops of the mix were then gently heat fixed and covered with a coverslip, so that they could be examined under the microscope. The slides were subsequently viewed with a light microscope at 40X to observe the fine structures of the fungi including the presence of conidia and hyphae and identified on the basis of standard taxonomic keys [15]. The Petri dishes were then sealed with Parafilm to prevent contamination and cultures were refrigerated.

Molecular Identification – Based on Polymerase Chain Reaction (PCR):

After extraction of DNA, the PCR was prepared according to the manufacturer's instructions (Table (1)) with a commercial kit (Promega USA). The reaction components were aliquoted into sterile PCR tubes,

spun down for 3 minutes at 3,000 rpm and then loaded into a thermal cycler (PCR machine). The amplification process consisted of three main steps:

Denaturation: Carried out at a temperature of 94-98 °C for 1-9 minutes, which causes the double-stranded DNA template to separate into two single strands. Annealing: The process of bringing the primers into close contact with their complementary sequences on the single stranded DNA template and is carried out at a lower temperature (50-65 °C) for 5 seconds.

Elongation (Extension): The last step of the cycle; the optimal working temperature for DNA polymerase enzyme to make the new complementary strand of DNA is 75-80 °C.

This three-step process is one single thermal cycle during which the target DNA is doubled. Thermal cycling is the basic process used in PCR, which is based on the thermodynamics of nucleic acid interactions through cycles of heating (denaturing) and cooling (annealing) nucleic acids to specific temperatures [16].

Table 1. PCR Reaction Mixture Components-used in the present study.

Master mix components	Volume
Master Mix	12.5
Forward primer	1.5
Reverse primer	1.5
Nuclease Free Water	5.5
DNA	4
Total volume	25

Gel Electrophoresis:

A 1.5% agarose gel was prepared by melting 1 g of agarose powder in 100 mL of 1X TBE buffer in a water bath until it was completely clear and free of suspended particles and other impurities and allowed to cool before use. After removing solution and letting it cool down until warm, 3 μL of ethidium bromide was added and the solution was gently swirled to mix without introducing aerosol. The molten agarose was then poured into a casting tray, that has a comb close to one end for creating slots for the loading wells, and allowed to set at room temperature for 30 minutes. The PCR product was carefully removed and placed into the wells and 100 bp molecular weight marker (5 μL) was loaded into the first well. The solidified gel was then immersed in 1X TBE running buffer and the electrophoresis machine was running at 1 V/2 cm for 1 hour. The gel was then viewed on a UV transilluminator upon completion of the run to identify band sizes and images were recorded with the camera system attached to the transilluminator [17].

Result

Isolation from the examined food samples (shawarma, fried chicken (Kentucky style), French fries, burger, appetizers (tabbouleh and tzatziki/cacik), vegetable salad, dessert (cake), potato chips and popcorn) showed the presence of 6 fungal genera, namely Aspergillus spp., Penicillium spp., Rhizopus spp., Saccharomyces spp., Mucor spp., Alternaria spp. In these, the most common and most dominant was the genus Aspergillus spp. found in shawarma and fried chicken, burgers, French fries, pizza, meat pastries (lahmacun), appetizers and vegetable salad. Penicillium spp. was the second most common genus found in shawarma, French fries, vegetable salad and desserts and potato chips. The fried chicken, pizza, meat pastries, desserts (cake) and potato chips were found to contain Rhizopus spp., while the French fries, burgers and appetizers (tabbouleh) contained Saccharomyces spp. As well, Mucor spp. was found in meat pastries and appetizers and Alternaria spp. in vegetable salads, only. However, no fungal growth was observed in the popcorn samples. Macroscopic and microscopic traits, as well as the scientific identification of the isolated fungi, based on the standard taxonomic books, are summarized in the following (2) table, and the food sources from which the samples were collected.

Table 2. Shows the macroscopic and microscopic features of the isolated fungi, their taxonomic reference and the appropriate food samples that were contorted.

No.	Macroscopic and Microscopic Characteristics	Fungal Genus	Reference	Food Source
1.	Macroscopic: Bluish-green colonies tending toward olive-green. Microscopic: Branched, multinucleate hyphae; conidiophores exhibiting a characteristic brush-like (penicillus) structure.	Penicillium spp.	[18].	Shawarma, French fries, salads, desserts (cake), potato chips
2.	Macroscopic: Rapidly spreading black colonies covering the entire plate. Microscopic: Septate hyphae; black globose conidial heads bearing spherical conidia (spores).	Aspergillus spp.	[19].	Shawarma, fried chicken, French fries, burgers, appetizers (tabbouleh, tzatziki), pizza, meat pastries (lahmacun), salads
3.	Macroscopic: White, smooth, glossy colonies with a mucoid consistency. Microscopic: Spherical to oval cells, with several cells exhibiting budding.	Saccharomyces spp.	[20].	French fries, burgers, appetizers (tabbouleh)
4.	Macroscopic: Grayish colonies with diffuse spreading structures across the plate. Microscopic: Non-septate (coenocytic) hyphae; globose to clavate (club-shaped) sporangia containing dispersed sporangiospores.	Rhizopus spp.	[21].	Fried chicken, pizza, meat pastries (lahmacun), desserts (cake), potato chips
5.	Macroscopic: White cottony or fluffy colonies that turn gray over time. Microscopic: Non-septate hyphae; unbranched sporangiophores bearing sporangia.	Mucor spp.	[22].	Appetizers (tabbouleh), meat pastries (lahmacun)
	Macroscopic: Olive-black colonies. Microscopic: Septate hyphae; geniculate (bent/zigzag)	Alternaria spp.	[23].	Salads

No.	Macroscopic and Microscopic Characteristics	Fungal Genus	Reference	Food Source
	conidiophores producing large conidia with longitudinal and transverse septa.			

The isolated fungi were examined macroscopically, as illustrated in Figure (1), and microscopically, as shown in Figure (1). The obtained results—depicted in both figures—were subsequently classified according to standard taxonomic keys [24].

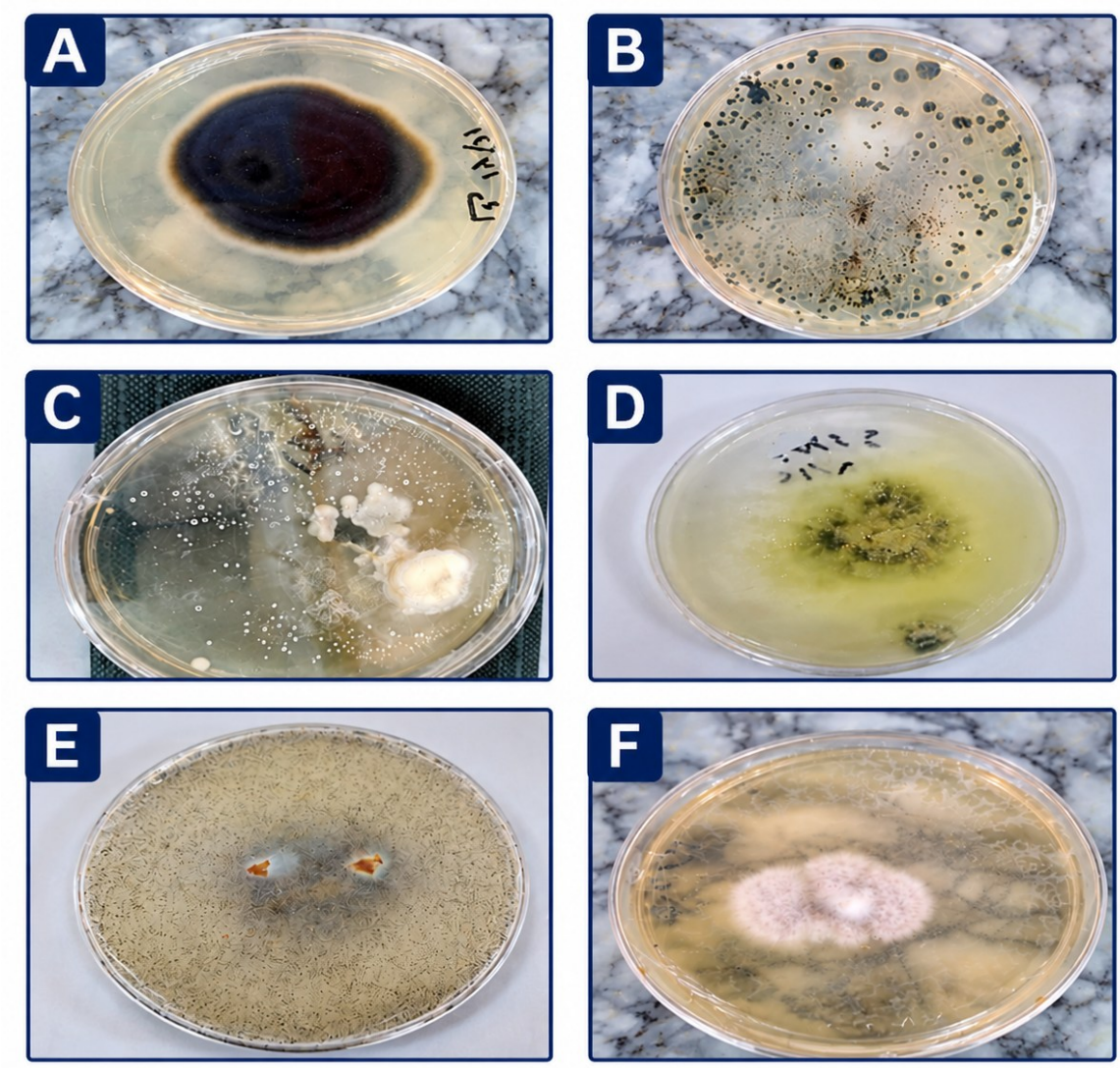

Figure 1. The macroscopic morphology of fungal genera grown on Potato Dextrose Agar (PDA) for 7 days at 25 °C are shown as follows: (A) Aspergillus spp., (B) Penicillium spp., (C) Saccharomyces spp., (D) Alternaria spp., (E) Rhizopus spp., and (F) Mucor spp.

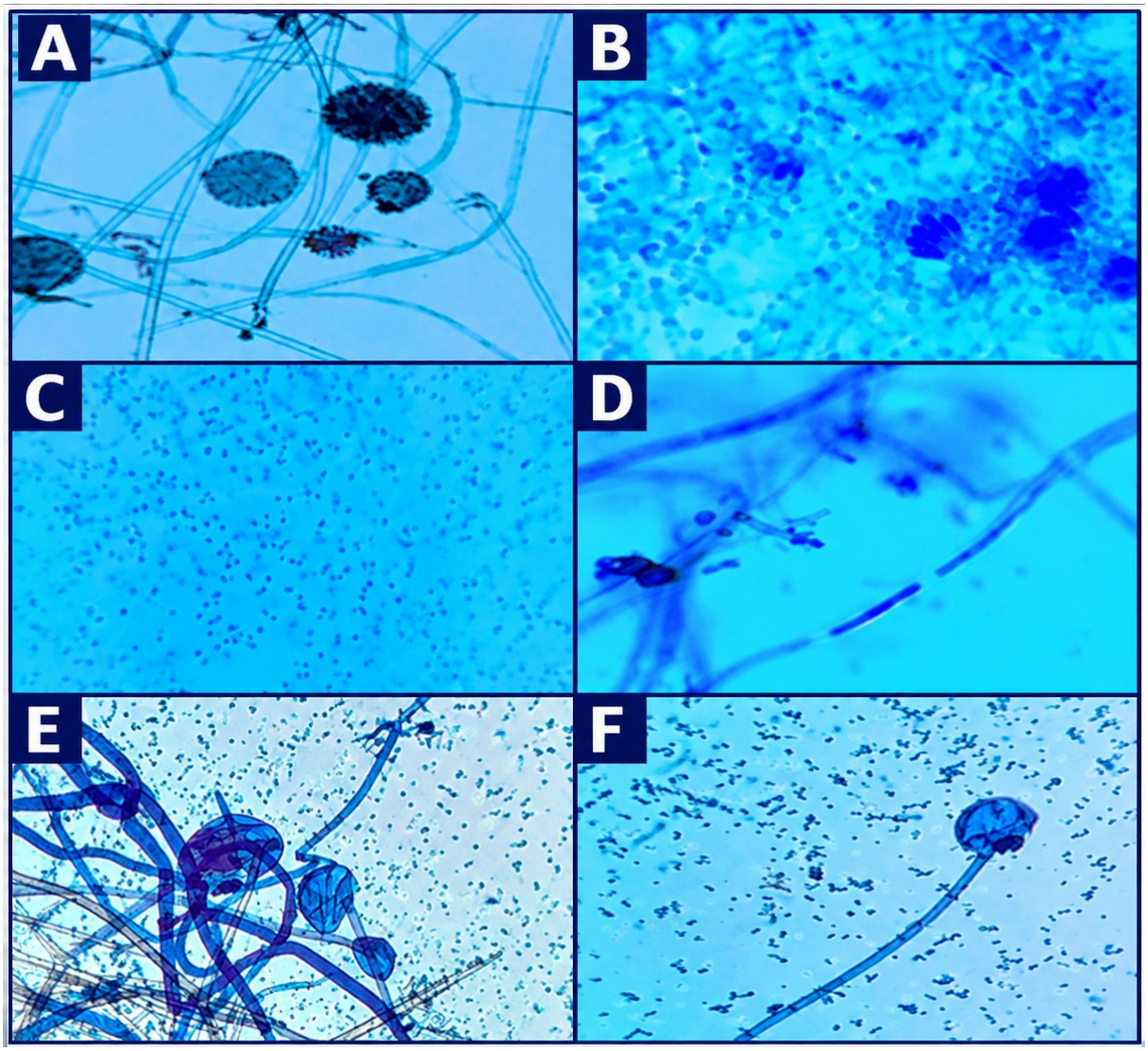

Figure 2. Microscopic morphology of the isolated fungal genera stained with Lactophenol Cotton Blue (LCB) under a light microscope (40× magnification), after 7 days of incubation at 25 °C: (A) Aspergillus spp., (B) Penicillium spp., (C) Saccharomyces spp., (D) Alternaria spp., (E) Rhizopus spp., and (F) Mucor spp.

The frequency of occurrence (percentage of appearance) of the isolated fungi was calculated according to the following equation [25].

Frequency of occurrence (%) = (Number of samples in which a particular species occurred / Total number of samples examined) × 100

Aspergillus spp. was the most prevalent genus with frequency of occurrence (34.2%) while Penicillium spp. was the second most prevalent genus (26.3%). Rhizopus spp. and Saccharomyces spp. were the third and fourth most frequently occurring fungi with occurrence rates of 23.1% and 13.1%, respectively. The fifth position was held by Mucor spp. which occurred 5.2%, while the lowest occurrence rate (2.6%) was obtained by Alternaria spp. of the fungi isolated, as shown in Figure (3). The results are in agreement with Easa (2011) who reported that the most common fungal genus is Aspergillus spp. which causes food spoilage [26].

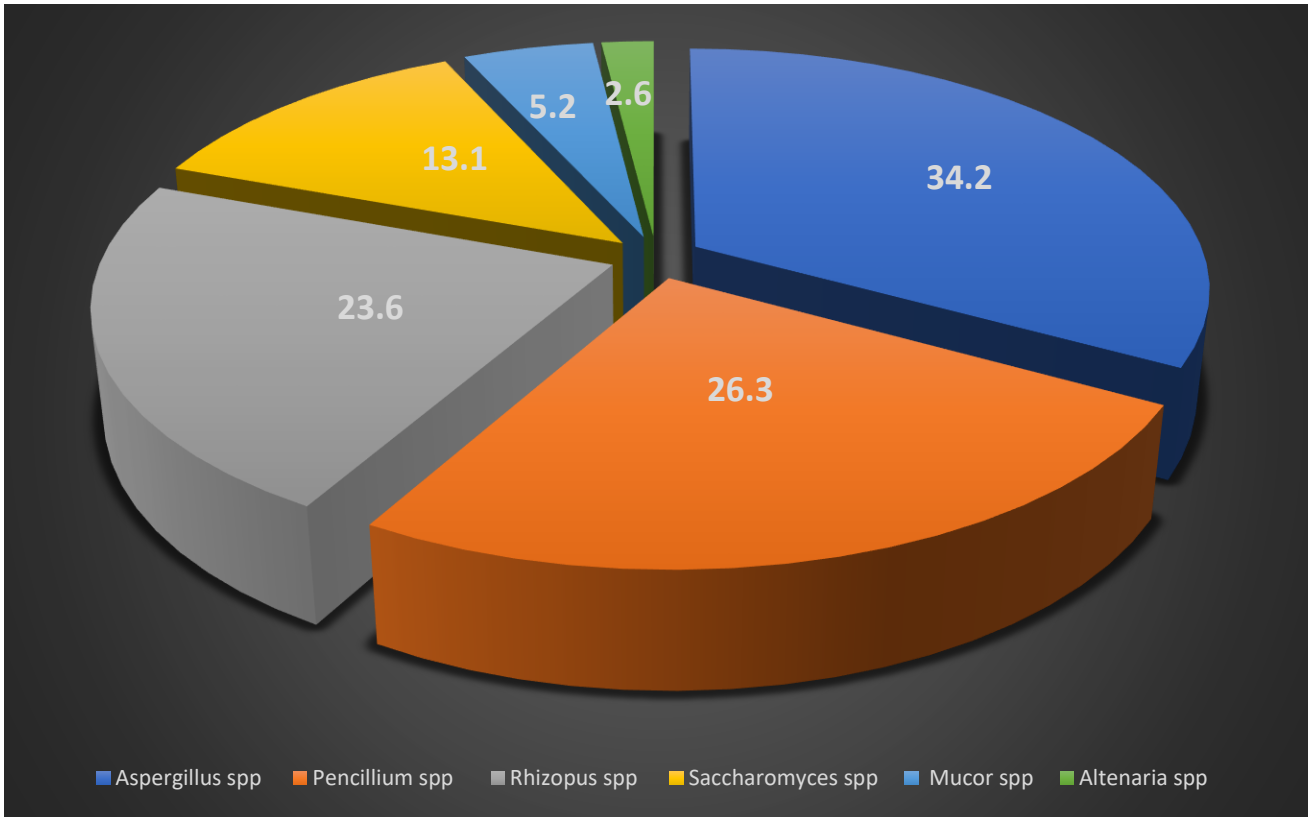

Figure 3. Frequency of occurrence (%) of the fungal genera isolated from food products available in local markets.

Conventional identification, only through phenotypic characteristics and colony morphology, is no longer enough for the identification of isolated fungal species. Hence, molecular identification was carried out for the accuracy of the morphological identification by Polymerase Chain Reaction (PCR) of the fungal region of Internal Transcribed Spacer (ITS) of 18S rRNA gene.

In the present study, six fungal genera were identified: Penicillium spp., Aspergillus spp., Saccharomyces spp., Rhizopus spp., Mucor spp., and Alternaria spp.

Fungal identification by conventional methods, solely based on phenotypic and morphological traits, is no longer enough as closely related species in the same genus often share the same phenotypic and morphological traits. It is also important to note that the intraspecific genetic variation (especially within the genus) and phenotypic plasticity to environment can make the classification difficult [27]. Researchers have therefore stressed on the need to use molecular methods, especially Polymerase Chain Reaction (PCR) technology which is considered as the "gold standard" because of its diagnostic and specific accuracy and sensitivity [28].

The 260nm/280nm ratios of extracted genomic DNA were evaluated using the NanoDrop spectrophotometer, which showed good DNA purity ratios between 1.82 and 1.98. A universal fungal primer pair (ITS1/ITS4) was used to amplify the Internal Transcribed Spacer (ITS) region, specifically designed to detect a wide range of fungal taxa, including ITS1, 5.8S and ITS2. Distinct PCR amplicons with different lengths ranging from 500 to 600 bp were obtained by amplification, which are the interspecific length polymorphisms of the ITS1-ITS4 region of various fungal species. Isolates were identified as species of Penicillium spp, Saccharomyces spp, Rhizopus spp, Mucor spp, Alternaria spp and Aspergillus spp as per the PCR amplification results as shown in Figure (4).

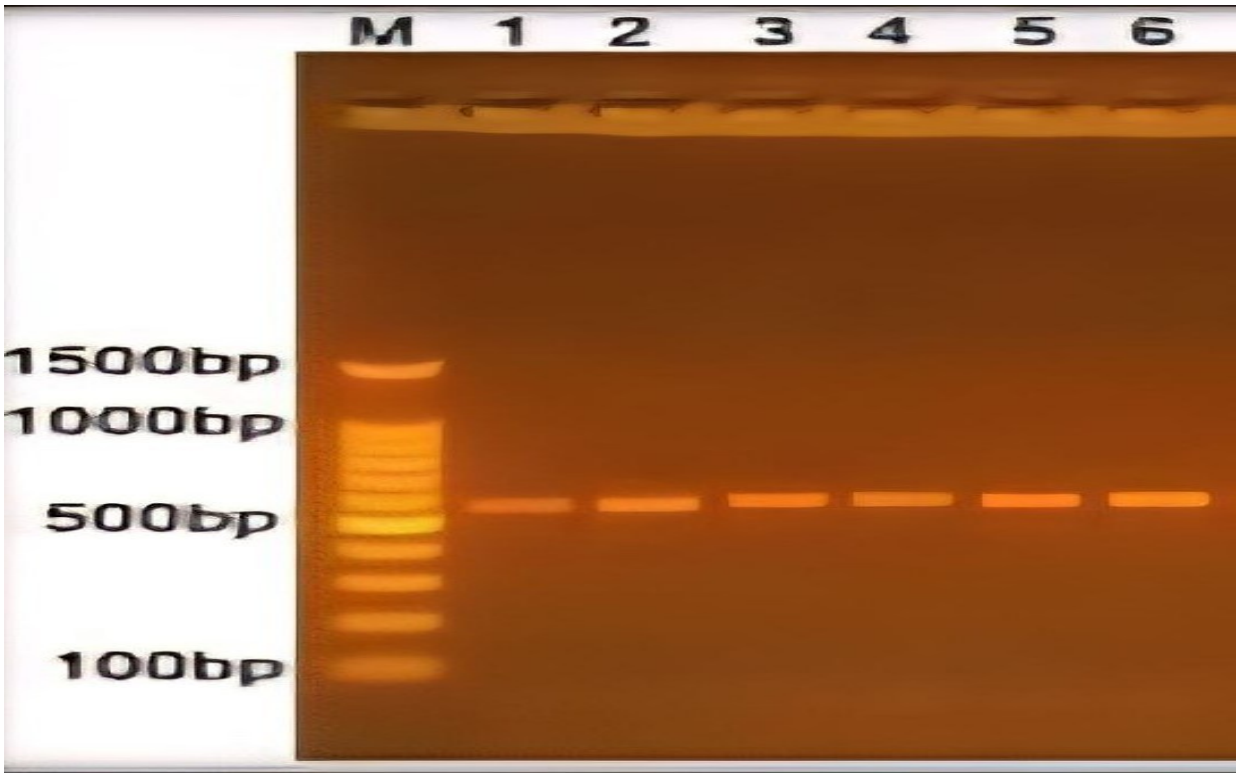

Figure 4. Agarose gel electrophoresis showing the PCR amplification products of the isolated fungal genera, where Lane M represents the 100–1500 bp DNA ladder (marker).

Table 3. Molecular sizes (bp) of the ITS1–ITS4 PCR amplicons for the isolated fungal genera.

Fungal Genus	Isolate No.	Amplicon Size (ITS1–ITS4) (bp)
Mucor spp.	1	520
Alternaria spp.	2	550
Penicillium spp.	3	570
Saccharomyces spp.	4	564
Rhizopus spp.	5	589
Aspergillus spp.	6	613

Discussion

The findings suggest that food products can be contaminated by a range of fungal species, because of the lack of proper hygienic practices during food preparation, storage and in local markets. In addition, the growth of fungi is blamed on poor personal and work hygiene practices, including the lack of dedicated restaurant uniforms and the use of disposable gloves when preparing food; and inadequate kitchen equipment and work environment sanitation practices. Other factors include cooking at an open and exposed place, preparing vegetables and meats on the same chopping board, and using old ingredients like sauces, mayonnaise, and ketchup [29].

Furthermore, fungal spores are ubiquitous in the air and can readily germinate and proliferate once they encounter a favorable environment. The specific fungal taxa present and their rate of growth depend largely on the composition and nature of the food substrate, as well as prevailing environmental factors such as temperature and relative humidity [30].

Conclusion

The present study suggests that the fungi found in the ready-to-eat food and local market food are filamentous molds and yeasts. Macroscopic, microscopic and molecular (PCR) analysis of the fungal genera, using internationally recognized taxonomic keys, revealed that six genera were isolated and identified accurately, with the highest frequency of occurrence being Aspergillus spp (34.2%) and the lowest being Alternaria spp (2.6%). Food safety and hygiene is a basic prerequisite for the protection of public health in all parts of the world so ensuring hygienic food handling is of paramount importance, by obliging food handlers to wear dedicated clothing and disposable gloves, strict quality assurance measures from the production point to the final consumption, proper sanitation of utensils and the prevention of raw meat and vegetables from coming into contact on the same cutting board, periodic inspections and laboratory tests by dedicated committees from the Ministry of Health to check the presence of fungi and mycotoxins in food and exploring solutions for the control of foodborne fungi with the help of natural plant extracts which are proven safe and do not pollute the environment.

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