

Application of Biotechnology for the Production of New Food Preservations

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ABSTRACT

The presence of pathogenic fungi has been a global concern has economical affects. Recently prevent the fungal growth using biological control using halal source become an important strategy for limiting mycotoxigenic fungi. Therefore the aim of this study that the use of some LAB bacteria and screen their antimicrobial activity against pathogenic fungi. Halal bacteria extracts were studied for the antimicrobial activity. The results indicated that two isolates were identified. Meanwhile a total of 90 fungal isolates were isolated from halal food samples. *Aspergillus* was the dominant fungi (65%) in five species namely, *parasiticus*, *flavus ochraceus*, *terreus*, and *niger*. *Fusarium* (*clomorum* and *graminerum*) and *Penicillium* were in lower frequency. Halal bacterial extracts were reduced the mycelium dry weight of all tested fungi and showed clear zone inhibition. Molecular identification of mycotoxin producing fungi was estimated. In conclusion, the study revealed the probability of using halal *Enterococcus* isolates in food preservation to ensure halal food safety. The partial or total sequencing of the 16s ribosomal DNA (rRNA) gene showed a fast technique used fungi classification

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1. Introduction

Different species of pathogenic fungi can produce mycotoxins which contaminate stored different halal agriculture commodity (Sabry et al., 2016). Some of these fungi can produce different mycotoxins as secondary metabolites (such as aflatoxins, fumonisins, ochratoxins, tricothethenes etc...). Aflatoxins are produced from some strains of *Aspergillus flavus* and *Aspergillus parasiticus* and contaminated food and agriculture commodity (Iqbal et al., 2013). Aflatoxin B₁ (AFB₁) has toxic, carcinogenic, mutagenic and teratogenic effects in laboratory animals (Abdel-Wahhab et al., 2010). In addition, AFB₁ and its metabolites can accumulate in animal tissues, and reached to human consumer through the food chain (Bintvihok et al., 2002).

Halal Lactic acid bacteria (LAB) have important role in food industry and that benefit humans and animals health. Moreover, their safety has been proved empirically and scientifically (Hathout and Aly, 2014). Some of probiotic bacterial strains like *L. reuteri* and *L. casei* inhibited the fungal growth and used to produce fermented Talpina as cereal- dairy product and Ras cheese (Sahab et al., 2011, Hathout and Aly, 2010). The most important LAB species are of the genera *Lactobacillus* and *Bifidobacterium*, due to their probiotic properties exhibited by some strains and generally

regarded as safe (GRAS) lactic acid bacteria. Abde-Shafi et al. (2014) reported that *Enterococcus faecium*, *Enterococcus faecalis* are important species of enterococcus genus with probiotic properties which used to suppress carcinogenesis, reduce cholesterol level, and prevent bacteria-associated diarrhea. So, these bacteria called halal food. LAB isolated from homemade fermented vegetables produce antibacterial substances against both gram-positive and importantly, gram-negative common foodborne bacterial pathogens. *Enterococcus faecium* and *enterococcus faecalis* *enterococci* are singular, double or short chained gram positive cocci. *Streptococcus faecalis* was defined by Felis and Dellaglio (2007). Halal bacteria in this genus were divided into various species such as; *E. faecium*, *E. durans*, *E. faecalis*, *E. casseliflavus*, *E. malodoratus*, *E. avium*, *E. pseudoavium*, *E. dispar*, *E. sulfureus*, *E. flavescens*, *E. saccharolyticus*, *E. columbae* ve *E. cecorum* (Giraffa, 2003; Khan et al., 2010).

Adimpong et al. (2012) examined the partial or total sequencing of the 16s ribosomal DNA (rRNA) gene for practical routine purposes. If two organisms present, a 16s rRNA gene sequence identity are higher than 97% they can be considered closely related and thus belonging to the same species (Větrovský et al., 2013). Identification based on the 16S rDNA sequence is of interest because ribosomal exists generally among bacteria and includes regions with species specific predictability. The aim this study was to isolate and identify the non halal isolates of fungi and control its growth using halal bacteria extracts as antimicrobial against pathogenic fungi and bacteria to produce halal food. The use of molecular protocol technique to identified mycotoxigenic fungi

2. Materials and methods

2.1. Samples collection

Twenty samples of different Egyptian cereal and its products were collected from local markets. While LAB bacteria, isolated from ten samples of traditional dairy product were collected.

2.2. Isolation of fungi using agar test

Fungi were isolated from samples using the agar test methods according to Sahab et al. (2014) using solid potato dextrose agar (PDA) halal media. The individual isolates were transferred to new PDA plates in order to obtain pure cultures. All isolates were maintained on PDA and kept at 4°C for further analysis.

2.3. Identification of fungi

The isolated fungi were identified according to colony morphology and microscopic examination according to the keys of Nelson et al. (1983), Barnett and Hunter (1986) and Leslie and Summerell (2006).

2.4. Ability of fungal isolates to produce mycotoxins

One mL of each of the fungal spore suspension was transferred into 250 mL conical flask containing 100 mL broth (yeast extract 2%-Sucrose 20%). The cultures were incubated for 7 days at 28°C. Aflatoxins, Ochratoxin A, were extracted from culture filtrates using chloroform. The culture filtrates were extracted three times with chloroform and the chloroform extracts were evaporated under nitrogen gas, the residue was dissolved in methanol, and then completely passed through immunoaffinity column (C18) at a rate of about 1-2 drops/second. After passing the samples, the immunoaffinity column was washed twice with 10 mL purified water at a rate of about 2 drops/second. Elution was performed with 1.0 mL methanol and then analyzed by HPLC.

The HPLC system used for mycotoxin analyses was an Agilent 1200 series system (Agilent, Berks, UK) with a fluorescence detector (FLD G1321A), an auto sampler ALS G1329A, FC/ALS thermal G1330B, Degasser G1379B, Bin Bump G1312A and a C18 (Phenomenex, Luna 5 micron, 150 × 4.6 mm) column joined to a pre-column (security guard, 4 × 3-mm cartridge, Phenomenex Luna). The mobile phase was water: acetonitrile: methanol (3:1:1, v/v/v) using an isocratic flow rate of 1 ml/min at 360 nm excitation and 420 nm emission wavelengths.

2.5. Molecular identification of mycotoxin producing fungi

Extraction of genomic DNA was carried out using Qiagen Kit (Qiagen Sciences, Maryland, USA) according to the manufacturer's instruction manual. The PCR reactions were done to amplify the ITS gene from the fungal isolates. The ITS region was amplified by using the primer set (ITS1) (5CTTGGTCATTTAGAGGAAGTAA-3) and ITS4 (5GCTGCGTTCTTCATCGATGC-3) according to White et al. (1990) with some modification. PCR cycles consisted of an initial denaturation step at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and amplification at 72°C for 2 min, with a final extension step at 72°C for 5 min. Amplification products for the ITS locus were separated on a 1% agarose gel by electrophoresis in 1X TBE buffer (Tris-borate EDTA, pH 8.0), using a 100-bp ladder DNA marker (Invitrogen, California, USA). The gel was visualized and photographed using TMXR + Gel Documentation System (Bio-Rad, California 94547, USA)

2.6. Molecular differentiation of isolated fungi using ISSR technique

Ten ISSR primers were used in the detection of polymorphism among five isolated fungi. These primers were synthesized by Metabion Corporation, Germany. The primers code and nucleotide sequences are presented in Table (1). The PCR amplification reactions were carried out as mentioned by Williams et al. (1990) with some modifications. Reactions were performed in 25 µl volume composed of 1x reaction buffer, 0.2 mM of dNTPs, 1.5 mM MgCl₂, 0.2 µM of primer, 0.5 unit of Taq polymerase (Qiagen Sciences, Maryland, USA) and 50 ng of template DNA, in sterile distilled water. ISSR-thermo-cycling profile the PCR amplification of the DNA was performed in a Perkin Elmer thermal cycler 9700. The temperature profile in the different cycles was as follows: an initial strand separation cycle at 94°C for 5 min followed by 40 cycles comprised of a denaturation step at 94°C for 1min, an annealing step at 45°C for 1 min and an extension step at 72°C for 1.5 min. The final cycle was a polymerization cycle for 10 min at 72°C. PCR products were mixed with 5 µl gel loading dye and resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 mg/mL in 1 x TBE buffer at 120 volts). A 100bp DNA ladder was used as molecular size standard. PCR products were visualized under UV light and documented using a TMXR+ Gel Documentation System (Bio-Rad, California 94547, USA. PCR fragment purification .The PCR products were eluted from agarose gels using Promega®'s Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's instructions.

Table 1. ISSR primers name and sequences

Primer	Sequence
ISSR- 1	5'-AGAGAGAGAGAGAGAGAYC-3'
ISSR- 2	5'-AGAGAGAGAGAGAGAGAYG-3'
ISSR- 3	5'-ACACACACACACACACYT-3'
ISSR- 4	5'-ACACACACACACACACYG-3'
ISSR- 8	5'-ACACACACACACACACYA-3'
ISSR- 11	5'-ACACACACACACACACYC-3'
ISSR- 12	5'-AGAGAGAGAGAGAGAGAYT-3'
ISSR- 13	5'-CTCCTCCTCCTCCTCTT-3'
ISSR- 15	5'-CTCTCTCTCTCTCTRG-3'
ISSR- 16	5'-TCTCTCTCTCTCTCA-3'

2.7. PCR fragment purification

2.7.1. Data analysis

The amplified fragments were scored as present (1) or absent (0). Ladder 100 bp DNA was used to identify the molecular weights of fragments. Similarity matrix among the fungi isolates was calculated and similarity coefficient was used to design the dendrogram using Unweighted Pair-Group Method with Arithmetical average (UPGMA) according to Rohlf (1993).

2.7.2. Isolation of lactic acid bacterial

The isolation of the LAB isolates was done according to Lavanya et al. (2011) as followed: 1ml (or 1g) of the product were homogenized with 9 ml of 0.85% w/v NaCl sterile saline. A 0.1 ml sample of suitable dilution was plated onto different types of media, i.e. MRS agar, and KAA agar. The plates were incubated at 30 for mesophilic LAB and 40 °C for thermophilic LAB for 2 to 3 days. Colonies were selected according to their shape. The purity of isolates was checked by continuous streaking on the respective media. The isolates which were considered as already purified was stored in medium containing 10% skim milk and 20% glycerol as isolate stock at freezing temperature (- 20 °C).

2.7.3. Preparation of bacterial culture extracts

The cultures were extracted from liquid cultures of LAB grown in MRS or M17 medium at optimum temperature for 24h. The cells were removed by centrifugation at 4600 rpm /15 min and the cell free supernatant was adjusted to PH7 and brought to final ammonium sulphate (concentration 60%) by and stirred at 4°C for 18 h. the supernatant fraction was decanted and the pellet was air dried.

2.8. Evaluation the Antifungal activity of isolated lactic acid bacteria:

2.8.1. Zone inhibition

The bacterial culture extracts were prepared and filter paper discs (5 mm diameter, Whatman No.1) were saturated with 10 µL from each extract. The paper discs were placed on inoculated agar plates with the tested fungi and incubated at the appropriate temperature five days. The diameter of the growth inhibition zones was measured averaged and the mean values were recorded.

2.8.2. Mycelium dry weight

Fungal spore suspensions (106 spores/mL) were prepared in an aqueous solution of 0.1% Tween 80. The lactic acid bacteria were grown in De Man Regosa Sharpe Broth at 37°C for 12 h. One mL of each of the bacterial suspension for each strain was transferred into 250 mL conical flask containing 100 mL broth (yeast extract 2%-Sucrose 20%) and inoculated with 1 mL fungal spore suspension. The cultures were incubated for 7 days at 28°C. The mycelium mats were collected by filtration through Whatman filter paper No.4, washed twice with water and dried in an oven at 95°C until constant weight and weighed.

3. Results and discussion

3.1. Fungal isolation

Analysis of different species isolated from cereal samples for morphological and cultural characteristics showed that there was variation in the colony color, margins, and texture and colony reverse colors. Results in Table (2) revealed that the percentage of contamination peanut samples reached to 93%. *Aspergillus spp* were observed on 83.5% of samples followed by *Fusarium* (10.67) while *Penicillium* was detected in few samples. Ninety isolates were counted from all samples belonging five genera and identified from different cereal samples. Table (2) also showed that 63 out 90 isolates were identified as *Aspergillus* species as the most predominant fungi (70%) followed by *Fusarium* (22.22%). Among the *Aspergillus* species (Table 3), *A. flavus* was isolated in higher incidence (18 out of 63) of all *Aspergillus* isolates followed by *A. niger*, *A. ochraceus*, *A. parasiticus* and *A. terreus* in descending order. These results were confirmed by Magnoli et al (2007) who reported that *A. flavus* is generally the most destructive species in Egyptian cereal and

cereal products. These fungi as well as other fungal species which increase the respiratory rate and accelerates deterioration in commercial and nutritional value. Nakai et al. (2008) and Fonseca (2012) explained that cereal and especially peanuts which a high-risk for contamination with these species because its harvest of rainy periods and have a long drying times. Also Marcos (2005) recorded higher incidence of these storage fungi. Moreover Sahab et al. (2014) also isolated *Fusarium species* in higher occurrence from Egyptian freshly harvested maize. The differences in finding due to the variation of weather conditions zone especially temperature and water humidity. Our results recorded higher occurrence *A. flavus* and *A.parasiticus* is vital observation because these species are known to produce deferent types of aflatoxins, (Pildain et al., 2008) especially aflatoxin B₁ which the most potent carcinogens and cause mycotoxicosis to human and animals.

Table 2. Percentage of infection and frequency occurrence (%) of fungal isolates

Fungal genera	% infection	*No of isolates	Occurrence (%)
<i>Aspergillus</i>	83.50	63	70
<i>Fusarium</i>	10.76	21	22.22
<i>Penicillium</i>	2.70	6	6.66

*Total number of isolates =90

Table 3. Frequency occurrence (%) of fungal species associated

Fungal species	No of isolates	Occurrence (%)
<i>A. flavus</i>	18	20.0
<i>A. ochraceus</i>	14	15.55
<i>A. niger</i>	17	18.88
<i>A.parasiticus</i>	8	8.88
<i>A terreus</i>	6	6.66
<i>F. clomorum</i>	12	13.33
<i>F.graminearum</i>	9	10.0
<i>Penicillium spp</i>	6	6.66

3.2. The Ability of fungal isolates to produce mycotoxins

Data in Table (4) showed that *A. parasiticus* produced higher concentrations of aflatoxins B₁ (10.7 ppb) than *Aspergillus flavus* (7.142 ppb). On the other hand, *A. ochraceus* produced ochratoxin A (OTA) at low concentration (5.086 ppb), whereas both isolated *A. niger* and *A. terreus* did not produce OTA or territrems respectively. The production high concentrations of aflatoxin B₁ consider highly dangerous thus the aflatoxin B₁ is the most potent carcinogen that causes mycotoxicoses to human and animals (Pildain et al., 2008). Previously, several researches (Sahab et al., 2011; Sabry et al., 2016) isolated mycotoxigenic fungi from different cereals commodities and identified their associated mycotoxins. They also reported that and *Aspergillus* genera is the most economically important genera especially those mycotoxin producing species.

Table 4. Ability of isolated fungi to produce mycotoxins

Fungal species	No of isolate	AflatoxinB ₁ (ppb)	Ochratoxin A (ppb).
<i>A. ochraceus</i>	I	-	0.432
	II	-	5.086
<i>A flavus</i>	I	7.142	-
	II	4.76	-
	III	ND	-
<i>A.parasiticus</i>	I	5.34	-
	II	3.67	-
	III	10.7	-
<i>A.niger</i>	I	ND	ND
	II	ND	ND

3.3. Antibacterial activity of LAB

Twenty local lactic acid bacteria (LAB) were isolated from milk and traditional dairy product samples (no tabulated data) and select two isolates named as isolate *E. faecalis* and *E. faecium*. Table (5) showed the antibacterial activity against the four pathogenic bacteria (*Escherichia coli*, *Staphylococcus aureus*, *salmonella typhi*, and *Bacillus cereus*). The mean values of zone inhibition diameter reached to 19.3 for isolate *E. faecalis* which consider a higher effect than *E. faecium*. The contamination food by pathogenic bacteria resulting not halal food and consequences in numerous foodborne diseases (Targowski and Płusa, 2015) which increase the estimated death worldwide especially in many developing countries (Abdhalai et al., 2015). In order to achieve improved food safety must be advanced by more application to produce halal food, without any haram ingredients, no cause any health hazards that applied a new innovation fermented food using LAB.

Table 5. Antibacterial activity of Bacteria extract against various Pathogenic Bacteria

Bacteria extract	Zone inhibition (mm)			
	<i>B. Cereus</i>	<i>E. Coli</i>	<i>S. aureus</i>	<i>S. typhimurium</i>
<i>E. faecalis</i>	19.0 ± 0.00	12.5 ± 0.70	15.0 ± 1.2	15.6 ± 1.3
<i>E. faecium</i>	13.2 ± 1.2	1.1 ± 9.6	19.3 ± 1.2	12.8 ± 1.5

Results in Figure (1) revealed that both *Enterococcus faecalis* and *Enterococcus faecium* greatly decreased mycelium dry weight, whereas *A. ochraceu* more sensitive to *E. faecium* (zero growth). The inhibition percentage ranged between 9.13% to 56.87% when using *Enterococcus faecalis* according to fungal species where *A. flavus* isolates showed the percentage of inhibited higher than *A. parasiticus* (Tables 6, 7). Our findings are in agreement with those reported by Lahtinen et al. (2004) and Niderkorn et al. (2006) who reported that LAB can contribute to the inhibition of molds development and production of mycotoxins. The antimicrobial activity of the two species due to the production of bacteriocin showed that *Enterococcus species* have a bactericidal and bacteriostatic effect (Hathout and Aly, 2010). Our results recorded variations of the antimicrobial effect due to the molecular characterization and biochemical analyses of each isolate which cleared the importance of these analyses to be sure of accurate name of the isolates. LAB have shown a major potential for use in biopreservation because of safety for human consumption (GRAS status) and the prevalent microflora during storage in many foods (Vignolo et al., 2008). Based on the previous studies, the end product of fermentation is depend on the substrate that *E. faecalis* FAIR-E 229 since lactate was the main end product and small amounts of ethanol, when lactose is the substrate. When citrate was used it was converted to acetate and formate, lactate, ethanol, and acetone. On the other side, *E. faecium* produces antibacterial peptides called bacteriocins.

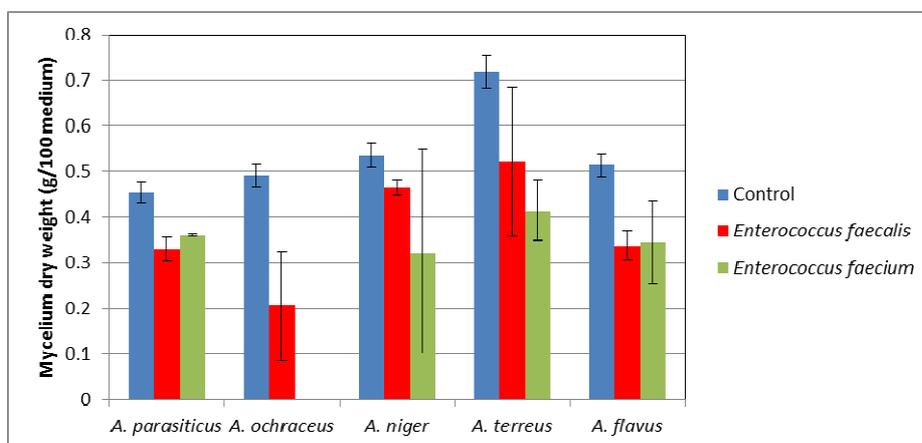


Fig 1. Effect of lactic acid bacteria on mycelium dry weight of *Aspergillus* spp. Results are mean ± SD (n=2).

Table 6. Antifungal activity of Bacteria extracts against various fungal isolates

Bacteria extract	Zone inhibition (mm)						
	A. <i>flavus</i>	A. <i>Parasiticus</i>	A. <i>ochraceus</i>	A. <i>niger</i>	F. <i>clomorum</i>	F. <i>graminerum</i>	F. <i>oxysporum</i>
<i>E. faecalis</i>	15.6 ± 1.2	16.2 ± 1.54	10.6 ± 1.5	22.3 ± 1.4	11.5 ± 1.4	11.7 ± 1.5	15 ± 2.1
<i>E. faecium</i>	17.4 ± 1.8	19.5 ± 1.19	21.2 ± 1.1	18.9 ± 1.3	14.7 ± 1.8	15.6 ± 1.3	9.2 ± 1.4

Table 7. percentage % inhibition and average growth of different fungal species affected by *Enterococcus spp.*

Fungal species	No of isolates	<i>Enterococcus spp.</i>			
		<i>Enterococcus faecalis</i>	avarege	<i>Enterococcus faecium</i>	
<i>A. flavus</i>	10	44.78-56.87	50.8	37.56-43.65	40.45
<i>A. ochraceus</i>	14	47.00-55.8	51.4	89.0-100	94.50
<i>A. niger</i>	25	31.24-36.9	34.7	42.73-51.76	47.20
<i>A. parasiticus</i>	8	22.12-34.7	28.4	13.52-21.85	17.68
<i>A. terreus</i>	6	33.36-37.8	35.58	50.17-23.0	36,58
<i>F. clomorum</i>	12	22.86-35.0	28.93	35.33-45.65	40.49
<i>F. graminearum</i>	9	9.13-22.7	15.9	44.7-56.8	50.75
<i>Penicillium spp</i>	6	14.8-34.7	24.75	22.05-34.6	28.32

3.4. Molecular identification of mycotoxin producing fungi

The ITS region is the official DNA barcoding marker for species-level Identification of Fungi. DNA barcoding systems employ a short standardized region (between 400 and 800 base pairs) to identify fungal species. ITS amplification products using the ITS1 and ITS4 primers are a unique band ranging from 500-600 bp was obtained for all isolated fungal strains (Fig 2). The amplicons of ITS regions were column purified and sequenced using a set of primers. The ITS region sequences were aligned using blast algorithm and compared with the published sequences of ITS region gene of different fungi (Table 8). The results revealed that the first fungal isolate of the sequenced 18S rRNA gene was identified as *Aspergillus ochraceus* and had (98%) similarity with *Aspergillus ochraceus* isolate Nitaf 24 (Fig 2). The second fungal isolate of the sequenced 18S rRNA gene was identified as *Aspergillus flavus* and had (99%) similarity with *Aspergillus flavus* strain UOA/HCPF 5774 (Fig 2). The high similarity between the fungal strains and their closest phylogenetic relative, indicating that 16S rRNA gene sequence data are helpful for identification of fungi. The gene sequence was deposited in GenBank database as; *Aspergillus ochraceus* Egy2 (Accession No. LC360803.1) and *Aspergillus flavus* Egy3 (Accession No. LC368455.1). Data in Figs (3,4) showed the phylogenetic tree based on ITS region sequences, the relationship between fungal isolates and other species. The tree was constructed using the neighbor-joining method.

The results indicated the importance of using molecular methods such as DNA barcoding systems (ITS region) for typing newly isolates microorganisms. Phenotypic and genotypic methods are part of the first step of identifications and selection of potential fungal isolates. These results are in agreement with Henry et al. (2000) who also used this method to identify *Aspergillus* at the species level and differentiate it from other true pathogenic and opportunistic molds using the 18S and 28S rRNA genes for primer binding sites. The contiguous internal transcribed spacer (ITS) region, ITS 1-5.8S-ITS 2, from referenced strains and clinical isolates of *Aspergilli* and other fungi, were amplified, sequenced, and compared with non-reference strain sequences in GenBank. ITS amplicons from *Aspergillus* species ranged in size from 565 to 613 bp .

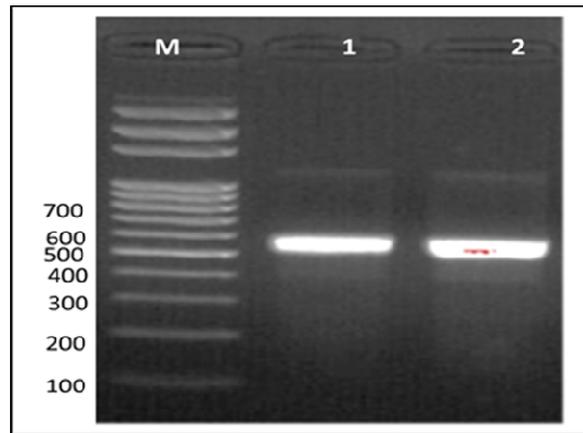


Fig 2. Polymerase chain reaction-amplification of 18S rRNA gene; Lane M: Gene Ruler DNA Ladder 100 bp, Lane 1: 18S rRNA gene fragment of isolate *Aspergillus ochraceus*, Lane 2: 18S rRNA gene fragment of isolate *Aspergillus flavus*. DNA molecular weight marker Band sizes: 100, 200, 300, 400, 500, 600 and 700 bp

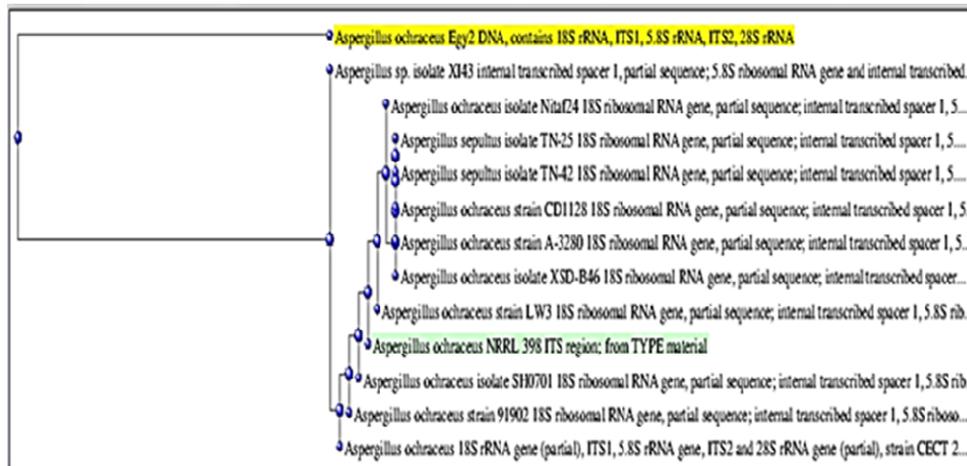


Fig 3. Phylogenetic tree showing relationship of closely related species constructed using the neighbor-joining method and based on 18S rRNA gene sequences. Isolate is closely related to *Aspergillus ochraceus*

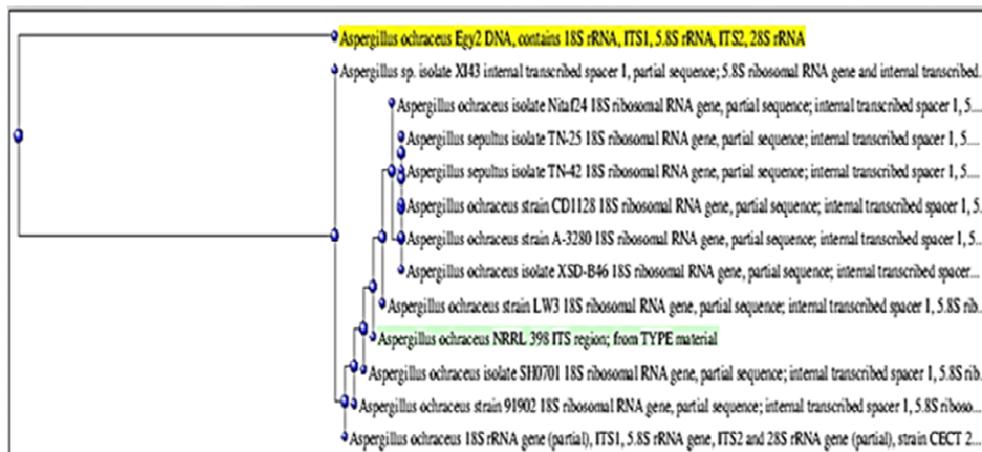


Fig 4. Phylogenetic tree showing relationship of closely related species constructed using the neighbor-joining method and based on 18S rRNA gene sequences. Isolate is closely related to *Aspergillus flavus*

Table 8. The nucleotide sequence of two fungal isolates

Strain	Aligned Sequence Data
<i>Aspergillus ochraceus</i>	<p>CGCGGCGCGCCCCCCCCCGCCCCCGATTACCCATTTATACCTCCA AACACCCCTTGACCCAAAAAATGCGCGCCTTTGTTCCGGGGGGGTGCGC GCGCTCAACTTTCCTTTCCTTAAGGGGAAACCCTGCGGAAGGATCATT CTGAGTGAGGGTCCCTCGGGGCCCAAACCTCCCCACCCCGTGGTATAC CGTACCTTGTGTTGCTTCGGGCGAGCCCCGCCCTTTTTCTTTAGGGGG CACAGCGCTCGCCGGAGACACCAACGTGAACACTGTCTGAAGTTTTGTC GTCTGAGTCGATTGTATCGCAATCAGTTAAAACCTTCAACAATGGATCT CTTGGTTCGGCATCGATGAAGAACGCAGCGAAATGCGATAATTAATGT GAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCACC CCCTGGTATTCCGGGGGGTATGCCTGTCCGAGCGTCATTGCTGCCCTCA AGCACGGCTTGTGTGTTGGGTCGTCTCCCCCCCCAGGGGGACGGGCC GAAAGGCAGCGGGCGGCACCGCGTCCGGTCCGAGCGTATGGGGCTTT GTCACCCGCTCTTGTAGCCCGGCCGGCTGCTGGCCGACGCTGAAAAGCA ACCAACTATTTTCCAGGGGACCTCGGATCAGGTAGGATACCCGCTGAAT TAGG</p>
<i>Aspergillus flavus</i>	<p>GTGTAACCTGCAGCATGATTACCGAGTGGTAGGGTTCCTTAGCGA GCCCAACCCTCCCCACCCCGTGTACTGTACTTTAATTGCTTCGGCGGG CCCCGCCATTCATGGCCGCCCGGGGTTTCAGCCCCGGGGCCCCGCGCC CCGCGGAGACACCACGAACCTCTGTCTGATCTAGTGAAGTCTGAGTTGA TTGTATCGCAATCAGTTAAAACCTTCAACAATGGATCTCTTGGTTCCGGC ATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAA TTCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCC GGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTG TGTGTTGGGTCGTCTCCCTCTCCGGGGGGGACGGGCCCAAAGGCAG CGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTGTACCCGCTC TGTAGGCCCGGCCGGCGCTTGCCGAACGCAAATCAACTTCCAGGTTGA CCTCGGATCAGGTAGGGAACCCGTGATTAGG</p>

3.5. Molecular differentiation of isolated fungi using ISSR technique

Inter Simple Sequence Repeat (ISSR) represent genome region between microsatellite loci. Sequences amplified by ISSR-PCR can be used for delimiting species. Inter-simple sequence repeat-PCR is a simple, not expensive, robust, multi-locus marker system which has been used in examines genetic variability among fungal pathogens. Primers depend on a sequence repeat and the resultant PCR reaction amplifies the sequence between two SSRs, yielding a multilocus marker system useful for fingerprinting, diversity analysis and genome mapping (Chadha and Gopalakrishna, 2007).

In this study, ISSR PCR technique was used to reveal the genetic diversity among different fungal isolates in order to search the genetic diversity between these isolates. Out of fifty ISSR primers, sixteen primers produced storable and reproducible banding patterns. Sixteen primers produced 388 band positions (loci) and out of these loci, 370 loci amplified were polymorphic. Majority of the primers which produced polymorphic bands had been GA or AG repeats followed by AC or CA repeats. Among the primers used, UBC-842 [(GA)₈ YG] produced most number of loci .

A total of 165 full bands were scored from the amplified products with the ten inter-Simple Sequence Repeat (ISSR) primer, 116 bands were polymorphic, 41 unique bands with average range size 143-1939 bp (Table, 9). All primers generated 100% polymorphism except primers ISSR-14 and ISSR-15 which generated 77, and 79% respectively. The obtained results revealed that the primers ISSR-1, ISSR-3 have amplified the maximum number of bands 21-23 respectively. On the other hand primers, ISSR-8 and ISSR-14 have amplified the lowest number of bands 13 (Fig 5). These results indicated that the primers ISSR-1 and ISSR-3 are the most repeated sequences in

these fungal isolates than other primers. On the other hand, the forty-one unique bands that were detected among the total bands could be considered for marker-assisted selection. The maximum numbers of unique bands were amplified by primer ISSR-3 which recorded 11 bands, whereas the lowest number of unique bands was amplified by ISSR-2, ISSR-8, ISSR-14, and ISSR-15.

These results showed that ISSR primers are robust, informative marker and would be a better tool for genetic divergence and phylogenetic studies. These data were used to create a similarity matrix for the construction of dendrograms by means of the UPGMA method. Mahmoud et al. (2016) used ISSR markers to analyze the genetic diversity of 30 *A. flavus* isolates from five agricultural crops and air, suggested that ISSR biotechnology is a highly useful tool for characterizing genetic diversity of *A. flavus* isolated from different sources. ITS species identification is nowadays performed by traditional techniques combined with molecular markers, resulting in a higher performance of isolate characterization. In the present study, internal transcribed spacer, inter-simple sequence repeats (ISSR), molecular markers were used, with the aim of genetically characterizing strains of *Aspergillus* species.

Table 9. The ISSR primer names and specific character of ISSR analysis

No	Name of primer	Monomorphic bands*	polymorphic bands**	Number of unique bands	Total bands	Polymorphism (%)	MW range (bp)	Mean of frequency
1	ISSR-1	0	16	5	21	100	214-1939	0.4
2	ISSR-2	0	17	2	19	100	323-997	0.5
3	ISSR-3	0	12	11	23	100	143-1730	0.4
4	ISSR-4	2	9	6	17	88	182-1338	0.5
5	ISSR-8	0	11	2	13	100	227-958	0.5
6	ISSR-11	0	11	3	14	100	145-1365	0.4
7	ISSR-12	0	9	5	14	100	182-1577	0.5
8	ISSR-13	0	14	3	17	100	197-1815	0.4
9	ISSR-14	3	8	2	13	77	278-1434	0.6
10	ISSR-15	3	9	2	14	79	905-1714	0.6
	Total	8	116	41	165	94.4		

The similarity matrix between isolated fungi revealed that isolates *A. parasiticus* and *A. niger* showed 59% similarity (Table 10). Results also showed that both *A. terreus* and *A. niger* had a similarity of 56%, followed by *A. flavus* and *A. niger* showing a similarity of 54%. Similarly, Yugander et al. (2015) used ISSR markers to examine the genetic variability of 24 strains of *Aspergillus* species isolated from paddy. Recently, Adss et al. (2017) used RAPD and ISSR to differentiate between 7 isolates of *A. solani* and their pathogenic capability. The resultant PCR reaction amplifies the sequence between two SSRs, yielding a multilocus marker system useful for finger printing, diversity analysis and genome mapping (Chadha and Gopalakrishna, 2007).

Table 10. Similarity matrix of isolated fungi based on ISSR analysis

Isolates	Matrix				
	1	2	3	4	5
<i>A. flavus</i>	1				
<i>A. parasiticus</i>	45	1			
<i>A. Terreus</i>	44	47	1		
<i>A. niger</i>	54	59	56	48	1

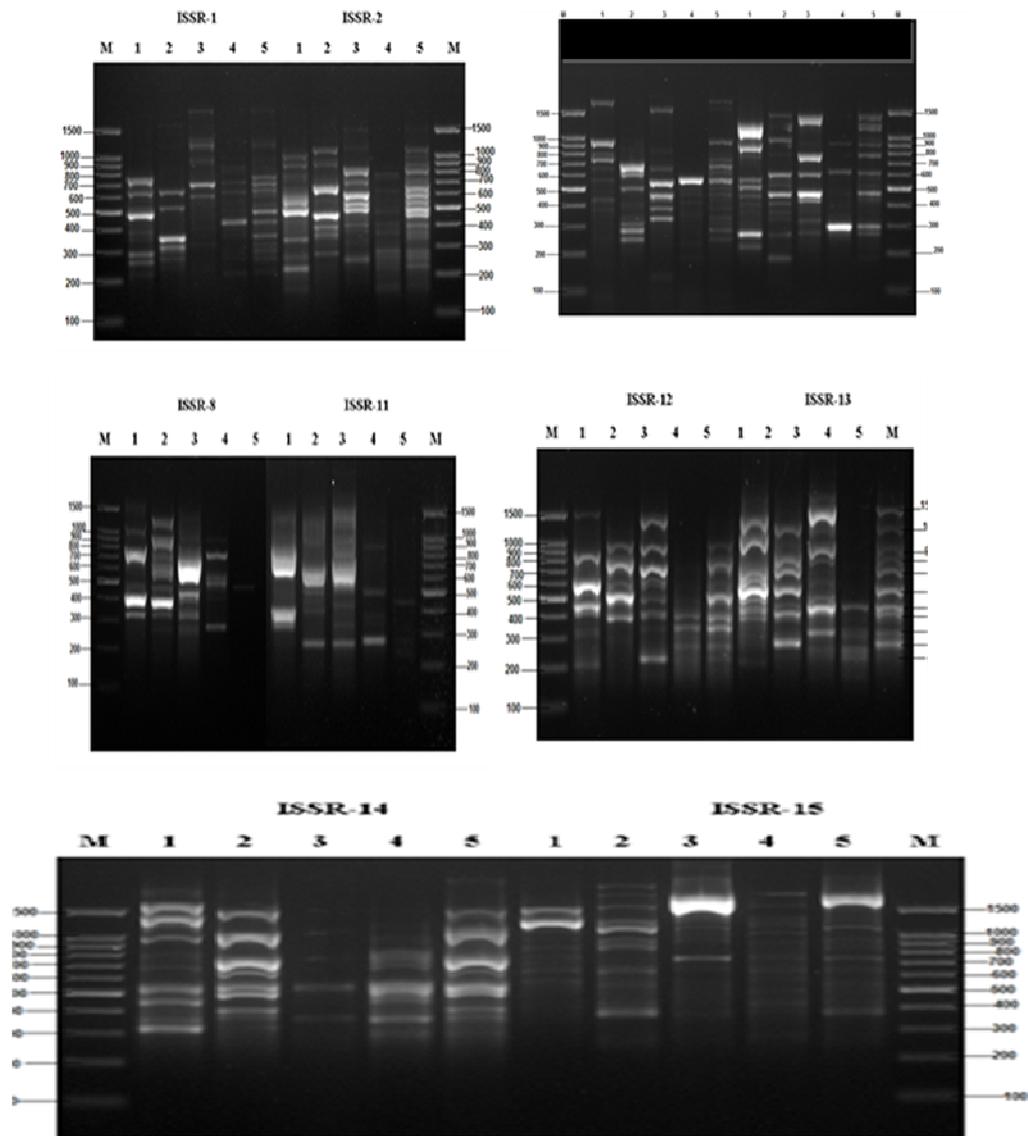


Fig 5. ISSR profile for five isolates of *Aspergillus* species. Lane 1: *Aspergillus flavus*; Lane 2: *Aspergillus parasiticus*; Lane 3: *Aspergillus terreus*; Lane 4: *Aspergillus ochraceus*; Lane 5: *Aspergillus niger*.

3.6. Cluster analyses

Dendrogram was constructed using UPGMA cluster analyses to reveal the genetic relationships among five fungal isolates. Fig (6) showed four major clusters, the first group included *A. flavus* isolate; the second group contained *A. terreus* isolate; the third group included *A. parasiticus* and *A. niger* isolates; the fourth group contained *A. ochraceus* isolate. Similar observations were reported by Abdulateef et al. (2014) who used ISSR-PCR based technology to document genetic diversity among some local Iraqi isolates through the determination of the ability *A. flavus* isolates to produce aflatoxin B₁.

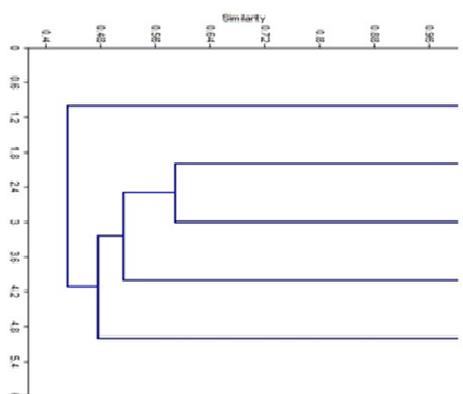


Fig 6. Dendrogram showing the relationships between isolated fungi based on ISSR analysis. Isolate 1: *Aspergillus flavus*; Isolate 2: *Aspergillus parasiticus*; Isolate 3: *Aspergillus terreus*; Isolate 4: *Aspergillus ochraceus*; Isolate 5: *Aspergillus niger*

4. Conclusion

These results recorded that the antimicrobial activity of bacterial isolate *Enterococcus faecalis* was higher than *Enterococcus faecium* isolate against most of microbial strains under study concluded that these isolated bacteria can be applied as halal food preservation. The present study indicated that ISSR is a suitable and effective tool to evaluate genetic diversity among *Aspergillus* genera.

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