Original Research

Journal of Advanced Veterinary Research
(2024) Volume 14, Issue 2, 261-264

Mold contamination and total aflatoxin content in marketed raw milk in Zagazig city, Egypt

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ARTICLE INFO

Recieved: 26 August 2023

Accepted: 14 September 2023

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Keywords:

Raw milk
Mold contamination
Aflatoxin
Egypt

ABSTRACT

Milk contains a lot of bioactive peptides, vitamins, and trace minerals including calcium and magnesium. Mold contamination of milk and aflatoxin formation are major concerns in the food industry. One of the primary tasks of the food safety and public health sectors is to ensure that the population receives safe animal products. Given these considerations, the current investigation attempted to examine into mold contamination of retailed raw milk from cattle, buffaloes, and sheep. Furthermore, the total aflatoxins in the analyzed samples were estimated, and their potential health risks were explored further. The obtained results revealed that cattle milk had the highest mold contamination, followed by buffalo and sheep milk, with 60%, 40%, and 35%, respectively. In the current study, the identification of distinct mold species indicated four mold genera recovered from the milk samples, namely *Aspergillus* spp., *Penicillium* spp., *Cladosporium* spp., and *Fusarium* spp. *Aspergillus* spp. was the most prevalent mold genera isolated from the milk samples of cattle, sheep, and buffaloes, with 34%, 13.2%, and 11.3%, respectively. The mean total aflatoxins (ppb) levels in the milk samples tested were 5.05±0.25 (cattle), 4.22±0.18 (buffaloes), and 3.1±0.11 (sheep), respectively. In conclusion, mold contamination was found in retailed raw milk from cattle, buffaloes, and sheep in Zagazig, Egypt. Aflatoxin was found in several samples. As a result, efficient heat treatment of milk to pasteurization temperatures and avoidance of raw milk consumption are strongly advised.

Introduction

Farm animals including cattle, buffaloes, and sheep are among the most important veterinary species in terms of economic importance in Egypt and worldwide. Such animal species, are exposed to a wide range of xenobiotics during their lives, including mycotoxins, heavy metals, antibiotics, and pesticides. Such pollutants have a number of negative effects on the animal and can enter the human body through ingestion of contaminated milk and other edible tissues (Giantin *et al*., 2008; Darwish *et al*., 2010).

Milk is high in bioactive peptides, vitamins, and vital trace elements like calcium and magnesium (Stadnik and Kska, 2015; Raslan *et al*., 2018). At the same time, milk is regarded as a bioindicator for animal exposure to xenobiotics because such chemicals are released into milk and then find their way into the bodies of people if contaminated milk is consumed (Thompson and Darwish, 2019).

Mold contamination of milk is of particular concern in the food business. Mold growth is influenced by a variety of parameters, including moisture, pH, oxygen, substrate, and interactions with other microbiological agents. Molds can grow in a variety of pH, temperature, and water activity (aW) conditions (Pitt and Hocking, 2009).

Mycotoxins are toxic metabolites and carcinogenic chemicals produced by several mold genera, including *Aspergillus* spp. and *Fusarium* spp. The high moisture content and fluctuating storage temperatures are two of the predisposing elements that promote the establishment of various mold genera (Darwish *et al*., 2016). Furthermore, the use of low-quality raw milk is one of the essential variables that contribute to higher initial mold counts in other dairies.

Aflatoxins (AFTs) are harmful secondary metabolites produced by a variety of fungi, including *Aspergillus flavus* and *Aspergillus parasiticus* (Alcaide-Molina *et al*. 2009). AFTs can enter the animal body primarily through the intake of contaminated feedstuffs, and then enter the human body through the consumption of contaminated animal products. AFTs can cause huge economic losses worldwide due to their negative health impacts on both human and animal bodies, as well as the condemnation of infected agricultural crops (Darwish *et al*. 2014). AFTs have been related to a number of toxicological consequences, including teratogenicity and immunosuppressive effects. AFTs are also linked to human hepatocellular cancer (Aljazzar *et al*., 2021; 2023).

One of the key responsibilities of the food safety and public health sectors is to ensure that the population receives safe animal products. Given these factors, the current inquiry sought to look into the mold contamination of retailed raw milk from calves, buffaloes, and sheep. In addition, the total AFTs in the examined samples were estimated, and their potential health hazards were discussed further.

Materials and methods

All experiments were carried out in accordance with the rules and regulations of Zagazig University in Egypt.

Collection of Samples

Sixty milk samples (20 of each cow, buffalo, and sheep milk) were acquired at random from marketplaces in Zagazig, Sharkia province, Egypt during March to June 2023. In Egypt, raw milk is offered in polyethylene bags, with each sample weighing 500 g. In a chilled container, samples

ISSN: 2090-6277/2090-6269/

were transported to the Milk Hygiene laboratory at Department of Food Hygiene, Safety and Technology, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Sample preparation

Fifty mL of each sample were aseptically mixed for 2 minutes at 2500 rpm with buffered peptone water 0.1% (450 ml) to generate a dilution of 10^{-1}, followed by decimal serial dilutions (APHA, 2001).

Total mold count (TMC) determination

Total mold counts (TMC) were obtained using the pour plate technique with malt extract agar media for conventional molds and Czapeck-Dox agar with 5% NaCl for xerophilic molds (Oxoid, Basingstoke, UK), followed by 5-7 days incubation in the dark at 25°C. The plates were checked every day for mold growth during the incubation period. TMC was estimated using a colony counter and direct counting of cultivated plates (APHA, 2001).
TMC/g = average number of colonies multiplied by the dilution factor

Identification of the isolated molds

Molds were identified using the macroscopical and microscopical properties of the mold colonies, as described by Pitt and Hocking (2009). During the incubation phase, the mold cultures were inspected daily to determine the rate and pattern of growth. Surface growth and folding consistency, colony edges, and surface and reverse pigmentation were also detected. The colonies' surfaces and backsides were both examined.

Estimation of total aflatoxins

The total aflatoxins were quantified using a Series-4EX Fluorometer (VICAM, Milford, USA) in accordance with earlier methodologies (Abd-El-ghany and Sallam, 2015; El-Ghareeb *et al*., 2013) with minor changes. In summary, 25 g of each sample was mixed with 5 g NaCl before being blended at high speed for 3 minutes in 100 mL methanol: water (4:1). The mixture was diluted four times with double-distilled water before passing through a 1.5 m glass microfiber filter. The filtrate was then run through the AflaTest®-P affinity column at a rate of 2 drops/s. Aflatoxin was eluted from the affinity column using HPLC grade methanol at a rate of one drop per second and collected in a glass cuvette (VICAM part # 34000). At 1.0 mL, AflaTest® Developer was applied to the cuvette and thoroughly mixed with the eluate. The cuvette was then inserted in a calibrated fluorometer. The AflaTest had a detection range of 0.1 (poor detection) to 300 (high detection) ng/g (ppb). The limit of detection (LOD) for all matrices investigated was 0.1 ng/g. The fluorimeter was calibrated with excitation at 360 nm and emission at 440 nm.

Statistical analysis

Mold counts were presented as mean±SE (log 10 cfu/g). Statistical testing was performed using Tukey–Kramer HSD test ($p < 0.05$) (Gomez and Gomez, 1984).

Results and Discussion

Molds can get entry to milk through the mammary gland, as well as during milking and storage. Therefore, examination of the retailed milk for mold contamination is of a high significance for the sake of the food safety. The results presented in Figure 1 showed the mold contamination rates and total mold counts in the examined raw milk samples. Cattle's milk had the highest mold contamination followed by that of the buffaloes and sheep at 60%, 40%, and 35%, respectively (Fig. 1A). In parallel, cattle's milk had significantly ($p < 0.05$) the highest total mold count 3.08±0.14 log 10 cfu/mL followed by buffalo's milk (2.44±0.16), and that of the sheep (2.42±0.10) (Fig. 1B). In agreement with such recorded mold contamination of dairies. In general, milk and other dairy products should be free from molds according to FAO/WHO (2003). Similarly, mold contamination was reported in raw cattle milk collected in Slovenia at 63.3% (Torkar and Vengušt, 2008), and in Latvia at 44% (Gulbe and Valdovska, 2014). Lower mold count was recorded in ovine milk collected in the Spanish region of Castilla La Mancha with a total mold count ranged between < 10 to 80 cfu/ml (Marín *et al*., 2015). Besides, the degree of microbiological contamination of raw cow milk produced in Bulgarian dairy operations with molds was assessed. Mold isolation and identification were carried out using traditional microbiological procedures. Mold contamination was found in 76.6% of raw milk samples, with an average of 3.4 $\log_{10}$ cfu/ml and a maximum of 4.8 $\log_{10}$ cfu/ml (Chipilev *et al*., 2016).

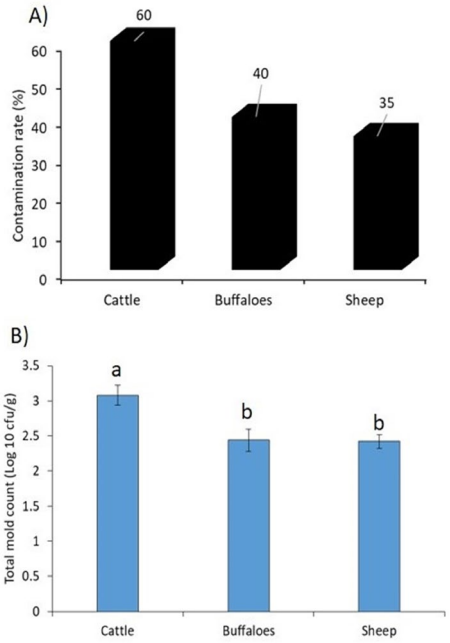

Fig. 1. A) Mold contamination rate (%) of the examined raw cattle, buffaloes, and sheep milk (n = 20 samples/each). B) Total mold count in the examined raw cattle, buffaloes, and sheep milk. Values represent means ± SD (n = 20 samples/each). Columns with different letter are significantly different at $p<0.05$.

The identification of different mold genera in the current investigation revealed four mold genera recovered from the examined milk samples, namely, *Aspergillus* spp., *Penicillium* spp., *Cladosporium* spp., and *Fusarium* spp. *Aspergillus* spp. was the most dominant mold genera where isolated at 34%, 13.2%, and 11.3% from the examined milk samples of cattle, sheep, and buffaloes respectively. *Penicillium* spp. came second to *Aspergillus* spp., where isolated at 13.2%, 5.7%, and 5.7% from the examined milk of the different animal spp., respectively. *Cladosporium* spp. was also recovered from the cattle's milk at 3.8%, and 1.9% from the milk of both buffaloes and sheep. *Fusarium* spp. was also recovered from the examined milk samples at 3.8% (cattle), 3.8% (buffaloes), and 1.9% (sheep) (Fig. 2). Further identification of the different *Aspergillus* spp. recovered from the examined milk samples in the present study revealed recovery of 5 *Aspergillus* spp., namely *A. niger*, *A. flavus*, *A. fumigatus*, *A. ochracous*, and *A. versicolor*. Both *A. niger* and *A. flavus* were the most predominant Aspergilli were recovered at 22.6% from the examined cattle milk samples, and at 9.7%, and 6.5% from that of the buffaloes, and at 6.5% and 9.7% for the recovered *A. niger* and *A. flavus* from the milk of the sheep, respectively. *A. fumigatus* was recovered at 3.2% from the milk of the cattle and sheep only, while *A. ochracous* was only recovered from the milk of the cattle at 3.2%. *A. versicolor* was recovered at 3.2% from milk samples of both of the buffaloes and sheep, and at 6.5% from the

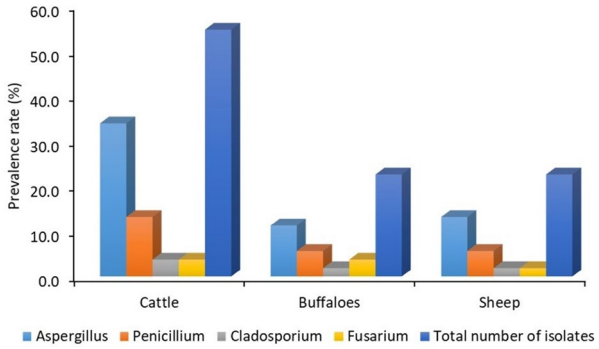

Fig. 2. Prevalence rates (%) of different mold genera recovered from the examined raw cattle, buffaloes, and sheep milk.

examined cattle milk samples, respectively (Fig. 3). The level of microbiological contamination with molds was assessed in 60 samples of raw milk gathered in Slovenia. Total mold count was with an average concentration of 0.6 $\log_{10}$ cfu/ml. Geotrichum (51.5%), *Aspergillus* (33.8%), Mucor (5.9%), *Fusarium* (2.9%), and *Penicillium* (2.9%) mold strains were isolated (Torkar and Vengušt, 2008). Likely, the mold genera identified in Latvian raw milk collected from organic farms belonged to 15 species, with the most common being *Absidia*, *Aspergillus*, *Apophysomyces, Mucor, Penicillium*, and *Rhizopus* spp., (Gulbe and Valdovska, 2014). Besides, mold species distribution was investigated at many locations along the manufacturing chain in a Manchego cheese plant and neighboring dairy farms, Spain. *Geotrichum* spp., and *Fusarium* spp., were the most often isolated genera from milk samples and cheeses aged for one month, indicating a direct transfer from raw milk. In contrast, the mycobiota of long-ripened cheeses was dominated by *Penicillium* species, which entered the cheese through the air of the ripening rooms (Marín *et al.*, 2015). The predominant mold genera recovered from raw cow milk samples collected in Bulgaria were *Aspergillus* (37.9%), *Geotrichum* (29.3%), *Mucor* (15.5%), *Cladosporium* (5.2%), and *Penicillium* (12.1%) were among the molds isolated (Chipilev *et al.*, 2016).

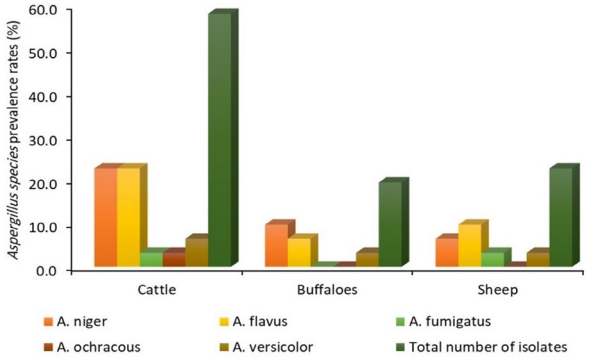

Fig. 3. Prevalence rates (%) of different Aspergilli recovered from the examined raw cattle, buffaloes, and sheep milk.

It is worth noting that the molds found in this study are of public health concern due to their ability to create secondary metabolites with a variety of negative health effects. *A. niger*, for example, is linked to pulmonary aspergillosis and produces poisonous metabolites such as kojic acid, oxalic acid, and malformins (Bennett, 1980). *A. flavus* has been linked to craniocerebral aspergillosis and allergic bronchopulmonary aspergillosis, as well as the production of toxic metabolites such as aflatoxins, aspergillic acid, kojic acid, asperotoxin, cyclopiazonic acid, and sterigmatocystin (Chakrabarti *et al.*, 2002; Hedayati *et al.*, 2007). *A. fumigatous* is linked to aspergillosis, aspergilloma, allergic responses, and the production of gliotoxin, a poisonous metabolite (Hohl and Feldmesser, 2007). Darwish *et al.* (2014) reported that *A. ochracous* generates ochratoxin A and citrinin. Sterigmatocystin is produced by *A. versicolor* (Kamei and Watanabe, 2005). Many hazardous metabolites of *Penicillium* spp. are produced, including meleagrin (mutagenic), roquefortine C (neuro

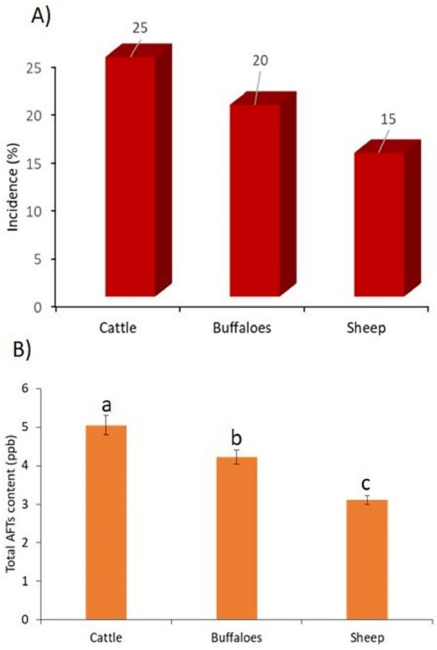

Fig. 4. A) Incidence (%) of total aflatoxin detection in the examined raw cattle, buffaloes, and sheep milk (n = 20 samples/each). B) Total aflatoxin content in the examined raw cattle, buffaloes, and sheep milk. Values represent means ± SD. Columns with different letter are significantly different at $p<0.05$.

toxic), mycophenolic acid (immunosuppressive), penitrem A (tremorgenic), and terrestric acid (cardiotoxic) (Pitt and Hocking, 2009). In addition, Darwish *et al.* (2014) mentioned that *Fusarium* spp. generates ochratoxins and deoxynivalenol mycotoxins. Several allergens are produced by *Cladosporium* spp. (Schoch *et al.*, 2006). Aflatoxins were detected at 25% (5 samples), 20% (4 samples), and 15% (3 samples) from the examined milk samples of cattle. Buffaloes, and sheep, respectively (Fig. 4A). The recorded mean values of the total aflatoxins (ppb) in the examined milk samples were 5.05±0.25 (cattle), 4.22±0.18 (buffaloes), and 3.1±0.11 (sheep), respectively (Fig. 4B). In comparison, aflatoxin M1 contamination at quantities greater than 50 ng/kg was found in 10% of cheese samples collected in Slovenia (Torkar and Vengušt, 2008). A total of 868 samples of raw cows' milk, pasteurized and UHT cows' milk, and dairy products were tested for AFM1 levels in a study conducted in Lebanon. Contamination levels in raw milk, pasteurized and UHT milk, and dairy products ranged from 0.011-0.440 µg/L, 0.013-0.219 µg/L, and 0.015-7.350 µg/L, respectively (Daou *et al.*, 2020). Aflatoxin M1 was also detected in the pasteurized milk samples in Mexico with a mean concentration of 31.3 ± 0.7 ng/L (Álvarez-Días *et al.*, 2022).

Conclusion

The obtained results of the present study revealed mold contamination of the retailed raw milk of cattle, buffaloes, and sheep in Zagazig city, Egypt. Several samples contained aflatoxins which carries significant health risks. Therefore, efficient heat treatment of the milk to pasteurization temperatures and avoidance of consuming raw milk are highly recommended.

Conflict of interest

The authors declare that they have no conflict of interest.

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