Diversity of Toxigenic Molds and Mycotoxins Isolated from Dairy Products: Antifungal Activity of Egyptian Marine Algae on Aspergillus and Candida Species

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Abbreviations: AFB1, Aflatoxin B1; AFM1, Aflatoxin M1; OTA, Ochratoxin A; TLC, thin-layer chromatography; DMSO, Dimethyl sulfoxide; GC–MS, Gas chromatography–mass spectrometry technique; CFU, Colony forming unit; aflR, Aflatoxin regulatory gene; AI, Acceptable Intake; ADI, Acceptable Daily Intake; DI, Daily Intake; WI, Weekly Intake; EDI, Estimated Daily Intake; EWI, Estimated Weekly Intake; PTWI, Provisional Tolerable Weekly Intake; EI, Estimated intake; MPL, permissible limits.

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Abstract

Fungal and mycotoxin contamination of milk products constitute a potential hazard to human health and food safety. Isolation and identifications of mold and yeast out of 140 milk products samples collected from dairy shops in Qena, Egypt were done through conventional microbiological methods. Aflatoxin-M1, aflatoxin-B1 and ochratoxin-A were characterized by thin-layer chromatography; aflR regulatory gene identified by using PCR. Marine algal extracts of *Halimeda opuntia*, *Padina pavonica* and *Turbinaria decurrens* species were studied for their antimicrobial activity. Overall of 80 and 64% dairy products samples were positive for mold and yeast contamination. A total of 38 mold and 15 yeast species were isolated. *Aspergillus* and *Candida* spp. were the most abundant isolated species. Furthermore 25, 40 and 27% of cheese and 71, 78 and 73.3 of dairy desserts samples were contaminated with AFM1, AFB1 and OTA, respectively; with average estimated dietary intake level much more than the acceptable daily intake for infant and adult. PCR identified aflR gene among four selected aflatoxigenic *A. flavus*. The major constituents of *H. opuntia* extract were 2,4-Decadienal, (E,E)-(21.56%) and 9,12-Octadecadienoic acid (Z,Z) -(36.16%). Ethyl acetate extract of *Halimeda opuntia* (3mg/ml) exhibited the strongest fungicidal activity with inhibition zones of 16.5 and 22.3 mm against *A. flavus* and *A. niger*. It exhibited potent candidacidal activity against *C. tropicalis*; 11 log10 orders of killing at 750 µg/ml. The discovered antimicrobial activity of *H. opuntia* is a promising candidate for designing novel antifungal agents which can be used in food preservatives and medicine industry.

Keywords: Milk products, *Aspergillus*, *Candida*, Mycotoxins, Algae, Antimicrobial activity

INTRODUCTION

Mold and yeast can invade the dairy products during unhygienic processing and handling conditions which constitute a public health hazard. Mold and yeast in milk and dairy products might act as allergen and an irritant to human health and may be the reason for the gastrointestinal disease. Species of *Aspergillus*, *Fusarium*, *Penicillium*, *Rhizopus* and *Trichoderma* are common contaminants of dairy products and known as spore formers. Mold species are also responsible for many serious diseases through production of toxic metabolites called mycotoxins. Thus, mold and yeast counts are considered the standard test of milk hygiene. *Aspergillus* and *Penicillium* species are common spore forming contaminants in the dairy derivatives.

Mycotoxins; aflatoxin-B1 (AFB1), aflatoxin-M1 (AFM1) and ochratoxin-A (OTA) are fungal toxic metabolites characterized by heat stable toxicity, mutagenicity, teratogenicity and carcinogenicity. Aflatoxins and ochratoxins are the major mycotoxins affecting our health. Aflatoxin is produced by several *Aspergillus* species including *Aspergillus flavus* and *Aspergillus parasiticus*. Major aflatoxins are B1, B2, G1, G2 and plus two additional metabolites; M1 and M2. AFB1 and its metabolite AFM1 in cow’s milk are human carcinogenic toxins. The AFB1 and AFM1 international permissible limits are 2.0 and 0.05µg/kg, respectively. Ochratoxin-A is produced by *Aspergillus* and *Penicillium* on the surface of cheese during ripening and is the most common and wide-scale carcinogenic toxin in the Ochratoxins’ family. The international permissible limit of OTA is 5.0µg/kg. Prevention of the toxigenic molds growth and mycotoxins synthesis in raw materials and end products to control its outbreak, is achieved traditionally by using of chemical preservatives. Despite of its proven efficiency, their repeated applications has resulted in side effects on human health, acquisition of microbial resistance to the applied chemicals and accumulation of chemical residues in food. The growing consumer demand for high quality food, safe, preservative free with extended shelf life has focused efforts in the discovery of new natural preservatives. On light of above, efforts have been directed to developing potentially effective, safer, healthy and natural food preservatives. Marine organisms including marine macroalgae (seaweeds) are source of various natural antimicrobial compounds with pharmacological and biological activities. Macroalgae contain many different secondary metabolites which have become recognized as potential sources of bioactive compounds, such as antimicrobial active compounds. The
existence of bioactive compounds with antifungal effect was recorded in crude extracts of different species of green, brown and red algae by many investigators. Utilization of algal extracts as antimicrobial agents for food preservation could be a fascinating alternative to chemical and physical methods, and it has received much attention recently. The algal extracts considered as natural antimicrobial agents, nutritionally safe and easily degradable. The antimicrobial activity exhibited by algal extracts against mold and yeast has been demonstrated by several researchers. The present study aimed to investigate the mold and yeast species present in dairy products, and estimate the levels of mycotoxins; AFB1, AFM1 and OTA using thin-layer chromatography (TLC) considering their permissible limits and acceptable daily intakes (ADI) for human taking the age into consideration. Furthermore, Aspergillus flavus strains would be molecularly identified for the presence of aflR regulatory gene. Furthermore, the antimicrobial activity of some algal extracts, i.e. Padina pavonica, Halimeda opuntia and Turbinaria decurrens against Aspergillus and Candida species were evaluated.

MATERIALS AND METHODS

All procedures of sampling, assays and analyses were strictly carried out according to the instructions and guidelines provided with the companies brochures for lab analysis.

Media and samples used for fungal isolation and identification

Sabouraud dextrose agar, Malt extract agar (MEA), Czapek yeast extract agar (CYA), 25% glycerol nitrate agar and TSB (pH 7.3) were used.

Samples

A total 140 random samples of cheese (Ras, Cheddar, Feta and Processed) and dairy desserts (Mahalabia, Custard and Rice Milk) were randomly collected from dairy markets. The samples were kept in polyethylene bags and preserved in ice box, then immediately transferred to the laboratory aseptically to be prepared for mycology.

Preparation of samples’ serial dilutions

The samples were released out aseptically from their packages and thoroughly mixed in a sterile mortar. From each prepared samples, 10 grams were transferred into sterile flask containing 90 ml sterile peptone water solution as a buffer. Ten-fold serial dilutions up to 10^8 from each sample were prepared.

Mold and yeast counting and identification

The developing yeast and mold colonies were counted and isolated for identification according to Chay et al. (2017) and Harrigan and MacCance (1966). The obtained results were compared with the maximum allowed mold or yeast counts of the Egyptian Organization for Standardization and Quality control (EQSOC). Mold isolates were inoculated in CYA, MEA and G25N (25%). Colony appearance, exudate production, reverse coloration and pigmentation were assessed. Colonies’ diameters were assessed after 7 days of growth at 25°C. The characteristics of yeast colonies including; the pattern and rate of growth, colony consistency, size and its surface color were studied. Vegetative reproduction was examined on corn meal agar. Yeast morphology was grossly and microscopically examined. Yeast growth was studied after isolates culturing on slopes of Sabouraud dextrose media and incubating it at 37°C for 3-5 days. Urease and germ tube tests were performed according to Cruickshank et al. (1975) and Koneman et al. (1992).

Detection of Mycotoxins Residues in the Dairy Products

Extraction of aflatoxins out of dairy products samples was done in accordance with the method mentioned by Roberts and Patterson (1975). The final samples’ filtrates were combined and evaporated to dryness in a rotatory evaporator and saved for analysis. Ochratoxin-A (OTA) was detected by thin-layer chromatography (TLC) according to AOAC (1980). Mycotoxins residues were qualitatively estimated according to the method proposed by (Scott, 1965; Howell and Taylor, 1981). The reference values, colors and intensities of unknown spots were compared to those standard reference values (Sigma, USA). Samples extracts which were found to contain mycotoxins by the qualitative technique were further calculated according to the standard formula:

\[
\text{µg/kg} = \frac{(S \times Y \times V)}{(X \times W)}
\]

\(S=\) µl of mycotoxin standard equal to unknown
\(Y=\) Concentration of mycotoxin in µg/ml

\(X=\) Volume of unknown sample in ml
\(V=\) Concentration of mycotoxin in µg/ml
V=µl of final extract dilution.
X=µl of the extract emitting a spot intensity equal to S. W= Mass (weight) of the sample, represented by the final extract in gram.

The obtained results were compared with the maximum permissible limits (MPL) of the local Egyptian Organization for Standardization and Quality control (EQSOC) and International organizations latest guidelines standards.

Health risk assessment: estimated daily intake and estimated weekly intake

Calculations of the estimated daily intake (EDI) and estimated weekly intake (EWI) of mycotoxins in the examined milk products samples were performed according to previous accredited equations. The obtained results were compared to the acceptable daily intake (ADI) according to the international standards organization.

Detection of Aspergillus flavus ability to produce aflatoxins using PCR

Genomic DNA was extracted from ground frozen Aspergillus flavus mycelium/spores using Spin Column DNaseasy plant minikit (Geneaid, USA). The target fragment of regulatory aflatoxin gene fragments of aflatoxigenic DNA was amplified by PCR. The forward-reverse primers aflR1 regulatory gene was; 5′ AACCGCATCCACAATCTCAT 3′, and 5′ AGTGCAGTTCGCTCAGAACA 3′, involved within 800-base pairs (bp) gene size16. The reaction mixtures consisted of extracted A. flavus target DNA, forward primer (F), reverse primer (R) and PCR Master Mix: DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, USA, Cat., No. K1081). Amplification of DNA was performed via optimized PCR reactions; denaturation, primer annealing and extension. Subsequently, 35 amplification cycles were done in a programmable thermocycler (Gradient Thermal cycler; 1000 S Thermal cycler BioRAD USA). The PCR-product was analysed by electrophoresis on agarose gel (1.5%) stained by ethidium bromide. The gel image was visualized by trans-illuminator. A 100 bp-size ladder is the marker for amplicons size.

Algal collection and extraction preparation

Three marine algae; Halimeda opuntia (Chlorophyta), Padina pavonica, and Turbinaria decurrens (Phaeophyta) were collected during October, 2018 from Hurghada coast, Red Sea Coast, Egypt. Seaweeds were collected in sterilized polyethylene bags, kept in ice box till reach the laboratory for identification, preparation and analysis17. Algal extraction was performed by using different solvents; ethyl acetate for Padina pavonica, and petroleum ether for Halimeda opuntia and Turbinaria decurrens. The extracts were suspended in dimethyl sulfoxide (DMSO) to a final concentration of 50 mg/ml, and then stored in airtight bottles at 4°C till used18.

Gas chromatography-mass spectrometry (GC-MS) analysis of algal extract

Algal extracts were identified by using GC-MS (Thermo Scientific Technologies, Trace-1310, capillary column TG-5; 30m×250μm×0.25μm). Split mode-mass detector and helium gas with flow rate carrier 1.5 ml/minutes (min) were used. Injector was operated at 230°C, and initial oven temperature 60°C for 2 min and ramp 10/min to 300°C for 8 min. Mass spectra were taken at 70 eV and the total GC running time was 35 min.

Microorganisms

Strains of Aspergillus flavus, Aspergillus niger and Candida tropicalis as identified by Mycology Department, Animal Health Research Institute, Cairo, Egypt were used to study the antifungal activities of algal extract. All isolates were cultured onto Sabouraud dextrose agar, 24 hrs prior to assay.

Antifungal assay

Inoculums of Aspergillus flavus and Aspergillus niger cultures were suspended in a sterile saline solution (0.85 %). Suspension turbidity was modified by the spectrophotometry at 530 nm till obtain a final concentration of 0.5 Mcfarland standards (0.5-2.5×10^3). The antifungal activity was evaluated by using well diffusion method on the agar plates of Sabouraud dextrose media19, where the inhibition zones were detected after 48 hours of incubation at 27°C. The assay was done in triplicates (n = 3).

Candidacidal assay

Candida tropicalis blastoconidia grown for 24 hrs in 1% TSB (pH 7.3) were rinsed, resuspended to 10^6 - 10^7 CFU/ml in Sabouraud dextrose broth, and then mixed with DMSO, in equal volume, containing series of the algal extracts; 3 mg/ml, at 2-fold serial dilutions. The mixture was plated on Sabouraud dextrose media and incubated at 28°C for 24 hrs followed by further incubation at 30°C for another 24 hrs. The algal colonies were finally counted in log CFU/ml in the assay media20.
Statistical analysis
The inhibition zones in response to treatment of the fungi with algae were analysed by using two-way analysis of variance (ANOVA) and Bonferroni was the post-hoc test. All data were set as mean ± standard error (SEM) for three replicate-assays. Graph-Pad Prism software, San-Diego, USA, v. 5 was used. The difference among groups was considered significant at P<0.05.

RESULTS
The examined dairy samples were variably contaminated with mold and yeast, ranged 50 - 100 % above the allowed limits; 10 and 400 CFU/g, respectively proposed by EOSQC (2005). The mean values of mold detected ranged from $1.84 \times 10^3$ up to $7.25 \times 10^4$ CFU/g (Table 1), and that of yeast ranged from $2.14 \times 10^3$ to $1.73 \times 10^5$ CFU/g (Table 1). Aspergillus spp. were mostly the dominant species isolated from cheese and milk desserts with 42 and 25 %, respectively, i.e. A. flavus, A. niger, A. ochraceus, A. fumigatus and A. parasiticus (Table 2). Also, the most predominant yeast species were those of Candida, i.e. C. tropicalis, C. krusei and C. parapsilosis (Table 3).

Dairy products showed heterogeneous mycotoxins that were chromatographically detected as shown in Table 4. The concentrations of aflatoxin-M1 (AFM1), aflatoxin-B1 (AFB1) and ochratoxin-A (OTA) in the examined samples ranged; 0.0-13.9, 11.1-13.8 and 4.5-13.9µg/kg, in 40-85, 15-85 and 15-80 %, respectively. Mostly all values of AFM1 and AFB1 estimated were exceeding their permissible limits according to Egyptian regulation permissible limits and EU regulation. However, 100, 100, 67, 88, 92, 6 and 94% of the examined ras, cheddar, feta, processed cheese, mahalabia, custard and rice milk had Ochratoxin A above the allowed limit (>5µg/g) according to Creppy (2002). The acceptable intakes (AI) compared to those estimated mycotoxins (EI) for children and adult human, either per day or week, were presented in Table 4 (B). The estimated daily intake (EDI) of mycotoxins from milk products was evaluated by using the consumed amount of the milk products and the average concentrations of mycotoxin estimated in each product type, considering the body weight average of the different groups. The current study indicated that all EDI levels of AFM1, AFB1 and OTA for infant and adult much exceeded over their acceptable daily intakes (ADI); 0.002, 0.0 and 0.014µg/kg b.w., respectively, according to the international regulation limits proposed by

### Table 1. Mold and yeast counts, presented as colony forming unit (CFU)/g, in different dairy products’ samples (n = 20), showing the safety from mold and yeast according to EOSQC

| A Milk products | +ve Mold (CFU/g) | No. | % | Min. | Max. | Mean±S.E | > 10 (%) |
|----------------|------------------|-----|---|------|------|----------|---------|
| Cheese         | Ras cheese       | 17  | 85| 1.00×10^1 | 1.60×10^4 | 3.54×10^3±1.14×10^3 | 80      |
|                | Cheddar          | 10  | 50| 3.00×10^1 | 1.10×10^4 | 1.84×10^3±5.43×10^2 | 50      |
|                | Feta             | 17  | 85| 1.00×10^1 | 3.00×10^4 | 4.85×10^3±1.58×10^3 | 85      |
|                | Processed        | 12  | 60| 7.00×10^2 | 2.50×10^5 | 3.30×10^4±1.29×10^2 | 50      |
| Dairy desserts | Mahalabia        | 18  | 90| 3.30×10^2 | 5.50×10^4 | 1.31×10^3±3.94×10^3 | 90      |
|                | Custard          | 20  | 100| 3.00×10^1 | 1.34×10^4 | 6.84×10^3±9.40×10^2 | 100     |
|                | Rice Milk        | 19  | 95| 1.30×10^1 | 3.30×10^4 | 7.25×10^3±2.43×10^3 | 95      |

| B Milk products | +ve Yeast (CFU/g) | No. | % | Min. | Max. | Mean±S.E | >400 (%) |
|----------------|------------------|-----|---|------|------|----------|---------|
| Cheese         | Ras cheese       | 15  | 75| 1.00×10^1 | 8.70×10^4 | 1.39×10^3±5.96×10^3 | 60      |
|                | Cheddar          | 11  | 55| 2.40×10^2 | 6.00×10^5 | 1.73×10^3±3.72×10^4 | 50      |
|                | Feta             | 10  | 50| 1.00×10^2 | 4.00×10^4 | 6.66×10^3±1.98×10^3 | 40      |
|                | Processed        | 12  | 60| 1.20×10^1 | 1.00×10^5 | 1.78×10^3±5.74×10^3 | 55      |
| Dairy desserts | Mahalabia        | 12  | 60| 2.00×10^2 | 5.00×10^4 | 9.58×10^3±3.19×10^3 | 35      |
|                | Custard          | 15  | 75| 3.00×10^2 | 4.40×10^4 | 5.75×10^3±2.32×10^3 | 60      |
|                | Rice Milk        | 15  | 75| 2.00×10^2 | 1.20×10^4 | 2.14×10^3±6.82×10^3 | 45      |
### Table 2. Percent of contamination of mold isolates in different dairy products samples (n = 20)

| Isolates of molds | Percent of contamination (%) | 
|-------------------|-----------------------------| 
| **Cheese samples** | Ras | Cheddar | Feta | Processed | Mahalabia | Custard | Rice Milk |
| Acremonium strictum | 0.0 | 0.0 | 0.0 | 0.0 | 3.7 | 8.3 | 0.0 |
| Arthrinium phaeospermum | 0.0 | 0.0 | 0.0 | 0.0 | 3.7 | 0.0 | 0.0 |
| Aspergillus flavus Link | 17.1 | 9.1 | 16.2 | 6.3 | 3.7 | 12.5 | 6.5 |
| Aspergillus fumigatus | 5.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Aspergillus niger Tiegh. nom. cons. | 11.4 | 9.1 | 18.9 | 43.7 | 14.8 | 14.6 | 12.9 |
| Aspergillus ochraceus | 5.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Aspergillus parasiticus | 2.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Aspergillus sydowii | 5.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Aspergillus terreus Thom | 0.0 | 0.0 | 5.4 | 0.0 | 0.0 | 0.0 | 6.5 |
| Aureobasidium pullulans | 0.0 | 0.0 | 0.0 | 0.0 | 3.7 | 8.3 | 3.2 |
| Botrytis cinerea | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Cladosporium cladosporioides G.A. de Vries | 17.1 | 9.1 | 24.4 | 18.7 | 22.2 | 8.3 | 32.3 |
| Cladosporium herbarum Link | 0.0 | 0.0 | 5.4 | 12.5 | 0.0 | 0.0 | 0.0 |
| Emericella nidulans | 0.0 | 0.0 | 0.0 | 0.0 | 7.4 | 4.2 | 3.2 |
| Eurotium chevaleri | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 18.8 | 0.0 |
| Eupenicillium spp. | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Fusarium chlamydosporum | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Fusarium poae Wollenw | 0.0 | 18.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Mucor plumbeus | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Paecilomyces variotii | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 6.5 |
| Penicillium aurantiogriseum Dierckx | 0.0 | 18.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Penicillium caseifulvum Lund, Filt. & Frisvad | 8.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Penicillium chrysogenum | 0.0 | 0.0 | 12.7 | 12.5 | 0.0 | 0.0 | 0.0 |
| Penicillium citreonigrum Dierckx | 0.0 | 0.0 | 0.0 | 0.0 | 3.7 | 0.0 | 0.0 |
| Penicillium citrinum Thom | 2.9 | 0.0 | 5.4 | 0.0 | 7.4 | 8.3 | 9.7 |
| Penicillium corylophilum Dierckx | 5.7 | 18.2 | 8.1 | 6.3 | 0.0 | 0.0 | 0.0 |
| Penicillium digitatum Sacc. Dierckx | 2.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Penicillium implicatum Biourge | 0.0 | 9.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Penicillium paneum Frisvad | 0.0 | 0.0 | 0.0 | 12.5 | 0.0 | 0.0 | 0.0 |
| Penicillium pxillii Bainier | 0.0 | 9.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Penicillium purpurogenum Stoll | 0.0 | 0.0 | 2.7 | 0.0 | 0.0 | 0.0 | 0.0 |
| Penicillium raistrickii G. Sm | 0.0 | 0.0 | 2.7 | 0.0 | 0.0 | 0.0 | 0.0 |
| Penicillium restrictum | 0.0 | 0.0 | 0.0 | 0.0 | 18.5 | 6.3 | 12.9 |
| Penicillium simplicissimum Thom | 0.0 | 0.0 | 5.4 | 0.0 | 0.0 | 0.0 | 0.0 |
| Penicillium variabile Sopp | 0.0 | 0.0 | 2.7 | 0.0 | 0.0 | 0.0 | 0.0 |
| Penicillium variable | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.1 | 0.0 |
| Rhizopus microsporus | 0.0 | 0.0 | 0.0 | 0.0 | 3.7 | 0.0 | 0.0 |
| Scopulariopsis brevicaulis | 0.0 | 0.0 | 0.0 | 0.0 | 3.7 | 0.0 | 0.0 |
| Total | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
Table 3. Percent of contamination of yeast isolates in different dairy products samples (n = 20)

| Isolates of Yeast         | Percent of contamination (%) | Cheese samples | Dairy desserts |
|---------------------------|-------------------------------|----------------|---------------|
|                           | Ras   | Cheddar | Feta  | Processed | Mahalabia | Custard | Rice Milk |
| **Candida famata**        | 0.0   | 0.0     | 0.0   | 0.0       | 11.8      | 0.0     | 5.0        |
| **Candida guilliermondii**| 0.0   | 0.0     | 7.7   | 6.7       | 5.9       | 0.0     | 5.0        |
| **Candida holmii**        | 6.7   | 0.0     | 0.0   | 0.0       | 0.0       | 0.0     | 0.0        |
| **Candida krusei**        | 6.7   | 0.0     | 15.4  | 0.0       | 5.9       | 4.3     | 0.0        |
| **Candida parapsilosis**  | 0.0   | 0.0     | 0.0   | 0.0       | 0.0       | 4.3     | 0.0        |
| **Candida tropicalis**    | 13.3  | 10.0    | 23.1  | 13.3      | 17.6      | 17.4    | 20.0       |
| **Cryptococcus albidos**  | 20.0  | 0.0     | 0.0   | 6.7       | 11.8      | 34.8    | 10.0       |
| **Debaryomyces hansenii** | 13.3  | 30.0    | 15.4  | 20.0      | 5.9       | 8.7     | 10.0       |
| **Geotrichum candidum Link** | 20.0 | 0.0     | 0.0   | 0.0       | 0.0       | 0.0     | 5.0        |
| **Pichia anomala**        | 6.7   | 0.0     | 7.7   | 0.0       | 0.0       | 0.0     | 0.0        |
| **Pichia membranaefaciens** | 0.0  | 10.0    | 0.0   | 0.0       | 0.0       | 0.0     | 0.0        |
| **Rhodotorula glutinis**  | 0.0   | 20.0    | 0.0   | 0.0       | 29.4      | 8.7     | 20.0       |
| **Rhodotorula spp.**      | 6.7   | 20.0    | 15.4  | 6.7       | 0.0       | 0.0     | 5.0        |
| **Saccharomyces cerevisiae** | 6.7  | 10.0    | 15.4  | 40.0      | 5.9       | 17.4    | 10.0       |
| **Trichosporon spp.**     | 0.0   | 0.0     | 0.0   | 6.7       | 5.9       | 4.3     | 0.0        |
| **Total**                 | 100   | 100     | 100   | 100       | 100       | 100     | 100        |

Fig. 1. Graphical abstract of the study
**Table 4A.** Maximum permissible limits (MPL; µg/kg) of mycotoxins in dairy products (n = 20); aflatoxin-M1 (AFM1), aflatoxin-B1 (AFB1) and ochratoxin-A (OTA) by the thin-layer chromatography (TLC). Letters (a, b, c, d) denote the international standards for MPLs. E.R.: Egyptian Regulation; I.S.: International Standards - a: EOSQC; b: European commission regulation; c: Creppy. (B) Acceptable and estimated intakes (µg/kg b.w) of mycotoxins in the dairy products consumed by children and adult (n = 20). Letters (a, b, c) denote the international standards for the acceptable daily intake (ADI). STD = Standard references - a: Kuiper-Goodman, b: Brera et al., c: JECFAd - ADI, acceptable daily intake; EDI, estimated daily intake; EWI, estimated weekly intake; PTWI, provisional tolerable weekly intake.

| Dairy Products | +ve Cases | Mean ± SE | E.R. | I.S. | MPL | MPL | STD | Children | Adults |
|----------------|-----------|----------|------|------|-----|-----|-----|----------|--------|
| AFM1 Ras Ch.   | 8         | 0.05     | 8    | 100  | 0.002 | 0.014 | 15.9| 40       | 111.8  |
| Ched. Ch.      | 0         | 0        | -    | -    | -    | -    | 0   | 0        | 0      |
| Feta Ch.       | 3         | 3.0      | 3    | 100  | 16.9 | 15   | 118.4| 15       | 45.6   |
| Proc. Ch.      | 9         | 13.9     | 9    | 100  | 25.0 | 45   | 175.1| 45       | 67.4   |
| Mahalabia      | 12        | 10.3     | 12   | 100  | 43.5 | 60   | 304.3| 60       | 117.0  |
| Custard        | 14        | 8.6      | 14   | 100  | 34.7 | 70   | 242.6| 70       | 93.3   |
| Rice Milk      | 17        | 13.6     | 17   | 100  | 45.1 | 85   | 315.8| 85       | 121.5  |
| AFB1 Ras Ch.   | 11        | 11.5     | 11   | 100  | 20.6 | 55   | 144.3| 55       | 55.5   |
| Ched. Ch.      | 0         | 1.0      | -    | -    | -    | -    | 0   | 0        | 0      |
| Feta Ch.       | 12        | 13.4     | 12   | 100  | 24.2 | 60   | 169.5| 60       | 65.2   |
| Proc. Ch.      | 6         | 13.7     | 6    | 100  | 24.6 | 30   | 172.3| 30       | 66.3   |
| Mahalabia      | 13        | 12.7     | 13   | 100  | 53.9 | 65   | 377.2| 65       | 145.1  |
| Custard        | 17        | 9.9      | 17   | 100  | 42.1 | 85   | 295.1| 85       | 113.4  |
| Rice Milk      | 17        | 11.1     | 17   | 100  | 46.9 | 85   | 328.2| 85       | 126.2  |
| OTA Ras Ch.    | 7         | 13.9     | 7    | 100  | 25.0 | 35   | 175.2| 35       | 67.4   |
| Ched. Ch.      | 4         | 0.9      | 4    | 100  | 17.8 | 20   | 124.6| 20       | 47.9   |
| Feta Ch.       | 3         | 6.2      | 3    | 100  | 11.2 | 15   | 78.4 | 15       | 30.1   |
| Proc. Ch.      | 8         | 8.0      | 8    | 100  | 14.4 | 40   | 100.8| 40       | 38.8   |
| Mahalabia      | 12        | 7.5      | 12   | 100  | 31.9 | 60   | 223.2| 60       | 85.8   |
| Custard        | 16        | 4.5      | 16   | 100  | 18.9 | 80   | 132.5| 80       | 51.0   |
| Rice Milk      | 17        | 8.1      | 17   | 100  | 34.3 | 80   | 240.4| 80       | 92.5   |
Electrophoretic banding of *afrR1* set was detected from four morphologically identified *Aspergillus flavus* as demonstration in connection with aflatoxin production which makes its detection is easily in compared to conventional plating techniques. The sizes of DNA fragments (800 bp) were estimated in accordance to commercial DNA ladder 100 bp (Fig. 2).

The antifungal activity of the algal extracts was reported in Fig. 3 and 4. The results revealed that *Padina pavonica* (petroleum ether extract), *Halimeda opuntia* (ethyl acetate extract) and *Turbinaria decurrens* (petroleum ether extract) were effective in suppressing the growth of *A.

### Table 5. Major bioactive chemical constituents identified in the algal extracts according to the gas chromatography-mass spectrometry (GC-MS) chromatogram analysis

| Algae           | Extract       | Sample No. | RT (min.) | Compound Name                              | Molecular Formula | Molecular Weight | %    |
|-----------------|---------------|------------|-----------|--------------------------------------------|-------------------|------------------|------|
| *Padina pavonica* | Petroleum ether | 1         | 7.30      | 4H-Pyran-4-One,2,3-dihydro-3,5-dihydroxy-6-methyl-cis-a-Farnesene | C6H8O4            | 144              | 1.66 |
|                 |               | 2         | 12.21     | Santalol                                   | C15H24O           | 204              | 1.24 |
|                 |               | 3         | 15.62     | Bisabolone oxide                           | C15H24O           | 236              | 1.77 |
|                 |               | 4         | 16.00     | Bisabolol oxide A                          | C15H26O           | 238              | 48.62|
|                 |               | 5         | 17.50     | En-in-dicycloether                         | C13H12O           | 200              | 27.63|
|                 |               | 6         | 20.23     | Hexadecanoic acid, ethyl ester             | C18H36O           | 284              | 5.22 |
|                 |               | 7         | 22.04     | Linoleate                                  | C20H36O           | 308              | 2.21 |
|                 |               | 8         | 24.98     | 8, 11, 14-Eicosatrienoic acid              | C20H34O           | 306              | 1.53 |
| *Halimeda opuntia* | Ethyl acetate | 1         | 14.46     | 2,4-Decadienal, (E,E)-                    | C10H12O           | 164              | 1.91 |
|                 |               | 2         | 15.07     | Eugenol                                    | C12H16O3          | 208              | 3.32 |
|                 |               | 3         | 20.62     | cis-Asarone                                | C18H30O           | 262              | 1.44 |
|                 |               | 4         | 21.73     | 9,12,15-Octadecatrienal                   | C15H20O           | 232              | 2.97 |
|                 |               | 5         | 26.91     | Alantolactone                              | C15H20O           | 232              | 3.39 |
|                 |               | 6         | 27.71     | Eudesma-5,11(13)-dien-8,12-olide           | C18H30O           | 232              | 3.39 |
|                 |               | 7         | 28.16     | Hexadecanoic acid                          | C18H32O           | 256              | 11.46|
|                 |               | 8         | 28.65     | Eremanthin                                 | C18H32O           | 230              | 2.76 |
|                 |               | 9         | 31.60     | 9,12-Octadecadienoic acid(2,Z)-            | C18H32O           | 280              | 36.16|
| *Turbinaria decurrens* | Petroleum ether | 10        | 31.71     | Oleic Acid                                 | C18H34O2          | 282              | 6.39 |
|                 |               | 11        | 31.95     | Octadecanoic acid                          | C18H36O2          | 284              | 3.23 |
|                 |               | 1         | 15.57     | 5-Hydroxymethylfurfural                   | C6H6O3            | 126              | 1.51 |
|                 |               | 2         | 20.23     | 2-Allyl-5-t-butyl-hydroquinone             | C13H18O2          | 206              | 2.61 |
|                 |               | 3         | 23.53     | Ar-tumerone                                | C15H20O           | 216              | 8.26 |
|                 |               | 4         | 24.34     | Curlone                                    | C15H22O           | 218              | 1.68 |
|                 |               | 5         | 28.73     | Hexadecanoic acid, methyl ester            | C17H34O2          | 270              | 13.47|
|                 |               | 6         | 29.93     | Hexadecanoic acid                          | C16H32O2          | 256              | 13.42|
|                 |               | 7         | 32.54     | Octadecanoic acid, methyl ester            | C18H38O2          | 298              | 5.31 |
|                 |               | 8         | 33.22     | Oleic acid                                 | C18H34O2          | 282              | 25.62|
|                 |               | 9         | 33.34     | Isochiapin B                               | C19H22O6          | 346              | 7.57 |
|                 |               | 10        | 38.82     | Dotriacontane                              | C32H66            | 450              | 11.63|
|                 |               | 11        | 46.56     | Lucenin 2                                  | C27H30O16         | 610              | 3.67 |
flavus, A. niger and C. tropicalis with variable potency. Ethyl acetate extract of Halimeda opuntia at the concentration of 3 mg/ml was the most effective retarding the growth of all tested pathogenic fungi with inhibition zones of 16.5 ± 0.6 and 22.3 ± 0.73 mm against A. flavus and A. niger, respectively. It is followed by petroleum ether extracts of Padina pavonica and Turbinaria decurrens with inhibition zones of 9.2 ± 0.6 and 6.8 ± 0.44 mm, against A. flavus and 10.0 ± 0.58 and 9.7 ± 0.33 mm against A. niger, respectively (P<0.5), and the later showed higher sensitivity response to all algal extracts rather than the former, A. flavus (Fig. 3).

The candidacidal activity of algal extracts by using the dilution method was reported in Fig. 4 (A and B). The algal extracts incubated with C. tropicalis for 24 hr exhibited strong candidacidal activity in dose-dependent manner (Fig. 4 A). Ethyl acetate extract of Halimeda opuntia was very effective against C. tropicalis resulting in severe reduction in CFU of yeast (11 log10 order of killing). Also, the extracts of P. pavonica and T. decurrens showed effective candidacidal activity (5-6 log10 order of killing power) (Fig. 4 B). The constituents of algal extracts detected by GC-MS were shown in Table 5 and Fig. 4. It showed presence of nine compounds in the petroleum ether extract of P. pavonica (Fig. 5 A), eleven compounds in the ethyl acetate extract of H. opuntia (Fig. 5 B), and eleven compounds in petroleum ether extract of T. decurrens. The major constituents of H. opuntia extract were 2, 4-Decadienal, (E, E; 21.56 %), hexadecanoic acid (11.46 %) and 9, 12-octadecadienoic acid (Z, Z; 36.16 %) (Fig. 5 C).
DISCUSSION

Unhygienic handling of milk products may lead to contamination by different fungi and propagation of mycotoxins. However, our results declared that milk products were contaminated with mold and yeast more than the permissible limit according to EOSQC (2005)\(^41\). A total of 80.7 and 64.3% of all samples were contaminated with mold and yeast, respectively. These results agreed with former findings reported by researchers in Egypt. They stated that milk samples are contaminated with mold and yeast in majority of the examined regions and most of samples didn’t comply with the permissible limit predetermined by National and International Standards Organizations\(^{47, 48}\). Furthermore, Italian researches found mold in 54 of the 122 analysed cheese samples (44.3%), stated that the potentially toxigenic fungal species were mainly detected in cheese samples\(^49\). Mold and yeast are widely distributed as environmental contaminants which can grow at variable temperature, so their presence in milk products could be attributed to unsanitary measures during manufacturing, processing and storage or using of bad quality raw ingredients. They induce undesirable changes such as off-flavor, color defects, rancidity and changes in texture\(^50\). Mold and yeast counts in dairy products are used as an index of the proper hygienic quality\(^51\). The mycotoxins-producing molds are potential hazard to food safety and human health\(^51\).

On the other hand, a total of 38 and 15 different species of mold and yeast were isolated and identified from examined milk products samples. *Aspergillus* and *Candida* spp. were the most dominant species of mold and yeast isolated from 32.2 and 29.2% of the milk products, respectively, which coincide with Khalifa et al. (2013)\(^52\) and ELbagory et al. (2014)\(^53\).

*Candida* species; *C. tropicalis*, *C. krusei* and *C. parapsilosis* are the most common human-specific fungi responsible for systemic and superficial infections originated from food or the environment\(^54\). Moreover, *C. tropicalis* is implicated in the higher mortality rates than other *Candida* spp. particularly in neutropenic and oncogenic patients. Several cases of *C. tropicalis* cross-resistance to antifungal agents have been reported in clinical isolates\(^55\). *Aspergillus* species including; *A. flavus*, *A. niger*, *A. ochraceus*, *A. parasiticus*, *A. fumigatus* have the potential to contaminate food and environment and...
linked to the life-threatening disorders, i.e. aspergillosis and mycotoxicosis\textsuperscript{56}. Aspergillosis causes wide scaled clinical manifestations, i.e. allergy, pulmonary, ocular infections, otomycosis, endocarditis, osteomyelitis, and skin infections, and so the invasive aspergillosis could lead to high mortalities\textsuperscript{57}. Moreover, \textit{Aspergillus} is an important genus in foods, causing more spoilage and biodeterioration than other fungi\textsuperscript{58}. Almost all food kinds including milk and its products are vulnerable to contamination by \textit{Aspergillus} species especially in tropical and subtropical climates resulted in huge agro-economic losses in the world\textsuperscript{59}.

\textit{Aspergillus} is the most significant genera of mycotoxigenic fungi, over 40 species of \textit{Aspergillus} have been known their ability to produce a wide range of mycotoxins having

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\textbf{Fig. 5.} GC-MS chromatogram analysis of the most effective extract constituents of algae; A) \textit{P. pavonica} (extracted by petroleum ether), B) \textit{H. opuntia} (extracted by ethyl acetate) and C) \textit{T. decurrens} (extracted by petroleum ether). RT = Retention time.
adverse effect on health of humans and animals consuming it. Aflatoxins are products of *A. flavus* and *A. parasiticus* and ochratoxin-A is produced by *A. niger*, *A. ochraceus* and *A. carbonarius*. Furthermore, Drusch and Aumann (2005) declared that Mycotoxins can diffuse into the food without any sign of mycelium growth. Consequently, the absence of mold does not guarantee mycotoxins free food.

The results showed heterogeneous mycotoxins mixtures; 25, 40 and 27.5% of cheese and 71.7, 78.3 and 73.3 % of dairy desserts were contaminated with mycotoxins, AFM1, AFB1 and OTA, respectively (Table 4). The concentrations of mycotoxins detected were exceeding more than the permissible limits declared by National/International Standards Organizations. These values are in accordance with the results reported by other Egyptian researchers who stated that, mycotoxins residues were contaminated milk products samples in various levels more than the permissible limit set by Egyptian and European regulation limits. They stated that the highest incidence of mycotoxins recorded in milk products may refer to unhygienic conditions in processing, package or storage which provide favourable conditions for mold growth and subsequently toxin production. Moreover, Turkish and Iranian researchers studied mycotoxins concentration in milk products samples, stated that mycotoxins concentrations were more than the maximum residue limits recommended by National and International standard Organizations. They concluded that the content of mycotoxins remains relatively stable during the different steps of dairy products production and storage and thermal processing used in dairy industry cannot inactivate it.

For what the data reported in this paper, the amounts of AFM1, AFB1 and OTA detected seem to be dangerous. In fact, the average consumption amount of cheese and dairy desserts for the adult and children assumed 45 and 106 g/ day, respectively (data referred to Egypt - Cairo Nutrition Institute, 1996; 2007), and the EDI levels of mycotoxins for infant and adult were much more than the ADI proposed by international regulation standards. The AFB1, the most dangerous mycotoxin, should be absent or the lowest recorded according to ALARA (as low as reasonably achievable) for food safety. Our data is agreed well with El-Badry, (2016) and Milicevic et al. (2017) whom found that most of analysed milk and milk products samples collected from different localities in Egypt and Serbia were above ADI of mycotoxins declared by International Standards Organizations. In contrast to our results, former international researchers concluded that collected milk samples containing mycotoxins level that were accepted for human as compared with recommended limits.

The carcinogenic properties of aflatoxins motivate us to develop rapid, sensitive and specific approach for the identification and detection of aflatoxin producing *A. flavus* from food samples. Aflatoxin regulatory *aflR1* gene forms the accurate and specific marker for aflatoxigenic strains of *A. flavus* in foods. In agreement with ElBagory et al. (2014), the *aflR*-specific primer designed for aflatoxigenic *A. flavus* was approved as an easily detecting method, so the detection of aflatoxigenic *A. flavus* from food using polymerase chain reaction (PCR) shows no false results. The PCR technique allows screening of many suspected samples in high sensitivity and accuracy, with the capacity to proceed a high number of samples in a short time.

Preventing food spoilage and protecting human health from the harmful effects of mold or yeast pathogens has become extremely challenging. The limited application of chemical preservatives, susceptibility, toxicity, microbial resistance and its adverse effects on human health increase the demand to search for natural, healthy, safer and potentially effective antifungal agents. Thus, antimicrobial activity of algal extracts can provides a key aspect of treatment of fungal infections and to be used as natural preservatives to ensure healthy and safe food. In the present study, the crude extracts of the three algae species including, *Halimeda opuntia*, *Turbinaria decurrens* and *Padina pavonica*, showed fungicidal activities against recovered three fungal pathogens, *A. flavus*, *A. niger* and *C. tropicalis* with various inhibitory actions depending on the seaweed species and the solvent used.

Various studies evaluated the antimicrobial activities of the marine algae viz., *H. opuntia*, *P. pavonica* and *Turbinaria* Species. Different solvents viz., petroleum ether, diethyl
ether, ethyl acetate, ethanol, chloroform, hexane and water were used for algae extraction to investigate the antimicrobial activity against mold and yeast including A. flavus, A. niger, A. fumigatus, Fusarium moniliforme, Penicillium herquei, Candida tropicalis, Candida albicans, Candida kefiri. Some Egyptian researches reported the fungicidal activities of H. opuntia, P. pavonica and Turbinaria Species which showed the inhibitory effect were 12 to 32 mm of H. opuntia and Padina pavonica extracts and 10-25 mm of methanolic H. opuntia extract against Candida spp.

Globally, Indira et al. (2013) stated that methanolic extracts of Halimeda species exhibit the highest antifungal activity against several fungal strains including Aspergillus flavus, Aspergillus niger, Candida albicans, Rhizopus spp. and Penicillium spp. in vitro using minimum fungicidal concentration and well diffusion method with the potential use of algal extracts as antimicrobial candidate. In addition, the ethanolic extract of Padina pavonica generated maximum inhibition zone against Candida spp. Moreover, it was reported that Turbinaria Species exhibited significant inhibition effect against Candida spp. (7.0±0.0 mm) and Aspergillus flavus (7.0±0.0 mm).

The current article identified the phytochemical algal constituents against the mycotic pathogens by using the GC-MS analysis, showing their active principles with retention times (RT), molecular formulae, molecular weight and relative concentration (%) in the different algal extracts. The GC-MS analysis reported the presence of 9 compounds in the extracted P. pavonica, eleven compounds in H. opuntia and eleven compounds in T. decurrens. The most predominant compounds in the P. pavonica extract were bisabolol oxide A (48.62%) and En-in-dicycloether (27.63%), whereas those in H. opuntia extract were 2, 4-decadienal, (E, E)- (21.56%), Hexadecanoic acid (11.46%) and 9, 12-Octadecadienoic acid (Z,Z) (36.16%). However, the T. decurrens mostly contained hexadecanoic acid, methylester (13.47%), hexadecanoic acid (13.42%), oleic acid (25.62%) and dotriacontane (11.63%).

The great efficacy of the seaweeds extracts against the pathogenic mold and yeast could be attributed to the active phytochemicals and metabolites compounds in addition to the fatty acids and their derivatives. Those abundant compounds had been previously identified and characterized from various herbal and algal sources. The in vitro data presented intensively in previous literatures showed that Octadecadienoic acid and Bisabolol oxide A poses potent fungicidal activities against wide range of fungal species. There are several reports regarding that bisabolol has strong fungicidal and bactericidal proprieties. Bisabolol produced nearly 98% loss in the viability of the germinating conidia of A. niger, A. flavus, A. fumigatus. In addition, bisabolol showed antimicrobial activity against A. niger at concentrations above 125µg/ml with hyphal growth and conidial production inhibition. Many of the studies in the scientific literature highlighted the antifungal activity Octadecadienoic acid (Z,Z)-. Furthermore, Ali et al. (2017) identified 9-octadecenoic acid (Z)-, methyl ester (10.27%) and 9-octadecenoic acid (Z)-, methyl ester (12.75%), that were found to be responsible for antifungal activity against various fungal genera. In addition to another study, their result indicated the abundances of methyl ester (Z,Z)-9,12-octadecadienoic acid, (E)- 9-octadecanoic acid, 9,12-octadecadienoic acid, which showed great antifungal agent against various fungus isolates. Bisabolol can be used to prevent microbial growth in wide ranging applications such as food, cosmetics and topical antifungal owing to its excellent nontoxic properties. Finally, it could be concluded that those identified molecules have potential antifungal activities and, notably, that the extracts of the algae where they are abundant also showed potent antifungal activity. The explored algal species could be more effective against the fungal infection rather than those traditional fungicidal agents. Therefore, it can be considered as natural preservatives providing healthy and safe food without the unpleasant effects of chemical one. The current study presented the ability of algae to promote as an antifungal agent via stable its biologically active compounds. Also, provides insights into designing of novel antifungal drugs for the clinical use or in food preservation.

CONCLUSION AND FUTURE TRENDS

Broad fungal diversity and heterogeneous mycotoxins mixture were detected in the locally...
produced dairy products which denote the unhygienic measures of either processing or preservation in dairy shops. Presence of mycotoxins in the dairy products despite of low levels represents public health hazards. Aspergillus and Candida spp. were mostly detected in 50 of 2001; 2009; 2010; 2016; 2014; 2003; 2010; 2010; 2003; 2001; 2001; 2001). 1. Kure CF, Skaar I, Brendehaug J. Mould contamination in production of semi-hard cheese. International Journal of Food Microbiology, 2004; 93(1): 41-49. https://doi.org/10.1016/j.ijfoodmicro.2003.10.005.
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CONFLICTS OF INTEREST
The authors declare that there is no conflict of interest.

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AUTHORS’ CONTRIBUTIONS
All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

DATA AVAILABILITY
All datasets generated or analysed during this study are included in the manuscript.

ETHICS STATEMENT
This article does not contain any studies with human participants or animals performed by any of the authors.

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