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ASSESSMENT OF FUNGAL CONTAMINATION IN SOME CHEESE VARIETIES WITH ATTEMPTS TO CONTROL ITS GROWTH

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ABSTRACT

The objective of this study was to isolate and identify molds in processed, kareish, and ras (roumy) cheese samples obtained from Kafrelshiekh city, Egypt. Additionally, the study aimed to conduct trials to control mold growth and measure the levels of aflatoxin M1 and ochratoxin A in some cheese samples. Out of 60 randomly selected samples, 31 (51.67%) were found to be positive for the presence of various mold species, with frequencies of 45%, 55%, and 55% in processed, kareish, and ras cheese samples, respectively. The mean mold counts were 2.51 ± 1.90 cfu/g, 3.42 ± 2.48 cfu/g, and 2.59 ± 1.79 cfu/g (log 10), respectively. Several mold species were identified in the positive samples, including Penicillium spp., Aspergillus spp., Mucor spp., Alternaria spp., Fusarium spp., *Geotrichum candidum*, *Chrysonilia sitophila*, and *Endomyces fibuligera*. PCR analysis and the detection of aflatoxin regulatory genes (*aflR*, *Ver1*, *Nor1*, and *omtA*) were employed to assess the afla-toxigenicity of three *Aspergillus flavus* isolates. All three isolates tested positive for the *aflR* and *Ver1* genes; one isolate was positive for the *omtA* gene, while the *Nor1* gene was not detected in any of the three isolates. Additionally, three *Aspergillus niger* isolates were tested for ochratoxin production using PCR to identify the *Pks* gene, revealing that the *Pks* gene was present in two of the isolates. The levels of AFM1 and ochratoxin A in the cheese samples under examination were found to be below the permitted limits outlined by ISO 14675 and EC 1881, respectively. Furthermore, the findings demonstrate that natamycin (0.015%) combined with thyme oil (2%) effectively inhibited the growth of *A. flavus* and *A. niger* in vitro, indicating their potential use in cheese production to prevent mold growth and the associated economic losses during storage.

Keywords: Aflatoxigenic and ochra-toxigenic genes, Aflatoxin M1 and ochratoxins, Cheese, Fungi, Natamycin and thyme oil.

INTRODUCTION

The microbial quality of milk, thermal processing, manufacturing temperature, humidity during the ripening process, degree of salting, and microbiological contamination during and after manufacturing all influence the fungal contamination that can occur in cheese (Torkar and Teger, 2006). These microorganisms negatively impact the biochemical characteristics, flavor, and appearance of the products, making them economically undesirable and often leading

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to lower grading of dairy products (Muir and Banks, 2000).

Fungal growth on cheese is a common issue during the ripening process and when it is stored in the refrigerator by both retailers and consumers. Cheese often contains Penicillium and Aspergillus species (Gandomi *et al*., 2009).

Mycotoxins, which are secondary metabolites produced by fungi through a series of enzyme-catalyzed reactions involving a few simple biochemical intermediates of primary metabolism, can affect the health of both humans and animals. These mycotoxins may contaminate food sources directly or indirectly (Bohra and Purohit, 2003).

Mycotoxins, particularly aflatoxins, are toxic metabolites that can cause mycotoxicosis—a condition affecting both animals and humans, characterized by immune system issues, liver tumors, growth problems in children, and even fatalities (Becker-Algeri *et al*., 2016). Aflatoxins, especially those produced by *Aspergillus flavus* and *Aspergillus parasiticus*, are the most common mycotoxins generated by fungi. According to Gürbay *et al*. (2006), AFM1 (the hydroxylated metabolite of aflatoxin B1) constitutes 1-3 to 6% of aflatoxin B1 in feed and can appear in milk within a few hours after the consumption of contaminated food, lasting up to two days following the cessation of the contaminated diet.

Aflatoxin M1 and AFB1 can both induce gene mutations, cause DNA damage, lead to chromosome abnormalities, and result in the metamorphosis of mammalian cells in vitro (Govaris *et al*., 2002). The presence of AFM1 in milk and dairy products raises public health concerns due to prolonged and continuous exposure to this carcinogenic compound. Additionally, several studies have shown that AFM1 binds to the casein in milk proteins (Prandini *et al*., 2009), which explains why cheese often contains higher levels of the toxin than the milk used to produce it.

On the other hand, several Aspergillus and Penicillium species produce the mycotoxin, ochratoxin A (OTA) (Bayman and Baker, 2006). Ochratoxin A has teratogenic, hepatotoxic, and nephrotoxic properties (Boudra and Morgavi, 2006). According to the International Agency for Research on Cancer (IARC), OTA is classified as a Group 2B carcinogen (Muscarella et al., 2004).

Fungal growth on cheese during ripening is a common problem for cheese manufacturers, as well as for retailers and consumers during refrigerated storage. To address this issue, natural preservatives with antibacterial properties that have no adverse effects on handlers or consumers are needed. *Streptomyces natalensis* produces the polyene macrolide natamycin (pimaricin), which inhibits microbial activity by binding to and altering the permeability of fungal cell membranes (Deacon, 1997). Natamycin is effective at very low concentrations, can suppress almost all yeast and mold growth for up to six months (Zeuthen and Sorensen, 2003), and does not affect the sensory characteristics of food products (Dzigbordi *et al*., 2013).

The FDA has classified natamycin as a generally recognized safe (GRAS) product for humans (Koontz et al., 2003). It is also categorized by the European Union (EU) as a natural preservative (EFSA, 2009). To prevent contamination by yeasts and molds, many researchers have recommended using natamycin in dairy-based foods as a naturally occurring antimycotic polyene (Dervisoglu et al., 2014). Additionally, thyme essential oil has strong radical scavenging activity, along with antibacterial and antifungal properties (Maksimov, 2017). The most prominent active components in thyme oil are carvacrol, thymol, and

rosmarinic acid (Baranauskiene et al., 2003). Such an environment is highly beneficial for both human health and the environment, as antioxidants help prevent cancer and coronary heart disease, while also reducing food deterioration caused by free radical-mediated processes.

The purpose of this research was to identify the frequency of various mold species in different types of cheese collected from several grocery stores in Kafrelshiekh city, Egypt, and to detect aflatoxigenic and ochra-toxigenic genes in some isolates. Additionally, the quantities of mycotoxins such as AFM1 and ochratoxin A in some of the analyzed samples were estimated. The study also evaluated the efficacy of natamycin combined with thyme essential oil against mold growth in some isolates.

MATERIALS AND METHODS

1. Samples collection

Sixty samples of locally produced cheese were randomly selected from various grocery stores and dairy departments in Kafrelshiekh city, Egypt. The cheese types collected included processed, kareish, and ras (roumy). The samples were transported to the lab in a cooler within 1-2 hours of collection and analyzed immediately.

2. Cheese sample preparation

Each 10-gram cheese sample was homogenized in 90 ml of sterile 2% sodium citrate solution using a stomacher. To create a 10^{-2} dilution, 1 ml of the original homogenate was added to a test tube containing 9 ml of sterile 0.1% peptone water. A tenfold serial dilution was then prepared following the same procedure (APHA, 2004).

3. Count, isolation and identification of mold species

According to APHA (2004), 0.1 ml of each dilution was added to a single Sabouraud Dextrose Agar (SDA) plate and evenly distributed using a sterile spreader. The inoculated plates were then incubated at 25°C for 5 to 7 days. After incubation, individual fungal colonies grown on SDA media were counted and isolated. Pure colonies were maintained on agar slants at 4°C for further identification. The isolated mold colonies were identified by examining their macroscopic and microscopic characteristics, following the methods outlined by Pitt and Hocking (2009).

4. Molecular identification of some Aflatoxigenic and Ochra-toxigenic genes in *Aspergillus flavus* and *Aspergillus niger* isolates

DNA was extracted using the QIAamp DNeasy Plant Mini Kit (Catalogue No. 69104) with some modifications according to the manufacturer's guidelines. Primers supplied by Metabion (Germany) were used, and their details are listed in Table 1. The traditional PCR master mix was prepared using the Takara Emerald Amp GT PCR Master Mix (Code No. RR310A), as described in Table 2. Electrophoresis was performed on a 1.5% agarose gel (Sambrook *et al*., 1989; WHO, 2002). The cycling conditions for each primer used in the conventional PCR are detailed in Table 3. Data analysis was carried out using computer software after the gel was photographed with a gel documentation system.

5. Detection of AFM1 and Ochratoxin A residues in some of the examined cheese samples

The competitive enzyme-linked immunosorbent assay (CELISA) was used to quantitatively measure AFM1 levels in the cheese samples. Most of the reagents used were part of the RIDASCREEN® test kit and were employed according to ISO 14675 (2003). The AFM1 standard solutions had concentrations of 0, 5, 10, 20, and 80 ppt.

Ochratoxin A (OTA) residue was determined using the RIDASCREEN® Ochratoxin A 30/15 test kit (Art No.: R1312).

Table 1: Sequences of oligonucleotide primers (Metabion, Germany).

Gene	Sequence	Amplified product	Reference
Aflatoxin *omtA*	GGCCCGGTTCCTTGGCTCCTAAGC	1024 bp	**Norlia *et al.*, 2019**
	CGCCCCAGTGAGACCCTTCCTCG		
Aflatoxin *Nor1*	ACCGCTACGCCGGCACTCTCGGCAC	400 bp	
	GTTGGCCGCCAGCTTCGACACTCCG		
Aflatoxin *Ver1*	GCCGCAGGCCGCGGAGAAAGTGGT	537 bp	
	GGGGATATACTCCCGCGACACAGCC		
Aflatoxin *aflR*	AAC CGC ATC CAC AAT CTC AT	800 bp	**Bintvihok *et al.*, 2016**
	AGT GCA GTT CGC TCA GAA CA		
Ochratoxin *Pks*	CTTCCTTAGGGGTGGCACAGC	400 bp	**Patiño *et al.*, 2005**
	GTTGCTTTTCAGCGTCGGCC		

Table 2: Conventional PCR Master Mix preparation.

Component	Volume/reaction
Emerald Amp GT PCR mastermix (2x premix)	**12.5*µl***
PCR grade water	**5.5 *µl***
Forward primer(20 pmol)	**1 *µl***
Reverse primer (20 pmol)	**1 *µl***
Template DNA	**5 *µl***

Table 3: Conditions for cycling the various primers in conventional PCR.

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	Cycles No.	Final extension
Aflatoxin *omtA*	5 min. 95˚C	30 sec. 94˚C	40 sec. 61˚C	1 min. 72˚C	35	10 min. 72˚C
Aflatoxin *Nor1*	5 min. 95˚C	30 sec. 94˚C	40 sec. 67˚C	45 sec. 72˚C	35	10 min. 72˚C
Aflatoxin *Ver1*	5 min. 95˚C	30 sec. 94˚C	40 sec. 67˚C	45 sec. 72˚C	35	10 min. 72˚C
Aflatoxin *aflR*	5 min. 95˚C	30 sec. 94˚C	40 sec. 50˚C	50 sec. 72˚C	35	10 min. 72˚C
Ochratoxin *Pks*	5 min. 95˚C	30 sec. 94˚C	40 sec. 59˚C	45 sec. 72˚C	35	10 min. 72˚C

6. Antifungal activity of natamycin and thyme essential oil against *A. flavus* and *A. niger*

This study tested the effectiveness of natamycin and thyme essential oil as antifungal agents against *A. flavus* and *A. niger*, which were previously identified from the examined cheese samples. Natamycin (Natamax), provided by Danisco (DuPont Nutrition Biosciences Aps, DK-7200 Grindsted, Denmark), was prepared at concentrations of 0.015% and 0.02% (Thomas and Broughton, 2001). Additionally, natamycin at 0.015% was tested in combination with thyme essential oil (Harraz, Planta Medical Group, Egypt) at concentrations of 1%, 1.5%, and 2%.

The agar dilution method was used to evaluate the antifungal activity of natamycin and thyme essential oil (Bansod and Mahendra, 2008). Sabouraud Dextrose Agar (SDA) was prepared as a liquid and poured into Petri dishes containing the specified concentrations of natamycin and thyme essential oil. The Petri dishes were shaken until the media and antifungal substances were thoroughly mixed and allowed to solidify. Discs (1 cm^2) of the previously isolated *A. flavus* and *A. niger* were placed in the center of the dishes (2 plates per mold). The plates were incubated for 5 to 7 days at 25°C. The parameter observed was the inhibition of Aspergillus spp. growth, with colony diameters compared to the control.

7. Statistical Analysis

All measurements were analyzed using SPSS 22.0 (IBM Corp., Armonk, NY, USA). Parametric data are presented as mean ± SE (standard error) and were subjected to ANOVA followed by the LSD (Least Significant Difference) test. Differences among groups were considered significant at $P < 0.05$.

RESULTS

Table 4: Incidence and statistical analysis of mold contamination in the examined cheese samples.

Samples type	Examined sample No.	Positive samples		Mean ± SE (Log 10, cfu/g)
		No.	%	
Processed cheese	20	9	45	2.51 ± 1.90 [b]
Kareish cheese	20	11	55	3.42 ± 2.48 [a]
Ras cheese (roumy)	20	11	55	2.59 ± 1.79 [b]
Total	60	31	51.67	

The means of various letters in the same column differ considerably at ($P< 0.05$)

Table 5: Frequency distribution of isolated mold species from positive cheese samples.

Identified fungi	Isolates		Processed cheese		Kareish cheese		Ras cheese (roumy)	
	No.	%	No.	%	No.	%	No.	%
Aspergillus flavus	3	7.69	1	6.67	1	9.09	1	7.69
Aspergillus niger	3	7.69	1	6.67	-	-	2	15.39
Aspergillus fumigatus	1	2.57	-	-	-	-	1	7.69
Alternaria spp.	7	17.96	5	33.33	-	-	2	15.39
Chrysoniliasitophila	1	2.57	1	6.67	-	-	-	-
Endomycesfibuligera	1	2.57	-	-	1	9.09	-	-
Fusarium spp.	1	2.57	-	-	-	-	1	7.69
Geotrichumcandidum	3	7.69	-	-	2	18.18	1	7.69
Mucor fragilis	1	2.57	-	-	1	9.09	-	-
Mucor irregularis	8	20.51	-	-	6	54.55	2	15.39
Penicillium chrysogenum	1	2.57	-	-	-	-	1	7.69
Penicillium hemtrachum	2	5.13	2	13.33	-	-	-	-
Penicillium pagulum	2	5.13	-	-	-	-	2	15.39
Penicillium caseifulvum	5	12.82	5	33.33	-	-	-	-
Total	39	100	15	38.46	11	28.21	13	33.33

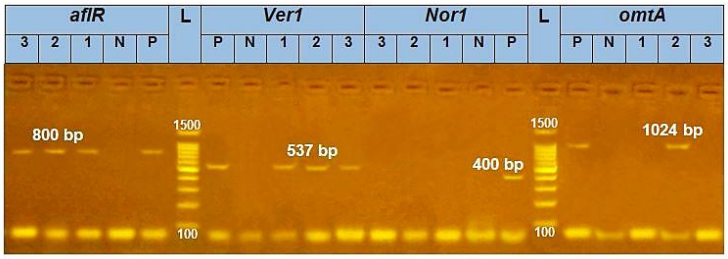

Photo (1): PCR for aflatoxigenic genes (*aflR, Ver1, Nor1, omtA*) in *Aspergillus flavus* isolates using agarose gel electrophoresis
L: 100-1500 bp molecular size marker.
P: Positive, control , **N:** Negative, control
Lane (1, 2 and 3): positive for *aflR* and *Ver1* (800 and 537 bp, respectively)
Lane (1, 2 and 3): negative for *Nor1* (400 bp)
Lane (2): positive for *omtA* (1024 bp)

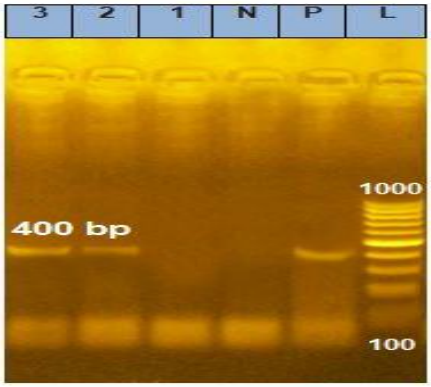

Photo (2): PCR for ochratoxigenic gene (*Pks*) in *Aspergillus niger* isolates using agarose gel electrophoresis
L: 100-1000 bp molecular size marker.
P: Positive, control, **N:** Negative, control
Lane (2 and 3): positive for *Pks* (400 bp)
Lane (1): negative for *Pks* (400 bp)

Table 6: Aflatoxins M1 level in some cheese samples.

Samples Type	Examined samples No.	Minimum	Maximum	Mean ± SE (ppt)	Permissible limit (ISO 14675:2003)
Processed cheese	4	< 5	6.14	5.69±0.26[b]	50 ppt
Kareish cheese	4	< 5	5.82	5.52±0.18[b]	
Ras cheese (roumy)	4	11.01	29.09	22.64±5.83[a]	

The means of various letters in the same column differ considerably at (P< 0.05)

Table 7: Ochratoxins A (OTA) level in some cheese samples

Samples Type	Examined sample No.	Minimum	Maximum	Mean ± SE (ppb)	Permissible limit (EC 1881: 2006)
Processed cheese	3	2	3.02	2.51±0.29	5 ppb
Ras cheese (roumy)	3	2.41	2.79	2.60±0.11	

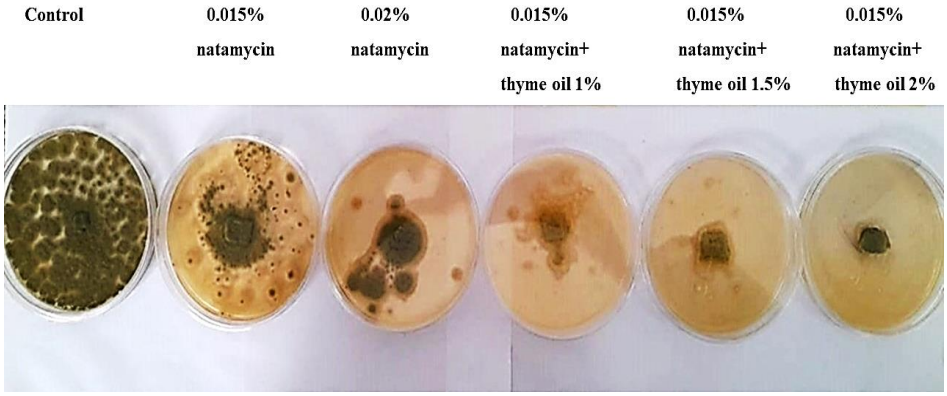

Photo (3): Effect of different concentrations from natamycin and thyme oil on growth of *Aspergillus flavus*

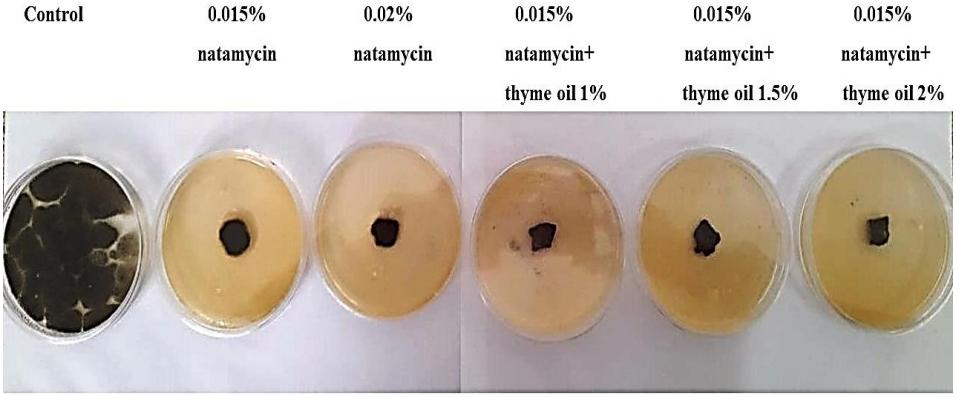

Photo (4): Effect of different concentrations from natamycin and thyme oil on growth of *Aspergillus niger*

DISCUSSION

Mold growth in cheese is unacceptable, even in small amounts, due to the significant economic losses it can cause. This issue impacts both food safety and quality, as various mold species can produce undesired changes that severely degrade the quality of the product (Kure and Skaar, 2019).

Table (4) shows the incidence of mold contamination in the examined cheese varieties. Molds were detected in 51.67% of all cheese samples, with contamination rates of 45%, 55%, and 55% for processed, kareish, and ras (roumy) cheeses, respectively. Kareish cheese had the highest mean mold count (3.42 ± 2.48 $\log_{10}$ cfu/g), followed by ras and processed cheese. There was no significant difference in the mean mold counts between processed and ras cheeses.

Abdel Hameed (2016) reported a lower incidence of mold contamination in processed cheese (20%), whereas Abd El Tawab *et al*. (2020) and Ahmed *et al.* (2020) found higher incidences (90% and 60%, respectively). For kareish cheese, Abdel Hameed (2016) and Eid *et al.* (2022) reported similar results (52% and 54%, respectively), while Salim *et al*. (2020) reported a higher incidence (86.67%) and ELbagory *et al.* (2014) found 100%. Conversely, Younis *et al.* (2016) reported a lower incidence (28%). For ras (roumy) cheese, Elramly *et al.* (2019) found a similar result (57.5%), whereas Ahmed *et al.* (2020) reported a higher incidence (85%) and Younis *et al.* (2016) found a lower incidence (48%).

Fungal contamination of raw milk and cheese occurs due to the widespread dispersal of spores in unhygienic environments, which contaminates dairy products during manufacturing,

transportation, processing, and storage. Factors such as using low-quality milk, inadequate sterilization, unsanitary conditions (including utensils, tools, personnel, or clothing), and reinfection during pasteurization, transportation, or storage can all contribute to fungal contamination (Younis *et al*., 2016).

Mold contamination in dairy products serves as an indicator of sanitary quality conditions (Varga, 2007). The distribution of isolated mold species from the positive cheese samples is shown in Table 5. In processed cheese, the most common mold isolates were Penicillium spp. (7/15, 46.66%), Alternaria spp. (5/15, 33.33%), Aspergillus spp. (2/15, 13.34%), and *Chrysonilia sitophila* (1/15, 6.67%). In kareish cheese, the most prevalent isolates were *Mucor irregularis* (6/11, 54.55%), *Geotrichum candidum* (2/11, 18.18%), *Aspergillus flavus* (1/11, 9.09%), *Endomyces fibuligera* (1/11, 9.09%), and *Mucor fragilis* (1/11, 9.09%). In ras cheese, the most abundant isolates were Aspergillus spp. (4/13, 30.77%), Penicillium spp. (3/13, 23.08%), Alternaria spp. (2/13, 15.39%), *Mucor irregularis* (2/13, 15.39%), Fusarium spp. (1/13, 7.69%), and *Geotrichum candidum* (1/13, 7.69%).

Similar findings were reported by Silva *et al*. (2015), who identified Aspergillus spp., Geotrichum spp., Penicillium spp., and Fusarium spp. as the primary fungi responsible for mycotoxin contamination in cheese and milk. ELbagory *et al.* (2014) found that the most prevalent genera among the four types of cheese samples examined were Aspergillus and Penicillium species. Additionally, Finne Kure *et al*. (2004) noted that Penicillium was the most common mold genus found in cheese samples, followed by Aspergillus, Geotrichum, Cladosporium, and Mucor species.

The proliferation of Penicillium, Cladosporium, Aspergillus, and Mucor species can lead to cheese rancidity and bitterness. Specifically, Penicillium species may cause the cheese surface to soften (Minervini *et al*., 2001). On the other hand, *A. niger* can result in allergic conditions and otomycosis. Certain Penicillium species have been linked to urinary tract infections, yellow rice disease, and pulmonary infections, which can be fatal. Additionally, some Fusarium species have been associated with allergic conditions and keratoconjunctivitis in humans (Nielsen *et al*., 1998).

Molecular analysis of toxigenic genes is gaining popularity due to its efficiency and quicker results compared to traditional morphological identification methods (Gil-Serna *et al*., 2009). For detecting toxins in food products, the genes *aflR1*, *omtA*, *Nor1*, and *Ver1* are frequently used for their precise, reliable, and rapid identification of aflatoxigenic Aspergillus spp., particularly in food samples (Sadhasivam *et al.*, 2017).

In this study, specially designed primers for the aflatoxin regulatory genes (*aflR*, *Ver1*, *Nor1*, and *omtA*) were used to detect aflatoxigenic strains among three *A. flavus* isolates. PCR results indicated that all tested isolates were positive for the *aflR* and *Ver1* genes (at 800 bp and 537 bp, respectively). One isolate was positive for the *omtA* gene (1024 bp), while all three isolates were negative for the *Nor1* gene (400 bp) (see Photo 1).

A. flavus is widely recognized as a potent liver cancer-causing agent and a primary source of aflatoxin B1, which transforms into aflatoxin M1 in milk. Cheese may have elevated levels of aflatoxin due to the high affinity of AFM1 for casein present in milk (Bakirci, 2001). To detect ochratoxigenic genes in three *A. niger* isolates, primers specifically designed for the ochratoxin regulatory pathway gene (*Pks*) were used. PCR results revealed that two strains were positive for the *Pks* gene (400 bp) (see Photo 2). According to Alsalabi *et al*. (2023), the presence of the polyketide synthase gene (*Pks*) in *A. niger* confirms the molds' ability to produce ochratoxin.

Mycotoxins are highly persistent and harmful, even in minute quantities, with only a few parts per billion being cause for concern. Research has shown that AFM1 levels are higher in colder months compared to warmer ones. This is because cows are

often fed compound diets during winter, which increases the concentration of AFB1 and, consequently, raises the level of AFM1 in milk (Azizollahi *et al*., 2012).

Egyptian researchers have reported that milk product samples contained mycotoxin residues exceeding the permissible limits set by Egyptian and European regulations. This suggests that elevated mycotoxin levels in dairy products may be linked to poor hygiene practices during manufacturing, packaging, or storage, which promote mold growth and the subsequent synthesis of mycotoxins (Khalifa and Shata, 2018).

According to Table 6, the levels of AFM1 in some processed cheese samples ranged from less than 5 up to 6.14 ppt, with a mean of 5.69 ± 0.26 ppt. In some kareish cheese samples, AFM1 levels ranged from less than 5 up to 5.82 ppt, with a mean of 5.52 ± 0.18 ppt. There was no significant difference between the mean AFM1 values of processed and kareish cheese samples. However, the mean AFM1 value in some ras cheese samples was 22.64 ± 5.83 ppt, which differed significantly from that of processed and kareish cheese samples. All investigated samples were below the maximum acceptable level of 50 ppt, as specified by ISO 14675 (2003) and Egyptian Standard (2007) for AFM1 in milk and its products.

Esam *et al*. (2022) reported an AFM1 mean level of 10.77 ± 1.39 ppt in processed cheese samples. In contrast, Ibrahim *et al*. (2016) found that AFM1 levels in kareish cheese samples exceeded the EU limit of 50 ppt. Younis *et al*. (2016) detected lower AFM1 levels (1 to 2.1 ppt) in ras cheese samples, whereas Elramly *et al*. (2019) obtained results that were notably similar to those found in this study, with AFM1 levels ranging from 17.1 to 30.9 ppt in ras cheese samples.

Aflatoxins are among the most potent hepatotoxins and carcinogens known, and their effects can vary depending on the duration of exposure and an individual's nutritional status. Acute aflatoxicosis can lead to cirrhosis or liver cancer, characterized by acute hepatic necrosis. This condition can result in symptoms such as mental disturbances, coma, edema, altered digestion, changes in nutritional absorption and metabolism, and hemorrhage (Barjesteh *et al*., 2010).

The concentration of ochratoxins (Table 7) in certain processed cheese samples ranged from 2 to 3.02 ppb, with a mean of 2.51 ± 0.29 ppb. In ras cheese samples, levels ranged from 2.41 to 2.79 ppb, with a mean of 2.60 ± 0.11 ppb. These values are below the maximum permitted limit of 5 ppb for ochratoxin in milk and dairy products, as stated in EC (1881: 2006).

The findings contrast with those of Ahmed *et al*. (2020), who reported that the majority of processed and ras cheese samples contained ochratoxin A levels exceeding the limits set by EC (1881: 2006) and EOSQC (2007). For ras cheese, our results were lower than those reported by Younis *et al*. (2016) but higher than those of Elramly *et al*. (2019), who found OTA levels in ras cheese samples ranging from 3 to 4.8 ppb and from 0.693 to 1.508 ppb, respectively.

The contamination of cheese with ochratoxin may arise from the use of milk contaminated with ochratoxin, which can be introduced through animals fed contaminated rations (Monaci and Palmisano, 2004) or through the presence of ochratoxin-producing species (Cabañes *et al*., 2010). Improper packaging or storage practices that promote mold growth on the cheese's surface can also contribute to ochratoxin contamination, as these conditions can facilitate toxin production. Ochratoxin A (OTA) is a potent nephrotoxin associated with kidney issues in both animals and humans. It is also immunotoxic, genotoxic, and carcinogenic (Clark and Snedeker, 2006).

The effects of various concentrations of natamycin and thyme oil on the growth of *A. flavus* and *A. niger*, cultured on SDA medium, are illustrated in Photos (3, 4). The results indicated that *A. niger* was more sensitive than *A. flavus*, as it was completely suppressed by a combination of 0.015% natamycin and 1% thyme oil. In contrast, *A.*

flavus was significantly reduced by 0.015% natamycin combined with 2% thyme oil.

Natamycin disrupts the permeability of fungal cell membranes by interacting with the sterols present in the membrane, leading to rapid leakage of essential peptides and ions and ultimately causing cell lysis (Jay, 2000). Khan and Ahmad (2011) demonstrated that essential oils derived from aromatic plants, including thyme, can damage the cell wall and cytoplasmic contents of fungal cells. According to Ultee *et al*. (2002), the lipophilic nature of essential oils allows them to penetrate the plasma membrane, leading to the accumulation of polysaccharides and the breakdown of the plasmalemma of fungal cells under drought-stressed conditions.

These findings are supported by several studies. Gonzalez-Forte *et al.* (2019) showed that natamycin effectively inhibits the growth of fungi isolated from cheese samples. Hassanin *et al*. (2021) found that essential oils, including thyme, clove, and peppermint, inhibited the growth of fungi isolated from ras cheese samples. Yangilar (2017) reported that applying a coating film supplemented with oregano and natamycin effectively prevented mold growth during the ripening of Kashar cheese without compromising quality.

CONCLUSION

The results of this study revealed a significant amount of mold contamination in the cheese samples, posing a risk to consumer health. The most effective strategy for minimizing mycotoxin levels in cheese is to prevent mold growth. This requires understanding and controlling the environmental factors that promote fungal development and toxin production in cheese-making facilities.

Applying natural antifungal agents such as natamycin and essential oils offers a promising approach to controlling fungal growth without compromising cheese quality. In this study, natamycin (0.015%) and thyme oil (1%, 1.5%, and 2%) were effective in inhibiting the growth of *A. flavus* and *A. niger* in vitro. Therefore, incorporating natamycin and thyme oil as natural preservatives in cheese production could help reduce spoilage and minimize economic losses.

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تقييم التلوث الفطرى فى بعض أصناف الجبن مع محاولات للسيطرة على نموه

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كان الغرض من هذه الدراسة هو عزل وتصنيف الفطريات في عينات الجبن المطبوخ والجبن القريش والجبن الرومى التي تم الحصول عليها من مدينة كفر الشيخ، مصر. علاوة على ذلك، تم إجراء تجارب للتحكم في نموها بالإضافة إلى قياس كمية الأفلاتوكسين M1 والأوكراتوكسين A في بعض عينات الجبن. من أصل ٦٠ عينة تم اختيارها عشوائياً، تبين أن٣١ (٥١,٦٧%) عينة كانت إيجابية لوجود العديد من أنواع الفطريات، مع نسبة حدوث ٤٥%، ٥٥%، و٥٥% في عينات الجبن المطبوخ، والجبن القريش، والجبن الرومى، على التوالى. مع متوسط عدد الفطريات ٢,٥١ ± ١,٩٠ ، ٣,٤٢ ± ٢,٤٨ ، و٢,٥٩ ± ١,٧٩ على التوالي. تم التعرف على أنواع مختلفة من الفطريات، بما في ذلك Aspergillus spp., Penicillium spp., Mucor spp., Alternaria spp., Fusarium spp., *Geotrichum candidum, Chrysonilia sitophila, Endomyces fibuligera* في العينات الإيجابية. تم استخدام PCR والجينات التنظيمية للأفلاتوكسين (*aflR*، *Ver1*، *Nor1* و*omtA*) لتقييم إنتاجية سموم الأفلاتوكسين لثلاث معزولات من فطر *Aspergillus flavus*. وكانت جميع المعزولات الثلاث التي تم اختبارها إيجابية لتواجد جين *aflR* و *Ver1*، وكانت معزولة واحدة إيجابية لجين *omtA*، ولم يتم اكتشاف الجين *Nor1* في أي من المعزولات الثلاث. بالإضافة إلى ذلك، تم اختبار ثلاث معزولات من *Aspergillus niger* لإنتاج سموم الأوكراتوكسين باستخدام تقنية PCR للكشف عن جين Pks، وأظهرت النتيجة أن معزولتين كانتا إيجابيتين لجين *Pks*. كانت مستويات الأفلاتوكسين والأوكراتوكسين في عينات الجبن قيد الفحص أقل من الحدود المسموح بها الموضحة في المواصفة ISO 14675 والمواصفة EC 1881، على التوالي. بالإضافة إلى ذلك، أظهرت النتائج أن الناتاميسين ٠,٠١٥% مع زيت الزعتر ٢% كان لهما تأثير كاف على التثبيط الكامل لنمو فطر*Aspergillus flavus* و *Aspergillus niger* في المعمل، مما يشير إلى إمكانية استخدامها في إنتاج الجبن لمنع نمو الفطريات التى قد تتسبب فى خسائر اقتصادية أثناء فترة التخزين.