



Effect of probiotic bacteria on the reduction of aflatoxin risks in milk

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ABSTRACT

Dairy products such as raw milk, Kariesh cheese, and yoghurt are considered as health food that supports part of the human needs of protein, minerals, and vitamins. This study was taken to investigate mold contamination in raw milk, Kariesh cheese, and yoghurt retailed in Zagazig city, Egypt. In addition, isolation and identification of the different mold genera were further conducted. Besides, total aflatoxins (AFTs) were estimated in the examined samples, and their daily intakes and health risk assessment were calculated. An experimental trial was conducted to investigate the antifungal activities of three probiotics strains, namely, *Lactobacillus acidophilus*, *L. rhamnosus*, and *L. plantarum*. The obtained results indicated that raw milk had the highest mold contamination rate at 40%, followed by Kariesh cheese at 30%, and yoghurt samples at 10%. The prevalent mold genera were *Aspergillus spp.*, *Penicillium spp.*, *Cladosporium spp.*, *Alternaria spp.*, *Mucor spp.*, *Rhizopus spp.*, *Absidia spp.*, and *Fusarium spp.*. The most dominant mold genera among the identified molds were *Aspergillus spp.*, (raw milk (20.82%), Kariesh cheese (15.62%), and yoghurt (6.25%). Further identification of the *Aspergillus* isolates revealed five *Aspergillus species*. The identified *Aspergilli* were *A. niger*, *A. flavus*, *A. fumigatus*, *A. ochraceus*, and *A. terreus*. AFTs were detected in raw milk samples at 20%, Kariesh cheese at 10%, and yoghurt at 6. None of the contaminated samples exceeded the international permissible limit of total AFTs. The tested probiotics strains exhibited clear antifungal activities.

Keywords: Molds; Dairy products; Egypt; Aflatoxin; Probiotics

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1. Introduction

Dairy products such as milk, Kariesh Cheese, and yoghurt are regarded as primary sources of high-quality protein with considerable concentrations of essential amino acid, minerals such as calcium, and magnesium, and vitamins such as vitamins A, and B12 (Gerosa and Skoet, 2013; Ma et al., 2020). However, raw milk, Kariesh cheese, and yoghurt of local produce are eligible for microbial contamination with vast array of microorganisms as bacterial, and mold. Consumption of inferior quality dairy products is associated with the onset of food poisoning cases (Becker-Algeri et al., 2016).

Fungal contamination of milk and other dairies is of a particular significance in the field of food industry. Molds can grow within a wide range of growth conditions such as temperatures, pH, and water activity (Pitt and Hocking, 2009). The use of inferior quality raw milk and the fluctuation of preservation temperature, in addition to improper sanitary measure during dairy processing facilitate mold growth. Mold contamination of dairy products is positively associated with their spoilage, lower keeping quality, and potential health risks. As mold

growth on the dairy products might lead to high economic losses in addition to formation of mycotoxins, particularly aflatoxins which are associated with several health risks (Darwish et al., 2014). However, the prevalence of different mold genera on the retailed dairy products in Egypt had received less attention.

Aflatoxins (AFTs) are secondary fungal metabolites produced by certain mold species, such as *Aspergillus flavus* and *Aspergillus parasiticus* (Alcaide-Molina et al., 2009). Contamination of milk with AFTs might start during the animal lifetime via ingestion of contaminated animal feed and water or as metabolites because of the growth of certain molds on the surface of dairy products. AFTs find their way into human body via ingestion of contaminated foods leading to several toxicological implications (Aljazzar et al. 2021). Such AFTs-related adverse effects include mutagenicity, carcinogenicity, particularly, hepatocellular carcinoma, and immunosuppressive effects (Abd-Elghany and Sallam 2015). However, little is known about the occurrence of total aflatoxins in the retailed dairy products in Egypt, and their associated health risk assessment.

Probiotics are living microorganisms that promote human health when administered in adequate amounts (Hill et al., 2014). In addition, probiotics were introduced to dairy industry in order to increase the shelf life of the final products via exerting antimicrobial properties. Of particular, dairy strains of *Lactobacilli* or lactic acid bacteria (LAB) such as *L. acidophilus*, *L. rhamnosus*, and *L. plantarum* are known for their antimicrobial activities (Faghihi Shahrestani et al., 2021). However, the anti-mold abilities of *L. acidophilus*, *L. rhamnosus*, and *L. plantarum* using raw milk as a substrate are less investigated.

In sight of the previous facts, the current study was conducted to estimate total mold counts on three dairy products, namely raw milk, Kariesh cheese, and yoghurt retailed in Sharkia Governorate, Egypt. In addition, the prevalence of different mold genera on such products was further examined. Furthermore, identification of the dominant *Aspergillus spp.* was additionally studied. The residual concentrations of total AFTs in the examined dairy products were additionally estimated. The estimated daily intakes and the associated health risks were calculated. Finally, the anti-mold abilities of some probiotic bacteria (*L. acidophilus*, *L. rhamnosus*, and *L. plantarum*) were further screened.

2. Material and methods

2.1. Sample collection:

A hundred and fifty samples including 50 samples from each of raw milk, Kariesh cheese, and yoghurt were randomly collected from different local vendors or grocery shops at Zagazig city, Sharkia Governorate, Egypt. Samples were transferred cooled with no delay and under aseptic conditions to the laboratory at Food Control Department, Faculty of Veterinary Medicine, Zagazig University, where mycological examination of samples was performed.

2.2. Sample preparation:

From each sample, twenty-five grams were aseptically homogenized in 225 mL of either buffered peptone water 0.1%, or sodium citrate 2% in case of cheese for 2 min at 2500 rpm to obtain a dilution of 10⁻¹, followed by preparation of decimal serial dilutions (APHA, 2001).

2.3. Determination of total mold count (TMC):

The pour plating method using both malt extract agar media for ordinary molds and Czapeck-Dox agar with 5% NaCl for xerophilic molds (Oxoid,

Basingstoke, UK) was done for determination of total mold counts (TMC). To one mL from each prepared dilution for each sample, 15 mL from the aforementioned media were poured, followed by incubation in dark at 25°C for 5-7 days. The culture plates were examined daily for mold growth and mold counts according to APHA (2001) using the following formula:

$$\text{TMC/g} = \text{average No. of colonies} \times \text{reciprocal of the dilution}$$

Counted colonies were expressed as log 10 cfu/g.

2.4. Identification of the isolated molds:

The mold identification key of Pitt and Hocking (2009) was used following the macroscopical and microscopical characteristics of the mold colonies. Both the surface and backside of the colonies were examined. Molds were identified to their genera level, while recovered *Aspergillus* isolates were identified to their species level (Pitt and Hocking, 2009).

2.5. Estimation of total aflatoxins in the examined samples:

Total AFTs was estimated using Series-4EX Fluorimeter (VICAM, Milford, USA) according to Abd-Elghany and Sallam (2015). In brief, 25 g of each sample was mixed with 5 g of NaCl and blended in 100 mL methanol: water (4:1 v/v) at a high speed for 3 min. The mixture was diluted 4 times in DDW and followed by filtration. The obtained filtrate (4 mL) was passed through AflaTest®-P affinity column and then eluted using methanol. One mL of the sample elute was mixed with one mL of AflaTest® Developer in a glass cuvette (VICAM part # 34000). Then, the cuvette was placed in the calibrated fluorimeter.

2.6. Dietary intakes:

Calculation of the estimated daily intakes (EDI) for AFTs was done according to USEPA (2010) from the following equation:

$$\text{EDI} = C * F_{\text{IR}} / \text{BW}$$

Where C is the concentration of total AFTs in the sample (ppb wet weight); FIR is the food ingestion rate in Egypt, which was estimated at 120 mL/day for milk, 65 g/day for Kariesh cheese, and yoghurt (FAO, 2003); BW is the body weight of Egyptian adults, which was set at 70 kg.

2.7. Health risk Assessment:

The cancer risk among the Egyptian population was evaluated using the margin of exposure (MOE) approach suggested by EFSA (2005), and FAO/WHO (2006). MOE is the ratio of the benchmark dose that causes a 10% increase in cancer incidence in fisher rats (BMDL10) to the average level of total intake in humans. The BMDL10 for AFTs was estimated to be 250 ng/kg body weight/day. MOE was calculated from the following equation (Benford et al., 2010):

$$\text{MOE} = \text{BMDL10/EDI}$$

MOE values lower than 10000 represent a major health concern (EFSA, 2020)

2.8. Reduction trial for *A. flavus* using probiotics:

As *A. flavus* is the major mold species which can produce AFTs, therefore, a reduction trial for *A. flavus* using probiotics strains was proposed.

2.8.1. Preparation of *A. flavus* spore suspension:

Aspergillus flavus previously isolated in the present study was cultured on malt extract agar supplemented with chloramphenicol (50 mg/L) and incubated at 37°C before each trial. After 72 h, Conidia were harvested by flooding the plates with sterile phosphate buffered saline containing 0.01% Tween 20, centrifuged at 3500 rpm for 30 min and quantified by counting with a hemocytometer (Melloul et al., 2014).

2.8.2. Preparation of bacteriocins' suspensions:

Pure probiotic bacteria (*L. acidophilus*, *L. rhamnosus*, and *L. plantarum*) were kindly gifted from Food Control Department, Faculty of Veterinary Medicine, Zagazig University, and Animal Health Research Institute, Zagazig Branch. Cell-free supernatant (CFS) was prepared according to Ibarra-Martínez et al. (2022) as follows: one ml of each *Lactobacillus* strains was cultured overnight in 20 ml M17 broth (HiMedia, Mumbai, India) then 1 ml of the obtained culture was sub-cultured overnight in 20 ml M17 broth. Once the probiotic strains reached 1×10^8 cells / ml, the medium was centrifuged at 5000 x g for 15 min at 4°C and the supernatant was recovered, filtered with a 0.22 µm nitrocellulose membrane (Millipore, Ireland). The supernatants were stored at -20°C until use.

2.8.3. Experimental groups

Milk samples (n = 25, 200 mL each) free from both mold contamination and AFTs residues were grouped into 5 groups. Group 1 was inoculated with 0.2 mL mixed probiotics strains (equal volume), group 2 was inoculated with 0.2 ml of purified *A. flavus*, group 3 was inoculated with 0.2 ml of purified *A. flavus* + 0.2 purified *L. acidophilus*, group 4 was inoculated with 0.2 ml of

purified *A. flavus* + 0.2 purified *L. rhamnosus*, and group 5 was inoculated with 0.2 ml of purified *A. flavus* + 0.2 purified *L. plantarum*. All groups were left to ferment at 25°C and changed to the yoghurt form. The formed yoghurt was stored at 4°C for 21 days and examined for mold growth on a weekly basis at zero, 7th, 14th. And 21st days of the chilling storage to evaluate the effect of the above-mentioned treatment on *A. flavus* growth. *A. flavus* counts were recorded, calculated, and expressed as log₁₀ cfu/g. Antifungal effects were calculated and expressed as inhibitory rates (%).

2.9. Statistical analysis

Statistical analysis was done using Tukey–Kramer HSD test where, $p < 0.05$ indicated statistical differences.

3. Results and Discussion

The obtained results in the present study indicated mold contamination of the examined raw milk, Kariesh cheese, and yoghurt samples at variable levels. Raw milk had the highest mold contamination rate at 40%, followed by Kariesh cheese at 30%, and yoghurt samples at 10% (Fig. 1). Total mold (log 10 cfu/g) were further counted among the examined samples. Raw milk had significantly ($P < 0.05$) the highest mean total mold count 4.15 ± 0.13 , followed by Kariesh cheese samples with 3.04 ± 0.12 , and yoghurt with 2.29 ± 0.14 log 10 cfu/g, respectively (Fig. 2). In agreement with the obtained results of the present study, Ibrahim et al. (2015) reported 100% mold contamination of both raw milk (average 3.5 log cfu/g) and Kariesh cheese (average 5.5 log cfu/g) retailed in dairy markets in Cairo city, Egypt. Kariesh cheese retailed in Alexandria city also had high mold contamination reaching to 94.44% with high average mold count reaching 7.95 log 10 cfu/g (Salem et al., 2016). Furthermore, Hassan et al. (2019) reported in Assuit city, Egypt, a high mold contamination for Kariesh cheese (100%), with high mold counts > 5.26 log 10 cfu/g. Internationally, molds were found in 63.3% and 60% of raw milk and soft cheese samples, with mean concentration of 0.6 and 2.1 log₁₀ cfu/g, respectively in samples collected from Slovenia (Torkar and Vengušt, 2008). Mold contamination of raw milk is possibly due to contamination of milk from the animal skin, contamination with animal excreta during milking process, or from milker's hands or equipment. Contamination of cheese and yoghurt by molds is possibly due to the use of inferior quality raw milk for the manufacture of such dairies, the poor hygienic measures adopted during the preparation and processing of cheese, and yoghurt, the fluctuation of the keeping temperature during distribution, and storage of these dairy products (Pitt and Hocking, 2009). In addition, improper hand washing alone accounts for more than 25% of all foodborne diseases. Besides, poor hygiene at dairy products processing plants due to dirty walls, cutting boards, unhygienic handling, and lack of knowledge of hygienic practices increases the transmission of foodborne pathogens and spoilage organisms (Tambekar et al., 2008).

Eight mold genera could be recovered and identified in the present study including *Aspergillus spp.*, *Penicillium spp.*, *Cladosporium spp.*, *Alternaria spp.*, *Mucor spp.*, *Rhizopus spp.*, *Absidia spp.*, and *Fusarium spp.* The most dominant mold genera among the identified molds were *Aspergillus spp.*, (raw milk (20.82%), Kariesh cheese (15.62%), and yoghurt (6.25%); followed by *Mucor spp.*, at 7.28%, 6.25%, and 2.08% in raw milk, Kariesh cheese, and yoghurt, respectively, and *Penicillium spp.*, at 7.28%, 4.16%, and 1.04% in the same samples, respectively (Fig. 3). Further identification of the isolated *Aspergillus* to their species level revealed identification of five *Aspergillus spp.*, namely, *A. niger*, *A. flavus*, *A. fumigatus*, *A. ochraceus*, and *A. terreus*. The dominant *Aspergillus spp.* among the examined samples were *A. niger* (raw milk (8.32%), Kariesh cheese (7.28%), and yoghurt (2.05%); followed by *A. flavus* at 6.24%, 4.16%, and 3.12% in raw milk, Kariesh cheese, and yoghurt, respectively, and *A. flavus* at 2.08%, 1.04%, and 1.04% in the same samples, respectively (Fig. 4). The high ability of *Aspergillus spp.*, *Mucor spp.*, and *Penicillium spp.*, to grow over a wide range of temperatures, and their minimum needs for oxygen for growth as well as spore germination explain their dominance as mold contaminants in the current study (Plahar et al., 1991). *Cladosporium spp.*, and *Fusarium spp.*, were also identified in the present study. These two genera could survive at severe adverse conditions such as low temperatures of up to -7°C and minimal water activity (0.85) (Jay, 2000). *A. niger* and *A. flavus* were the dominant *Aspergillus*. It notes worthy to mention that that *A. flavus* is one of the major aflatoxigenic molds. In agreement with the obtained results in the current investigation, Spanish raw milk was contaminated with molds such as *Geotrichum spp.*, (76.5%), *Fusarium spp.*, (45.3%), and *Aspergillus spp.*, (31.2%), the latter was further identified to *A. flavus*, and *A. glaucus* (Jordal et al., 1993). Moreover, raw milk and soft cheese were contaminated with yeast and mold in Slovenia, the isolated mold genera were *Geotrichum spp.*, (51.5%), *Aspergillus spp.*, (33.8%), *Mucor spp.*, (5.9%), *Fusarium spp.*, (2.9%), and *Penicillium spp.*, (2.9%) (Torkar and Vengušt, 2008).

It is necessary to confirm that the identified molds in the present study are of public health concern due to their abilities to produce some secondary metabolites known as mycotoxins that have several adverse health effects. Therefore, this study was extended to examine the occurrence of total AFTs in the examined samples, and to estimate their potential health risks as shown in Table 1. The recorded results showed that 10 out of 50 raw milk samples (20%) were contaminated with AFTs with a mean value of 4.62 ± 0.11 ppb, followed by Kariesh cheese samples where 5 out of 50 samples (10%) were contaminated with AFTs with an average of 2.90 ± 0.22 ppb, and yoghurt samples with 3 out of 50 samples (6%) were contaminated with AFTs with a mean value of 1.17 ± 0.18 ppb, respectively. None of the contaminated samples exceeded the international permissible limit of total AFTs (25 ppb, EC, 2006). Calculation of the EDI values (ppb/Kg BW) for total AFTs based on the recorded average concentration of total AFTs revealed values of 7.92, 2.69, and 1.09. Further calculation of the margin of exposure values demonstrated potential health risks for Egyptian if consumed dairies contaminated with AFTs as the recorded MOE values were far below 10000 (EFSA, 2020) reaching 31.56 (raw milk), 92.74 (Kariesh cheese), and 228.92 (yoghurt) (Table 1). Higher residual concentrations of total AFTs or AFM1 were recorded in raw milk samples marketed in Slovenia (50 ppb) (Torkar and Vengušt, 2008), and Italy (10.3 ppb) (Serraino et al., 2019). EDI value of AFM1 for the average consumption of milk in Italy ranged between 0.025-

0.325 ppb (Serraino et al., 2019). Moreover, AFM1 was detected in raw milk retailed in Bangladesh at 71.4% with a concentration range of 5.0-198.7 ppb (Sumon et al., 2021).

In a trial to investigate the antifungal activities of three probiotics strain using yoghurt (made from raw milk, specifically for this study) as a food substrate, the obtained results showed that *L. Acidophilus* had the highest antifungal activities achieving reduction rates of 50.66% on the 7th day, 40.43% on the 14th day, and 27.87% on the 21st days of refrigeration. *L. rhamnosus* came second with inhibitory rates of 43.63% on the 7th day, 35.43% on the 14th day, and 41.86% on the 21st days of refrigeration. *L. plantarum* had the lowest inhibitory rates at 27.87%, 25.69%, and 27.42% on the examined periods, respectively (Table 2). All examined samples were apparently normal with no visible mold growth by eye till the 21st day, where some mold growth started to be observable by eye. In agreement with the obtained results of the present study, Lactobacillus isolates including *L. alimentarius*, and *L. delbrueckii* showed significant antifungal activities against *Penicillium notatum*, and *Aspergillus fulvovus* on the agar-based settings (Karami et al., 2017). Moreover, a combination of *L. rhamnosus*, and *L. plantarum* showed clear antifungal activities as demonstrated by their direct effects to delay the growth of *Penicillium commune*, and *Mucor racemosus* using either yoghurt or semi-hard cheese as a food matrix (Leyva Salas et al., 2018).

Table 1: Total AFTs content, dietary intakes, and their health risk assessment

Sample	N	Positive samples (%)	Total AFTs content (ppb)	%	EDI (ppb/Kg BW)	MOE
Raw milk	50	10 (20%)	4.62 ± 0.11^a	0%	7.92	31.56
Kariesh cheese	50	5 (10%)	2.90 ± 0.22^b	0%	2.69	92.74
Yoghurt	50	3 (6%)	1.17 ± 0.18^c	0%	1.09	228.92

N: Refers to the total number of the examined samples

%: Refers to the percentage of samples exceeding MPL (25 ppb, EC, 2006)

Total AFTs content are represented by mean \pm SE, mean values carrying different superscript letters are significantly different at $p < 0.05$.

Table 2: Antifungal effects of the tested probiotics strains

Day	Control	<i>L. acidophilus</i>	<i>L. rhamnosus</i>	<i>L. plantarum</i>
0	0	0	0	0
7	0	50.66%	43.63%	27.87%
14	0	40.43%	35.43%	25.69%
21		27.87%	41.86%	27.42%

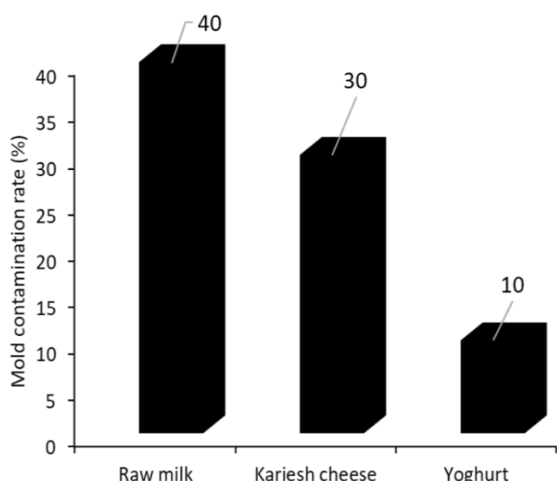


Fig. 1: Mold contamination rates (%) among the retailed raw milk, Kariesh cheese, and yoghurt samples in Zagazig city, Egypt (n = 50)

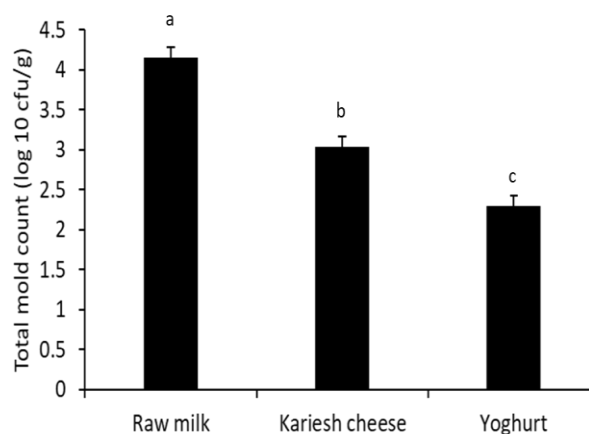


Fig. 2: Total mold counts (log 10 cfu/g) in the raw milk, Kariesh cheese, and yoghurt samples retailed in Zagazig city, Egypt. Values represent means \pm SD (n = 50). Columns with different letter are statistically different at $P < 0.05$.

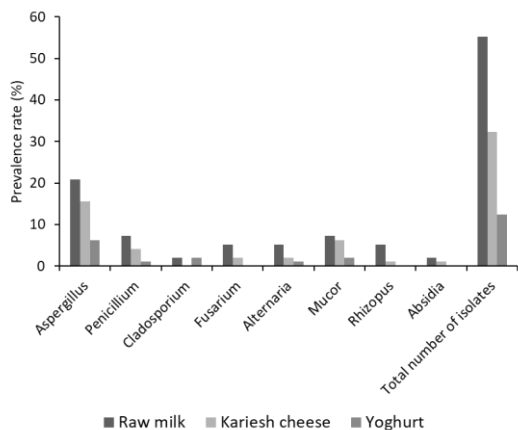


Fig. 3: Prevalence rates (%) of the identified mold genera among the examined raw milk, Kariesh cheese, and yoghurt samples retailed in Zagazig city, Egypt

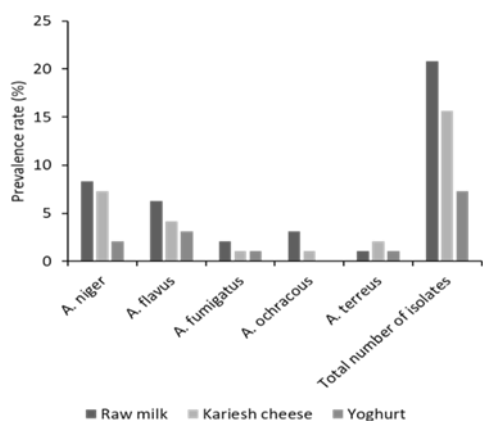


Fig. 4: Prevalence rates (%) of the identified *Aspergillus spp.*, among the examined raw milk, Kariesh cheese, and yoghurt samples retailed in Zagazig city, Egypt

4. Conclusion

The obtained results of the present study demonstrated mold contamination raw milk, Kariesh cheese, and yoghurt retailed in Zagazig city, Egypt with molds of a public health concern. *Aspergillus spp.*, particularly *A. niger* and *A. flavus* were the most dominant mold species in the present study. Several samples of the examined dairies were contaminated with total AFTs, indicating potential health risks for Egyptian population if such contaminated samples were consumed based on the recorded MOE values. Probiotics strains such as *L. acidophilus*, *L. rhamnosus*, and *L. plantarum* had clear antifungal activities as demonstrated in an experimental trial. Therefore, strict hygienic measures should be adopted during processing and handling of dairy products with selection of raw materials with high quality. The use of probiotics in dairy industry is also highly recommended.

Conflict of interest: None.

Authors' contributions: All authors contributed equally.

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