

*Research Paper*

**STUDIES ON CONTAMINATION OF DAIRY PRODUCTS BY AFLATOXIN M<sub>1</sub> AND ITS CONTROL BY PROBIOTICS**

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**Abstract**

Aflatoxins are carcinogenic compounds produced predominantly by certain strains of the *Aspergillus spp.* Aflatoxin M<sub>1</sub> (AFM<sub>1</sub>), a carcinogenic metabolite in milk and milk products resulting from aflatoxin B<sub>1</sub> ingestion by dairy animals, is considered a potential long lasting biohazard. This metabolite is relatively stable during milk pasteurization and storage as well as during the preparation of various dairy products. In this study, 30 samples from raw as well as pasteurized milk and 20 samples from different dairy products (processed, kariesh, mozzarella, akawi and roumy cheese and yoghurt) were randomly obtained from great Cairo district markets, samples were tested for mould contamination, toxigenicity of isolated *Aspergillus flavus* strains, AFM<sub>1</sub> contamination using ELISA technique as well as ability of some dairy strains of lactic acid bacteria to reduce the risk of aflatoxin M<sub>1</sub>. The obtained data pointed out the percentage of mould contamination in different examined samples. *Aspergillus* species were the most prevalent in the examined samples followed by *Penicillium* species. Screening of isolated strains of *Aspergillus flavus* for aflatoxin production by culturing on coconut medium revealed that only one (11.1%) out of 9 isolates of *Aspergillus flavus* produced aflatoxin. Results showed presence of AFM<sub>1</sub> in 73% of raw milk samples by average concentration of  $200.25 \pm 66.66$  ppt, 5 samples (22.7%) exceeded the maximum tolerance limit (500 ppt) accepted by Codex Alimentarius Commission and National Agency for Food and Drug Administration. Moreover, 50% of UHT milk samples tested were positive for AFM<sub>1</sub> with concentration of  $60.58 \pm 12.23$  ppt, none of these samples exceeded the permissible limit. Results revealed that 75%, 100%, 100%, 80%, 75%, and 100% of processed, kariesh, mozzarella, akawi, roumy cheese and yoghurt samples were positive for the presence of aflatoxin M<sub>1</sub>, with a maximum and a minimum aflatoxin levels of 1622 ppt and 3.57 ppt in kariesh and mozzarella cheese samples respectively. It is worthy to mention that among all the dairy product samples, mozzarella cheese proved to have the highest rate exceeding the permissible limit. The experimental use of probiotic showed logarithmic clearance ability of the previously contaminated milk from its aflatoxin contaminant. The highest degradation rate (96.2%) of aflatoxin was

observed at 72h during cold storage by combined use of *Lactobacillus Acidophilus* and *Bifidobacterium lactis* (1% v/v). So, this preliminary study warrants the public against the potential risk of AFM<sub>1</sub>contamination in dairy products with counteract amelioration by the convenient use different probiotics.

Key words: Mould; Aflatoxin M<sub>1</sub>; milk; dairy products; *Lactobacillus Acidophilus*; *Lactobacillus lactis*; ELISA; probiotics.

## INTRODUCTION

Food, the fuel of life, is of major concern for its quality and safety. Harmful components in plant derived foods can be either produced by the plant itself, or are contaminants derived from man-made sources or from microorganisms. Among these microorganisms, toxin producing fungi are ubiquitous in the environment and can invade our crops and produce toxic secondary metabolites known as mycotoxins. Many mycotoxins are highly resistant, and survive food processing, and therefore enter the food chain and provide a threat to human health. Worldwide, millions of tons of crops are destroyed every year due to fungal growth and spoilage. These fungi grow under particular conditions of temperature and humidity and can occur in a wide range of agricultural commodities. The problem of fungal contamination of foods and feeds has been already discussed [1, 2 and 3]. Aflatoxins (AFs) are highly toxic secondary metabolites produced by the species of *Aspergillus*, especially *A. flavus* and *A. parasiticus*. These fungi can grow on a wide variety of foods and feeds under favourable temperature and humidity. Contamination by aflatoxins can take place at any point along the food chain from the field, harvest, handling, shipment and storage [4].

Aflatoxins are common contaminants of foods, particularly in the staple diets of many developing countries, and are categorized as class 1 A human carcinogen by the International Agency for Research on Cancer [5]. Low level chronic aflatoxin exposure is linked to the development of "occult" conditions, such as impaired growth and immune function and chronic diseases, such as liver cancer in areas where the aflatoxin producing *Aspergillus* fungi are prevalent. It is therefore of major interest, to prevent formation of aflatoxins in the first place, or to reduce its bioavailability from foods to prevent their harmful effects. Aflatoxin M<sub>1</sub> (AFM<sub>1</sub>) is the hydroxylated metabolite of aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) under the influence of cytochrom p450 oxidase system found in the rumen microflora and the animal's own cells and can be found in milk and subsequently in other dairy products when lactating animals are fed with contaminated feedstuffs [6, 7, 8, 9, 10, 11, 12, 13 and 14].

AFM<sub>1</sub> could be detected in milk 12-24 h after the first AFB<sub>1</sub> ingestion, reaching a high level after a few days. The ratio between AFB<sub>1</sub> ingested and AFM<sub>1</sub> excreted has been estimated to be 1-3% [10, 15 and 16]. This toxin has been categorized by the International Agency for Research on Cancer as a class 2B toxin, a possible human carcinogen [17]. Although, AFM<sub>1</sub>, is less carcinogenic, hepatogenic and mutagenic than AFB<sub>1</sub>, it exhibits a high level of genotoxic activity and certainly represents a health risk because of its possible accumulation and linkage to DNA [18 and 19]. In the assessment of cancer risk, the infants are more exposed to the risk because the milk is a major constituent of their diet. Therefore, the presence of AFM<sub>1</sub> in milk and milk products is considered to be undesirable [20 and 21].

AFM<sub>1</sub> is a very stable aflatoxin, so that it is not destroyed by storage or processing, such as pasteurization, autoclaving or other methods used in the production of fluid milk, and if present in raw milk it may persist into final products for human consumption [22]. Due to toxicity, most countries have set up maximum admissible levels of AFM<sub>1</sub> in milk, which vary from the 50 ng/kg established by the EU to the 500 ng /kg established by US FDA [23 and 24]. The contamination of milk and milk products with AFM<sub>1</sub> displays variations according to geography, country and season. The pollution level of AFM<sub>1</sub> is differentiated further by hot and cold seasons, due to the fact that grass, pasture, weed and rough feeds are found more commonly in spring and summer than in winter [25, 26 and 27].

Milk and dairy products represent fundamental items in the human diet and can be the principal way for aflatoxins to be ingested, [28 and 29]. Milk is the most important source of calcium and

phosphorus of human body and due to having essential amino acids, has an important status in supplying the body's protein needs. Studies have shown that there is a close relationship between consumption of milk and health status of people in terms of efficiency, intelligence quotient (IQ), reducing the risk of infectious diseases, regulation of metabolic activities, decreasing blood pressure, increasing beneficial blood lipids (High-density lipoprotein), preventing from colon cancer and osteoporosis [30]. Due to a close relationship between livestock feed with health and safety of milk, various researches have been conducted on livestock feed. The researches have shown that contamination of livestock feed with certain types of moulds such as *Aspergillus* causes aflatoxin production and its transfer to milk [31].

Aflatoxin contamination of milk and milk products is produced in two ways: either toxins pass into milk by ingestion of feeds contaminated with aflatoxins, or it results as subsequent contamination of milk and milk products with fungi [22 and 25]. To reduce human exposure to mycotoxins, technologies are available to minimize fungal growth and contamination during harvest, processing and storing of crops, but these methods are only available in developed countries, resulting in a reduced prevalence of mycotoxin exposure. Low level mycotoxin exposure occurs in parts of the world where food is available in higher quality and variety, whereas high level exposure causes acute disease which may result in death and is prevalent in areas where populations depend on a single staple food commodity. Microorganisms, especially bacteria, have been studied for their potential to either degrade mycotoxins or reduce their bioavailability [32]. Among these bacteria, probiotic lactic acid bacteria have been identified as a safe means to reduce availability of aflatoxins in vitro [33]. Furthermore, probiotic bacteria exert a number of other beneficial health effects, which make them even more suitable additives to food and feed.

The food industry has been put under pressure to find how to inhibit the growth of toxigenic moulds and the synthesis of mycotoxins in raw materials and end products, while the general public requires high quality, preservative free, safe but mildly processed food with extended shelf life. Bio-preservation, the control of one organism by another, could be an interesting alternative to physical and chemical methods, and it has received much attention lately [34]. On the other hand, it is known that lactic acid bacteria (LAB) are capable to bind AFs in liquid media, apparently to cell wall components, polysaccharides and peptidoglycans of LAB [35], and therefore could be used as potential mycotoxin decontaminating agents [33, 35, 36, 37, 38, 39 and 40]. The inclusion of appropriate microorganisms in the contaminated diet could prevent the absorption of mycotoxins during their passage in the gastrointestinal tract and eliminated them in the faeces [41, 42, 43 and 44]. Moreover, the binding of AFB<sub>1</sub> to the surface of LAB reduced their adhesive properties, and the accumulation of aflatoxins in the intestine may therefore be reduced via the increased excretion of an aflatoxin-bacteria complex [45]. These considerations encouraged the recent emphasis on biological methods, but mainly focused on preventing AFs absorption in the gastrointestinal tract of the consumers, including these microorganisms in the diet and so prevent the aflatoxicosis effects. Many probiotic organisms have their origins in fermented foods, and their "history of safe use" in human consumption allows the status of generally recognized as safe (GRAS) [46]. On the other hands, some strains of LAB have been shown to inhibit both growth of moulds and the production of mycotoxins [47].

Specific dairy strains of lactobacilli can remove aflatoxins from aqueous solutions [48, 33]. In addition, specific dairy strains of lactic acid bacteria also removed aflatoxin M<sub>1</sub> from reconstituted milk [49]. The removal of aflatoxin involves physical binding of the toxin probably to the bacterial cell wall or cell wall components [50, 36].

Therefore, the purpose of this study was to examine the level of aflatoxin M<sub>1</sub> in milk and some dairy products and to investigate the ability of some dairy strains of lactic acid bacteria to reduce aflatoxins M<sub>1</sub> in milk.

## 2 MATERIALS AND METHODS

### 2.1. Collection of samples:

One hundred and eighty random samples each of 30 raw and pasteurized milk and 20 each of processed, Kariesh, Mozzarella, Akawi and Roumy cheese and yoghurt were collected from different retail markets and shops in Cairo and Giza Governorates. Samples were aseptically transported to the laboratory in a cooler with ice packs for isolation of moulds then stored at -4°C until analysis of AFM<sub>1</sub>.

### **2.2. Preparation of samples:**

Ten millimeters of milk samples and ten grams of cheese samples were transferred aseptically into a sterile blender jar, to which 90 ml of 1% peptone water were added and homogenized in a sterile warring blender for 2 minutes. One millimeter quantities of the previously prepared dilutions were inoculated separately into Petri dish plates and mixed with rose Bengal agar medium. The plates were left to solidify after mixing [51].

### **2.3. Isolation and identification of moulds:**

The isolates were sub-cultured onto malt extract agar and Czapek-Dox agar then incubated at 28 °C for 7 days [52]. The isolated fungi were identified individually by macro- and microscopic characteristics of the mould colonies according to the keys of [53, 54, and 55].

### **2.4. Determination of toxigenic potential of *Aspergillus flavus*:**

The isolated strains of *A. flavus* were inoculated at the center of a coconut agar medium plate and incubated at 28°C for 3 days. The plates were inspected daily for a blue fluorescent zone around the suspected colonies when exposed to along wave of UV light (365nm) [56].

### **2.5. ELISA test procedure:**

#### **2.5.1. AFM<sub>1</sub> determination:**

The quantitative analysis of AFM<sub>1</sub> in examined samples was performed by competitive ELISA (RIDASCREEN AFM<sub>1</sub>, Art No.R1111-R-Biopharm GmbH, and Darmstadt, Germany) procedure as described by R-biopharm GmbH [57].

#### **2.5.2. Samples preparation for analysis:**

Milk samples were skimmed following the test procedure and used directly in the test. Concerning the solid samples, two grams of triturated and homogenized composite samples of cheese or yoghurt were weighed and extracted with 8 ml dichloromethane by shaking for 30min. on a heated shaker at 50 °C. The following steps were done as suggested by RIDASCREEN instructions.

#### **2.5.3. Evaluation of AFM<sub>1</sub>:**

The absorbance values obtained for the standards and the samples were divided by the absorbance value of the first standard (zero standards) and multiplied by 100 (percentage maximum absorbance). Therefore, the zero standard is thus made equal to 100%, and the absorbance values were quoted in percentages. The values calculated for the standards were entered in a system of coordinates on graph paper against the AFM<sub>1</sub> concentration in ppt (Figure 1).

#### **2.5.4. Statistical Analysis:**

Data were analyzed and results reported as mean ± SD. The calibration curve and trend line equation were prepared using available software, percentage, minimum, maximum and mean± SD were carried out [58].

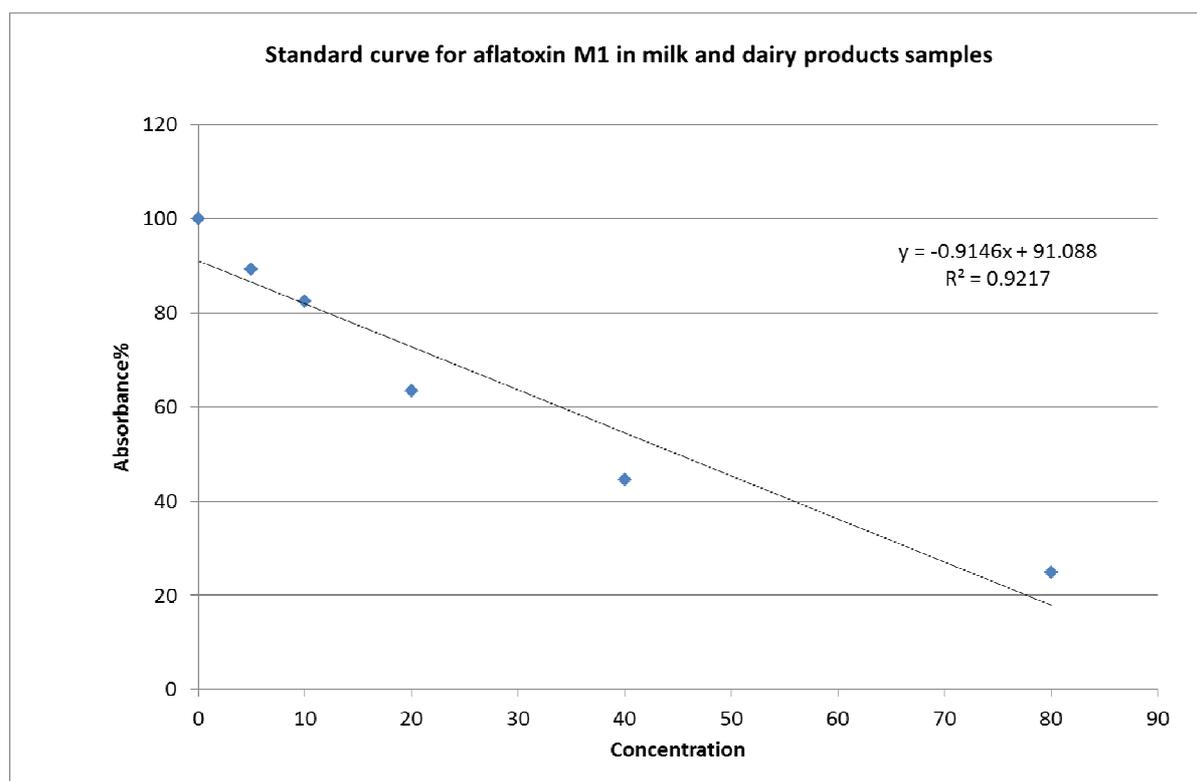


Figure 1: Calibration curve of aflatoxin M<sub>1</sub>

## 2.6. Detoxification of AFM<sub>1</sub> in Yoghurt by Lactic acid Bacteria:

### 2.6.1. Cultures activation:

Lactic acid bacteria were obtained from Ch. Hansen's Laboratories, Copenhagen, Denmark. The cultures were activated in 11 % reconstituted skim milk for several times and the last 3 times were in specific medium at 37 °C for all strains.

### 2.6.2. Preparation of lactic acid bacteria (LAB) inoculum:

*Lactobacillus acidophilus* was originally obtained from Chr. Hansen's Lab. (Denmark), and cultivated in 25 ml De Man Regosa & Sharp medium (MRS) broth and Agar (Oxoid CM 359) at 37°C for 24 h. On the other hand, *Bifidobacterium lactis* was collected from Australian Research center and cultivated in 25 ml MRS broth (Oxoid 358) at 37°C for 24 h. The suspensions were centrifuged at 1.700 X g for 15 minutes. The supernatant was discarded and the bacterial pellets were washed twice with phosphate buffered saline (PBS; pH 7.3, 0.01 M) and the concentration of LAB and *Bifidobacterium* was adjusted to 3 X 10<sup>8</sup> and 7.6 X 10<sup>6</sup> bacteria per 4 ml PBS (per tube) respectively.

### 2.6.3. Binding ability of LAB in AFM<sub>1</sub> contaminated milk:

In order to study the binding ability of *Lactobacillus acidophilus* and *Bifidobacterium lactis*, 1 ml of each pure culture of *Lactobacillus acidophilus* (2%), *Bifidobacterium lactis* (2%) and 1ml of a combination of L.A (1%) and B.L 1% (0.5 ml each ) were suspended separately in a Falcon tube containing 49 ml of naturally contaminated commercial UHT skim milk with AFM<sub>1</sub> at a concentration of 50.2 ppt and incubated at 37°C for 5 h. Unbound AFM<sub>1</sub> content was determined by ELISA analysis after 24 hrs, 48 hrs and 72 hrs during storage period at 4±1°C. The toxin was measured using ELISA technique, cell-free milk contaminated with AFM<sub>1</sub> was used as positive control. Bacteria suspended in non-contaminated skim milk were used as negative control (pure species) and all assays were performed in triplicate.

## 3. RESULTS AND DISCUSSION

Mould contamination not only causes deterioration of food and feed but also can adversely affect the health of humans. Moreover, fungi influence the biochemical characters and flavour of

the product and its appearance is commercially undesirable and often results in down grading of the product.

Many types of cheese are an excellent substrate for mould growth. Important fungi growing on cheese include *Penicillium*, *Aspergillus*, *Cladosporium*, *Geotrichum*, *Mucor* and *Trichoderma* species. It was stated that, incidence of moulds in cheese indicates that the predominant flora belong to the genus *Penicillium* [2].

Results given in Table (1) revealed that moulds were isolated from 76.6 % of the examined raw milk samples. Nearly similar finding were reported by [59], while no moulds were isolated from UHT milk samples. Moulds were isolated from 90 %, 90 %, 75%, 65%, 40% and 0% of the processed cheese, kariesh, mozzarella, akawi, roumy and yoghurt samples, respectively.

Mould contamination in some cheese types can periodically cause both economic and sensory problems. Since moulds do not survive pasteurization; their presence in pasteurized milk and other milk products is caused probably by re-infection during manufacturing [60 and 61]. It was reported that the contamination of milk products, particularly cheese is due to surrounding environment [62, 63].

In accord with these facts our results agree for certain extent with the results of other authors [64], but show higher values than that reported by others [65 and 66]. The present findings confirm the poor hygienic conditions during handling and processing. Regarding yoghurt, our results differed from that reported by one of the authors, who proved contamination of yoghurt with 50% with mold [67].

Consistent with previous studies [68 and 69], different mould species were isolated from milk and dairy products with various percentages in this study including *Aspergillus*, *Cladosporium*, *Mucor*, *Penicillium*, *Rhizopus*, *Fusarium* and *Dematiaceous* Fungi (Table 2).

Screening of obtained isolates of *A. flavus* for aflatoxin production revealed that only one (11.1%) out of 9 tested isolates was found to be aflatoxin producer, this was in lower extent compared with some authors [70 and 71].

Although moulds have little practical importance in raw milk, they are important in pasteurized milk, particularly when it is used for the manufacture of cheese and other dairy products. The characteristic feature of some mould-ripened cheese types is extensive proteolysis and lipolysis. The presence of wild types of moulds is undesirable as they may influence the organoleptic characteristics of the cheeses, they can produce mycotoxins and represent a potential health risk [61, 72].

Furthermore, milk and dairy products that being good sources of bioavailable calcium and proteins for all age groups are always at risk of being contaminated with AFM<sub>1</sub>. AFM<sub>1</sub> in dairy products is a serious health hazard for consumers specially children who are more sensitive to adverse effects of aflatoxin than adults [73]. Therefore, the current study was undertaken to examine the presence of AFM<sub>1</sub> in milk and other dairy products with further attempt trial for its detoxification. Table 3 shows the results of analysis of 180 samples of milk and dairy products for AFM<sub>1</sub> contamination. From the results, it is clear that aflatoxin M<sub>1</sub> was found in 73% of raw milk samples with a mean concentration of 200.25 ± 66.66 ppt, whereas 15 (50%) UHT milk samples were found to contain AFM<sub>1</sub> with a mean value of 60.58 ± 12.23ppt.

However, many of the previous studies has indicated the presence of AFM<sub>1</sub> at high concentrations in milk samples [74, 75, 76, 77, 78,79, 80, 81, 82 and 25] as the incidences of AFM<sub>1</sub> ranged between 92.3% and 100%, also our data agree with another, who reported the incidence of AFM<sub>1</sub> in UHT milk samples (55.2%) and mean concentrations of AFM<sub>1</sub> levels of 61 milk samples in the range of 35.8–58.6 ppt [18].

Regardless to variations that may be attributed to differences in region, season, and especially analysis method, the higher concentration of AFM<sub>1</sub> in the milk samples might be due to feeding the animals with AFB<sub>1</sub>contaminated feeds in Egypt, which is characterized by higher temperature and humidity.

With a view of the fact that milk is used by all the age groups including infants and children all over the world, even the low amount of aflatoxin M1 in milk can be a serious public health problem. Since the commission of the European Communities stated that even if aflatoxin M1 is

regarded a less dangerous more genotoxic and carcinogenic substance than Aflatoxin B1, it is necessary to prevent the presence of AFM1 in milk and its products [8].

Despite of low levels of aflatoxin M1 in milk and its products in many European countries [80] as a result of stringent regulations of aflatoxin B1 in complementary feedstuffs for dairy cattle, other European studies indicated relative higher values. An example of other countries, like Syria, 80% of tested raw milk samples collected from the Syrian market was contaminated with various levels of aflatoxin M1 ranging from 20 - 765 ng/L [83]. This variation, however, can be related to dairy feed quality [84].

On the other hand, Table 3 showed that aflatoxin M<sub>1</sub> residue was detected in 75% ,100%, 100%, 80%, 75% and 100% of our processed, kariesh, mozzarella, akawi and roumy cheese and yoghurt samples, respectively with mean levels of  $304.78 \pm 119.5$ ,  $528 \pm 140.8$ ,  $549.7 \pm 129.9$ ,  $875.77 \pm 115.7$ ,  $288.6 \pm 100.6$  and  $72.9 \pm 1.7$  ppt.

These findings are within the range of previously performed studies [85, 86, 87, 9, 88, 10, 89, 90 and 91] and are even lower than data indicating concentrations of 2610 , 4500 and 5730 ppt in soft , hard and processed cheese[92] , also AFM1 was detected in Brazilian cheese with range of 20 – 6920 ppt [93], while in a later study AFM1 was detected in amounts of 6300 , 5100 , 3300 , 2999 , 2099 , 2340 , 3300 and 3400 ppt in fresh Roumy , aged Rowel , fresh Domiati , aged Domiati , cream processed, Karish , Feta and Cheddar cheese, respectively[94]. AFM1 was also detected in different cheese types ( Cheddar , Feta , processed , aged Ras , fresh Ras , aged Domiati , fresh Domiati , spread and mish ) with average of 3500 , 6200 , 3600 , 5000 , 4900 , 5200 , 1000 , 2000 and 5600 ppt, respectively [95]. In Turkey, cream cheese samples were reported to contain AFM1 with range of 0- 4100 ppt [96].

Another study in Egypt, the range of AFM1 in Karish cheese samples was 5000 to 35000 ppt with mean value 17500 ppt[97] , also soft cheese (fresh Karish and Domiati ) samples were examined and found that the mean value were 3600 and 67000 ppt. [98], however, low amount of AFM1 was detected by other authors [99, 100, and 101], While no detection for aflatoxin in some cheese samples were reported by others [102, 103 and 104].

Studies have reported that the concentration of AFM<sub>1</sub> in cheese and dairy products varied depending on the type of cheese, water content, variation in the original milk contamination, and production technologies [105]. This contamination was probably caused by only a few contaminated milk samples entering the bulk milk supply. Such contamination levels may be a serious problem for public health, since all the age groups, including infants and children, consume these products worldwide.

With respect to yoghurt, several surveys were performed in order to determine the AFM<sub>1</sub> levels in yogurt. About 80% of all yogurt samples in Italy were contaminated with AFM<sub>1</sub>, ranged between 1- 3.1 ng/kg [28]. Later, 61.0% yogurt samples were contaminated with AFM<sub>1</sub> at lower levels than those in previous survey [29]. In Portugal, 48 samples of yogurt were tested and only 2 (4.2%) contained AFM<sub>1</sub> at levels of 0.45 ng/kg [106]. However ,in Brazil; there was no detection for AFM<sub>1</sub> in 30 of tested yogurt samples [103], most of the yogurt samples (62.88%) purchased at different markets in Ankara were free of AFM<sub>1</sub>[107]. Also in Turkey, it was revealed that 65.38% of ordinary yogurt samples, 33.33% of fruit yogurt samples, and 55.77% of strained yogurt samples contained the aflatoxin [108]. AFM<sub>1</sub> occurrence in 2.8% of yoghurt samples was determined [75].

According to observations, the levels of contamination of local yogurt by AFM<sub>1</sub> seem to vary in many studies. These variations may be related to different reasons such as yogurt manufacturing procedures, different milk contaminations, type of yogurt, conditions of yogurt ripening, geographical region, the country, the season and the analytical methods employed [6, and 13].

Some recent studies were performed in different milk and dairy products with different incidence of AFM<sub>1</sub> [109, 110 and 111].

Occurrence of AFM<sub>1</sub> in dairy products can be due to three possible causes: (1) AFM<sub>1</sub> present in raw milk because of carryovers of AFB1 from contaminated cow feed to milk, (2) Synthesis of AF (B1, B2, G1 and G2) by *A. flavus* and *A. parasiticus* growing on cheese [112], and (3) Occurrence of these toxins in dried milk used to enrich the milk which it is being used in the

production of cheese [113]. However, the increase in AFM<sub>1</sub> concentration in cheese has been explained by the affinity of AFM<sub>1</sub> for casein [114,115, 116]. Some previous studies exhibited contradictory data on the behavior of AFM<sub>1</sub> during cheese making, as it was found that AFM<sub>1</sub> distribution during cheese making had a reduction of about 60% compared to the milk [117].

For all practical purposes, AFM<sub>1</sub> is stable in unprocessed milk and processed milk products, and is unaffected by pasteurization or processing of milk into cheese or yogurt.

Therefore, the second part of the current work was our trial to minimize the risk of AFM<sub>1</sub> contamination by the virtue of the probiotic use. Since there is no current procedure for destroying AFM<sub>1</sub> in milk without destroying the milk, lactic acid bacteria (LAB) afford a good alternative to compete with the AFM<sub>1</sub> in milk and its products. This is due in large part, generally to their recognition as safe (GRAS) status and their use as probiotics, is of particular interest for reducing the bioavailability of AFs. A number of studies have screened these microorganisms for their ability to bind to AFs and have reported a wide range of genus, species and strain specific binding capacities. Additionally, it is accepted that daily intake of these probiotics contributes to improving and maintaining well balanced intestinal flora, and prevents gastrointestinal disorders [118]. Various species of genera *Lactobacillus* and *Bifidobacterium* mainly have been used as probiotics over the years [119, 120,121].

Previously, the ability of dairy strains of lactobacilli has been proved to remove aflatoxin from aqueous solution [33, 36, 48 and 49]. This removal of aflatoxin involves physical binding of the toxin probably to the bacterial cell wall or cell wall components. The principal reason of that may be due to the binding properties of AFM<sub>1</sub> to milk casein. Earlier report addressed that the average of 30.7% more AFM<sub>1</sub> was found in milk once treated with proteolytic enzyme than in untreated milk and suggested that AFM<sub>1</sub> is bound to milk protein [122].

The cell wall polysaccharide and peptidoglycan are the two main elements responsible for the binding of mutagens to lactic acid bacteria. This perturbation of the bacterial cell wall may allow AFB<sub>1</sub> to bind to cell wall and plasma membrane constituents that are not available when the bacterial cell is intact [123,124,125].

In Figure (2) the two strains of (LAB), *Lacobacillus acidophilus* and *Bifidobacterium lactis* were tested for aflatoxin M<sub>1</sub> reduction in naturally contaminated milk with 50.2 ppt AFM<sub>1</sub>. It is clear from the figure that there was a gradual reduction as a function of time with complete elimination by the end of storage period (3 days) at refrigerator, where *Lactobacillus acidophilus* and *Bifidobacterium lactis* showed significantly ( $p < 0.05$ ) more ability for removing of AFM<sub>1</sub>. After one day, the concentration of aflatoxin M<sub>1</sub> decreased to 34.7 ppt (30.9%), 22.7ppt (54.8%) and 18.8ppt (62.5%) in the presence of *L. acidophilus* (2%), *B. lactis* (2%) and combination of *L. acidophilus*(1%) and *B. lactis* (1%) %, respectively. Meanwhile the most extensive reduction of AFM<sub>1</sub>concentration of 10.9ppt (78.3%), 4.2 ppt (91.6%) and 1.9 ppt (96.2%) was achieved by using the same concentrations of lactic acid bacteria after 48 h. No aflatoxin M<sub>1</sub> was detected in the third day.

Concerning the effect of lactic acid bacteria on reducing the concentration of AFM<sub>1</sub>, the obtained results came in agreement with the author, who measured a reduction of aflatoxin M<sub>1</sub> in yoghurt made by *L. acidophilus* and *Bifidobacterium bifidum* of 95.3 and 84.7% for AFM<sub>1</sub> after 5 days [126].

The ability of dairy strains of lactic acid bacteria to remove aflatoxin M<sub>1</sub> from contaminated phosphate buffer saline, skim and full cream milk was investigated [49].All tested strains, whether viable or heat-killed, could reduce the AFM<sub>1</sub>content of a liquid medium, which indicates that bacterial viability is not prerequisite to toxin removal and suggests involvement of a cell wall-related physical phenomenon instead of a metabolic degradation reaction.

Also, the same conclusion was reached when different spp. of lactic acid bacteria were used the reduction level by these strains ranged from 26.2- 34.0%, depending upon the bacterial isolates [127 and 128].

On the other hand, several studies investigating antimutagenic and anticarcinogenic effects of probiotics [129,130, 131]

Opposite results were obtained by other authors, who observed variable increases of AFM<sub>1</sub> content in yoghurt related to the milk [132, and 133]. As regards AFM<sub>1</sub> stability during cold

storage of yoghurt at 7 °C, it was found that no reduction of AFM<sub>1</sub> in yoghurt during the storage period [133, 134, and 135]. These differences in results might be explained by the differences in extraction procedures, concentration of toxin, time elapsed before analysis, storage temperature, milk contaminating method, variability in composition of milk, or differences in the behavior of cultures used to make the yoghurt [136].

The beneficial effects of food with added live microbes (probiotics) on human health showed a significant ( $P < 0.05$ ) improvement in liver functions and in particular by milk products on children and other high-risk populations who are being increasingly promoted by health professionals. It has been reported that these probiotics can play an important role in immunological, digestive and respiratory functions and could have a significant effect in alleviating infectious disease in children. As there is no international consensus on the methodology to assess the efficacy and the safety of these products, at present, it was considered necessary to convene an Expert Consultation to evaluate and suggest general guidelines for such assessments.

Regarding to compare our results to maximum tolerated limits of AFM<sub>1</sub> in milk and dairy products (Table 4), it is of importance to emphasize that 21, 9, 8, 17, 14, 16, 13 and 20 samples of raw milk, UHT milk, processed, Kareish, Mozzarella, Akawi, Roumy cheese and yoghurt, respectively contained AFM<sub>1</sub> residues more than EU (50 ppt). While 5, 0, 4, 8, 6, 10, 3 and 0 samples of the above analyzed milk and dairy products, respectively contained AFM<sub>1</sub> residues more than the limit established by Codex Alimentarius Commission and National Agency for Food and Drug Administration (500ppt).

As aflatoxins pose more serious risks for public health, certain limits of AFM<sub>1</sub> in milk and dairy products were determined in different countries as shown in (Table 5).

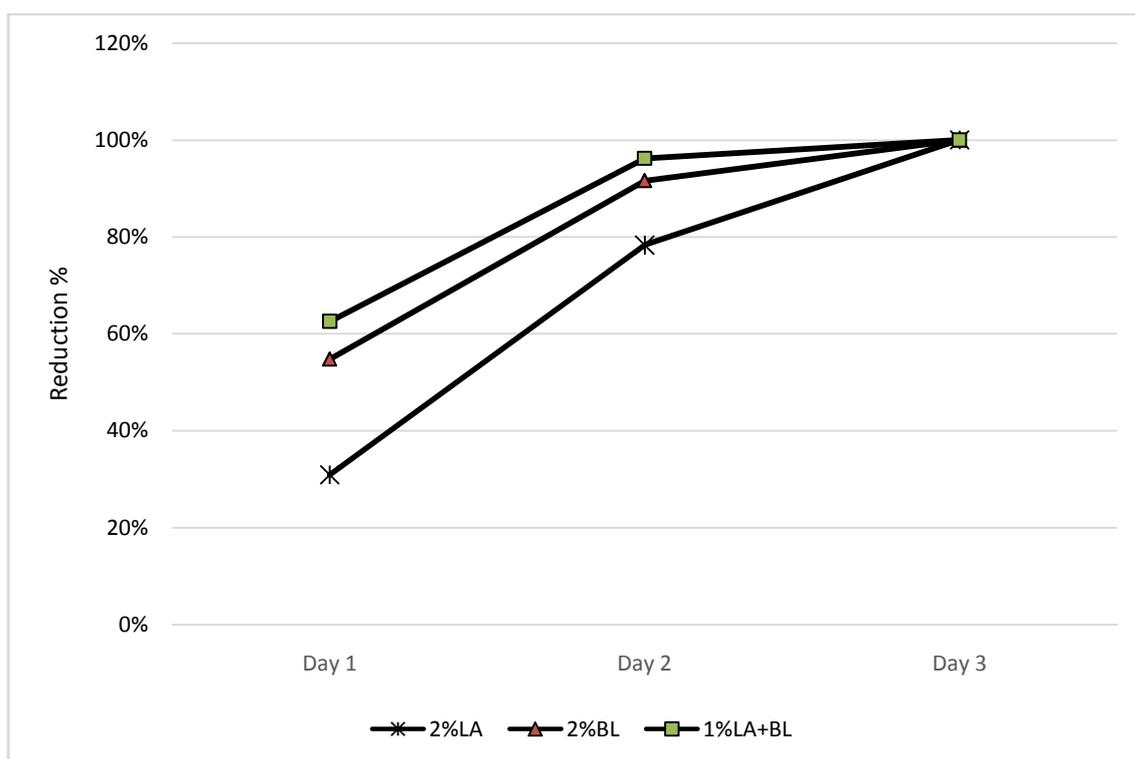


Figure (2): Reduction of aflatoxin M<sub>1</sub> in milk using *Lactobacillus acidophilus* (LA) and *Bifidobacteria lactis* (BL).

Table 1: Incidence of isolated moulds from the examined milk and dairy products samples.

Examined samples	Total no. of examined samples	Moulds			
		Positive		Negative	
		NO.	%	NO.	%
Milk					
• Raw milk	30	23	76.6%	7	23.3%
• UHT milk	30	0	0%	30	100%
2. Dairy products					
• Processed cheese	20	18	90%	2	10%
• Karesh cheese	20	18	90%	2	10%
• Mozzarella cheese	20	15	75%	5	25%
• Akawi cheese	20	14	65%	7	35%
• Roumy cheese	20	8	40%	12	60%
• Yoghurt	20	0	0%	20	100%

Table 2: Incidence of identified mould genera isolated from examined milk and dairy product samples.

Examined samples	Total no. of examined samples	genus <i>Aspergillus</i>		genus <i>Rhizopus</i>		genus <i>Fusarium</i>		Genus <i>Cladosporium</i>		genus <i>Penicillium</i>		<i>Dematiaceous</i> Fungi		genus <i>Mucor</i>	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
1. Milk															
• Raw milk	30	10	33.	1	33.	1	3.	0	0	7	23.	1	3.3	1	3.
• UHT milk	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2. Dairy products															
• Processed cheese	20	1	5	0	0	0	0	0	0	0	0	0	0	0	0
• Karesh cheese	20	0	0	1	5	0	0	0	0	0	0	0	0	0	0
• Mozzarella cheese	20	15	75	0	0	0	0	2	10	5	25	0	0	0	0
• Akawi cheese	20	7	35	0	0	2	10	0	0	5	25	3	15	0	0
• Roumy cheese	20	0	0	0	0	0	0	0	0	6	30	0	0	0	0
• Yoghurt	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 3: incidence and statistical analysis of AFM<sub>1</sub>residue levels (ppt) in examined milk and dairy product samples.

Examined samples	Total no. of examined samples	Positive samples (%)	Min.	Max	Mean ± SD
1. Milk					
• Raw milk	30	22 (73)	4.9	666.56	200.25 ± 66.66
• UHT milk	30	15 (50)	2.04	110	60.58 ± 12.23
2.Dairy products					
• Processed cheese	20	15 (75)	7.24	1601	304.78 ± 119.5
• Kariesh cheese					
• Mozzarella cheese	20	20 (100)	26.35	1622	
• Akawi cheese	20	20 (100)	3.57	1491	528 ± 140.8
• Roumy cheese					
• Yoghurt	20	16 (80)	58.23	1550	549.7 ± 129.9
	20	15 (75)	45.87	1027	
	20	20 (100)	62.35	81.14	875.77 ± 115.7
					288.6 ± 100.6
					72.9 ± 1.7

Table 4: Comparison of AFM<sub>1</sub> levels of analyzed samples with Codex Alimentarius and European Union legal limits.

Examined samples	N (%)	N1 (%)	N2 (%)	N3 (%)	N4 (%)
1. Milk					
• Raw milk	22 (73)	17 (77)	5 (23)	1 (5)	21 (95)
• UHT milk	15 (50)	15(100)	0 (0)	6 (40)	9 (60)
2.Dairy products					
• Processed cheese	15 (75)	11 (73.3)	4 (26.6)	7(46.6 )	8(53.3)
• Kariesh cheese	20 (100)	12(60)	8(40)	3(15)	17(85)
• Mozzarella cheese	20 (100)	14(70)	6(30)	6(30)	14(70)
• Akawi cheese	20 (100)				
• Roumy cheese		6(37.5)	10(62.5)		16(100)
• Yoghurt	16 (80)	12(80)	3(20)	0(0)	13(86.6)
	15 (75)	20(100)	0(0)	2(13.3 )	20(100)
	20 (100)			0(0)	

N: AFM<sub>1</sub> positive samples.

N1: samples below the limits of codex (500ppt)

N2: samples exceeding the limits of codex (500ppt)

N3: samples below the limits of EU (50ppt)

N4: samples exceeding the limits of EU (50ppt)

Table 5: International legislation on AFM<sub>1</sub> in milk and dairy products for human consumption. [137]

Country	Raw milk (µg/kg)	Dairy products (µg/kg)
Argentina	0.05	0.50 (milk products)
Australia	0.05, 0.01 (pasteurised infant milk)	0.02 (butter), 0.25 (cheese), 0.4 (powdered milk)
Egypt	0	0
European Union	0.05	0.05
Honduras	0.05	0.25 (cheese)
Rumania	0	0
Switzerland	0.05	0.025 (milk whey and products), 0.25 (cheese), 0.02 (butter)
Turkey	0.05	0.25 (cheese)
USA		0.50

## CONCLUSION

In conclusion, this study has shown the serious risk for public health since all age groups, including infants and children, consume milk and its products worldwide. For this reason, milk and milk products have to be controlled continuously for presence of AFB<sub>1</sub> contamination. It is also extremely important to maintain low levels of AFB<sub>1</sub> in the feeds of dairy animals. In order to achieve this, dairy cow feeds should be kept away from contamination as much as possible, should be checked regularly for aflatoxins and particularly important, storage conditions of feeds must be strictly controlled. Also widespread and continuous training and surveillance programs must be arranged for both the producers and consumers.

It is necessary to apply an ideal recommended limit to minimize the health hazard from aflatoxin M<sub>1</sub> contamination in milk. Application of good agricultural and veterinary Practices and also the Hazard Analysis and Critical Control Points (HACCP) system as a draft code of practice for preharvest and postharvest control of dairy cow's feed and in milk and dairy products processing is effective. The high detoxification rates by *Lactobacillus* and *Bifidobacterium* indicate potential for application in food and feed processing industries.

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