EFFECT OF MICROBIAL CONTAMINATION WITH FUNGI, AFLATOXIN M_1 AND ENTERIC GRAM NEGATIVE BACTERIA ON MILK AND SOME DAIRY PRODUCTS

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Abstract

Milk and milk products are excellent high quality foods, providing nutritional values through important elements in the healthy human diet. In this study, 75 samples of dried milk, raw milk, butter, cream and cooked (processed) cheese (15 of each) were collected randomly from various dairy shops and supermarkets in Giza Governorate, and tested microbiologically for fungal and enteric gram negative bacterial contamination; detection of AFM_1 residues using ELISA technique as well as detection of lipolytic and proteolytic activities of the most isolated fungi and bacteria. Six genera of moulds were recovered from the examined samples and three genera of yeasts. The most isolated moulds were species of genera *Penicillium* followed by *Aspergillus, Cladosporium, Geotrichum, Mucor* and *Scopulariopsis,* while the most isolated yeasts were species of genera *Candida* followed by *Rhodotorula* and *Saccharomyces.* AFM_1 levels were detected in all analyzed samples of raw milk and milk products. AFM_1 levels exceed EU legal limits (50 ppt) (0.05 µg/L/kg) while the detected limits were below the international legal limits of USA (FDA) (500 ppt) (0.50 µg/L/kg) in raw milk and dairy products for human consumption. The highest lipolytic and proteolytic activities were detected in *A. niger* and *Mucor* spp. (100%) while *Cladosporium* spp. possessed the lowest activities (50%), *Candida albicans* had activities (80%) and *Rhodotorula* had activities (62.5% and 75%). *Pseudomonas* spp. isolates were examined for proteolytic and lipolytic activities; three isolates had lipolytic activity (27.27%), also three isolates had proteolytic activity (27.27%). On the other hand, the most isolated enteric gram negative bacteria from the examined samples were identified as *E. coli* followed by species of *Psudomonas, Klebsiella, Shigella* and *Salmonella*. Moreover, no any bacteria were isolated from butter and dry milk samples. The economic importance and public health significance to the present results as well as apply the proposed sanitary measures to reduce microbial contamination and food safety for human health were discussed.

Key Words: Fungi, ATM_1, gram negative bacteria, milk products, proteolytic and lipolytic activities, ELISA.

INTRODUCTION

Milk and dairy products contains carbohydrates, protein, fats, vitamins, mineral elements and water. Milk is the most important source of calcium and phosphorus for human body and having essential amino acids (Huth *et al.,* 2006).

However, raw milk and milk products can pose a serious public health risks due to their contamination with many food borne pathogens (FDA, 2013). These pathogenic bacteria and fungi can originate from dairy animal farms through contaminated water, utensils used for collection, storage and transportation of milk. Contamination of raw milk and dairy products takes place from several types of M.O. which originate from the soil, water, skin and hair of the animals or from milk handlers (Lendenbach and Marshal, 2009).

Milk can be fermented by bacteria, yeasts and filamentous fungi to produce a variety of products such as cheese, butter and yoghurt. The main source of microorganisms of butter is cream, whether sweet or sour as well as, raw or pasteurized (Jay J., 1996). Yeasts and moulds are important spoilage microorganisms of butter and could be resulted in a surface discoloration and off-flavor (ICMSF, 2005). Cooked butter is one of the most popular types of fat consumed in Egyptian houses, which is made from milk or cream or both. It is eaten as table butter, used as oil for food preparation or for cooking, and of high nutritive value, but if contaminated, it could constitute a public health hazards besides economic losses. Moulds and yeasts in butter and cheese are growing at a wide range of a temperature and pH values, resulting in spoilage of the product (Pitt and Hocking, 1999). Cheese is a milk concentrate that consists mainly of protein (casein), fat and contains all essential fatty and amino acids. Also it is a source of vitamins and minerals and considered one of the most important consumed foods in Egypt. Spoilage of cheeses by yeasts appeared as visible growth of yeast colonies on the surface of cheese, poor appearance, changes in color, smell or taste and texture (Effat, 2000). Dairy products as cheese and yoghurt are probably most often spoiled by mould growth during ripening, processing, after cutting and slicing and during storage in shops or at home and may constitute a public health hazards (Hassan and Hammad, 2001).

Aflatoxin M_1 (AFM_1) is a highly toxic undesirable secondary metabolite produced mainly by *Aspergillus flavus* and *A. parasiticus* in milk and milk products, causing indirect milk contamination resulting from aflatoxin B_1 ingestion of mouldy feed that contains mycotoxins which pass into the milk such as aflatoxin M_1 by lactating cows (Tajkarimi *et al*., 2008) or direct mould growth on dairy products (spoilage agents) or due to the growth of moulds which secrete aflatoxins B_1, B_2, G_1

and G_2 (Sengun *et al*., 2008). Aflatoxin B_1 (AFB_1) present in feed of lactating animals transformed to 4- hepatic hydroxylated metabolites in liver and is excreted in milk as aflatoxin M_1 (AFM_1). Price *et al.* (1985) determined that 1.6% of ingested AFB_1 is converted to AFM_1, which could be detected in milk 12-24 hr_s after the first AFB_1 ingestion. AFM_1, is less carcinogenic, hepatoxic and mutagenic than AFB_1, the presence of AFM_1 in milk and milk products is considered to be undesirable (Elzupir and Elhussein 2010). AFM_1 is a very stable aflatoxin; it is resistant to thermal inactivation and not destroyed completely by pasteurization, autoclaving and heating processing (Sadeghi *et al*., 2009). The growth of fungi on a rich nutrient source as milk and milk products and production of mycotoxins by toxigenic fungi are favored under different environmental conditions including temperature, relative humidity, water activity, carbon dioxide, pH and oxygen concentration (Bullerman *et al*., 1984).

The most important mycotoxins occasionally found in milk and cheese products are aflatoxin M_1 and sterigmatocystin. Aflatoxin M_1 is the result of biotransformation of aflatoxin B_1 in cows, and sterigmatocystin is produced mainly by *Aspergillus versicolor, A. nidulans* (Van Egmond *et al*., 1997). Carcinogenic aflatoxin B_1 and ochratoxin A are not considered a problem in cow milk since it is cleaved in the rumen (Engel, 2000). Aflatoxin B_1 is the most known potent liver carcinogen (Pitt and Hocking, 1999). Worldwide, Aflatoxins are the most important mycotoxins in foodstuffs and they can produce acute and chronic toxicity in animals and humans.

In cheese, the most hazardous mycotoxins are OTA and AFM_1. Standards limits of Aflatoxins M_1 in many countries ranged between 0 to 0.5 ppb, in milk and dairy products. In cheese, aflatoxin M_1 (AFM_1) is the only mycotoxin for which maximum levels (0.05 and 0.5 ppb in the milk used for cheese-making in the EU; US and China, respectively). The European Union (EU) has the lowest maximum allowable level for AFM_1 in milk of 50 ng/L (Commission Regulation, 2006) while the level for AFM_1 in fluid milk in the United States (US) is tenfold higher at 500 ng/L (FDA, 2013).

Therefore, feeding on a low quality food contaminated with moulds more than 10^6 /g and kept under humid conditions cause a potential of mycotoxin production and may result in intoxication to both animals and human when consumed these animal products (Khalifa *et al*., 2013).

Moulds and yeasts might cause gas and off flavor in cheese and rancidity or other flavor defects in butter due to their proteolytic activity (Viljoen and Greyling, 1995).

The species *Geotrichum candinum* is one of the most undesirable contaminants of cheese, similar to *Listeria monocytogenes* (Hubecova *et al.,* 2009).

Pseudomonas spp. plays an important role in milk spoilage during the storage of raw milk they produce many thermo-tolerant lipolytic and proteolytic enzymes that reduce both the quality and shelf life of processed milk (Wiedmann *et al.,* 2000). Psychotrophic Gram negative bacteria may developed and resulted proteolytic (digest protein) and lipolytic (decomposing fats, damage to foods such as butter, raw milk, fish, meat and edible vegetable oils) changes (ICMSF, 2005).

The aim of this study was to examine fungal and enteric gram negative bacterial growth, measure aflatoxin M_1 existing in milk and some dairy products and detection the lipolytic and proteolytic activities of isolated fungi and enteric gram negative bacteria as well as the economic importance of the isolated organisms and their public health significance to assess health risk for consumers.

MATERIALS AND METHODS

1- Samples collection:

A total of 75 samples of dried milk, raw milk, butter, cream, and cooked (processed) cheese (15 of each) were randomly collected from different dairy shops and supermarkets in Giza Governorate, in clean, dry and sterile polyethylene plastic bags and containers and aseptically transported under refrigerated condition in ice box rapidly to the laboratory. Samples were maintained at 4 °C until analysis without any delay.

2- Isolation and Identification of fungi:

2- 1- Samples preparation for fungi:

Ten grams of dried milk samples and 10 millimeters of raw milk samples were transferred aseptically separately into a sterile blender jar, to which 90 ml of 1% sterile peptone water were added and homogenized in a sterile warring blender for 2 minutes. Ten grams from the centre of each cooked (processed) cheese sample were aseptically removed and homogenized with 90 ml sterile 0.2 % sodium citrate solution in a Stomacher bag (Lab-Blender 400, Seward, UAC House Friars Road, London SE19UG. Model No. 6021) at 1400 rpm for 2.5 min. Ten grams of each of the prepared butter and cream samples were transferred separately into a sterile flask containing 90 ml of warm sterile peptone water 1% (40±1°C). One ml from each original sample homogenate was added to a sterile test tube containing 9 ml 0.1% sterile peptone water to provide a dilution of 10^2. Similarly a tenfold serial dilution up to 10^3 was prepared (APHA, 2003).

From each previously prepared dilution, 1 ml was inoculated separately into sterile Petri dish plates and mixed with sterile SDA (Sabouraud's dextrose agar**)** and Dichloran Rose Bengal agar medium (containing antibiotic 0.05 mg of chloramphenicol

/ ml) and left to solidify at room temperature after mixing then incubated at 25°C for 5 - 7 days.

2- 2- Isolation and identification of moulds:

The mould isolates were sub-cultured onto malt extract agar and Czapek-Dox agar then incubated at 25 ^{O}C for 5-7 days. The isolated mould colonies were selected, purified, identified individually by macroscopic (based on colony morphology such as pigmentation, shape and coloration on the dorsal side) and microscopic characteristics under oil immersion. The isolated mould genera and species were identified according to Pitt and Hocking (1999).

2- 3- Isolation and identification of yeasts:

The isolated yeast colonies with yeast-specific morphology were identified using tests for growth on rice agar and SDA, formation of ascospores, vegetative reproduction, fermentation and assimilation of sugars, nitrates assimilation and Urease hydrolysis. The isolated yeast genera and species were identified according to Kreger-Van Rij (1984).

3- Isolation and Identification of Enteric gram negative bacteria:

It was done according to ICMSF (2005) and Quinn *et al.* (2002).

4- Serological Identification:

- For *Salmonella* spp*.* according to Minor and Popoff (2000) by using slide agglutination technique according to Kauffmann White Scheme.
- For *E.coli* according to Neville and Bryant (1986) by using slide agglutination technique.
- For *Pseudomonas* spp. according to Homma (1982) by using slide agglutination technique.

5- Lipolytic activity of isolated bacteria and fungi:

Tributyrin agar medium (Merck Darmstadt, Germany) was used according to Koburger (1972). Screening of lipase producers on agar plates is frequently done by using Tributyrin or Tween 80 as a substrate, (to detect bacterial lipase in a medium containing trioleoylglycerol and rhodamine B) was used according to Kouker and Jaeger (1987) and incubated at 37°C for 24-48 hr$_s$ for bacteria or (use antibiotic (0.05 mg chloramphenicol/ ml) and incubated at 25°C for 5 days for fungi). The zones of hydrolysis surrounded lipolytic colonies but medium appeared opaque. The lipolytic activity was determined by measuring size of zone around each bacterial or fungal colony (mm). The extent of activity was calculated as: (-) negative; (+) positive zone of 1 mm; (++) positive zone of 2 mm and (+++) positive zone of 3 mm or higher**.**

6- Proteolytic activity of isolated bacteria and fungi:

A casein substrate was used according to Koburger, J.A. (1972) and O'Reilly and Day (1983). The most isolated bacteria or fungi (mould or yeast) were separately inoculated on the surface of Skim Milk agar plates and were incubated at 37°C for 24-48 hr_s for bacteria or at 25°C for 5 days for fungi. The clear transparent zones of hydrolysis around bacterial or fungal colonies mean positive results (degradation of milk protein around bacterial or fungal colonies) leading to protease production.

7- Procedure of ELISA test:

Enzyme immunoassay for the quantitative analysis of AFM_1 in examined samples was performed by competitive ELISA test kit (including the calibration curve) (RIDASCREEN IMMUNOLAB AFM_1, Art No. R1111- R- Biopharm Gmb H, and Darmstadt, Germany) procedure as described by R- biopharm Gmb H (Anonymous, 1999).

7- 1- Preparation of samples for AFM_1 analysis:

Raw milk samples were skimmed following the test procedure or dried milk and used directly in the test while the solid samples, two grams of grinded and homogenized composite samples of butter or cream or cooked (processed) cheese were weighed and extracted with 8 ml dichloromethane by shaking for 30 min. on a heated shaker at 50 $^{\circ}C$. The following steps were done as RIDASCREEN instructions.

7- 2- Evaluation of AFM_1:

The absorbance values obtained for the standards and the samples were divided by the absorbance value of the first standard (zero standard) and multiplied by 100 (percentage maximum absorbance). The zero standards are equal to 100%, and the absorbance values were recorded in percentages. The values calculated for the standards were entered in a system of coordinates on graph paper against the AFM_1 concentration in ppt (Fig. 1).

The calibration curve and line equation were prepared, data were analyzed and results recorded.

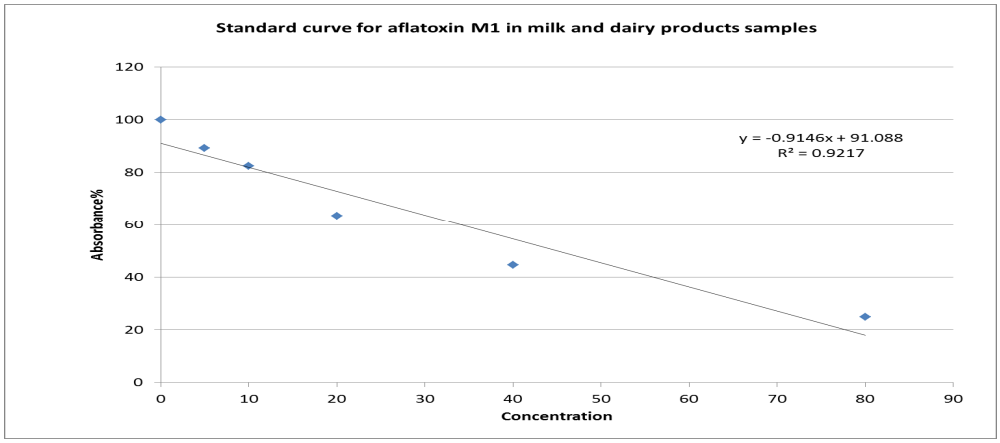

Fig. (1). Standard calibration curve of AFM_1.

RESULTS AND DISCUSSION

Table 1. Prevalence of isolated fungi from examined milk and some dairy products samples. (15 samples of each product were examined).

Isolated Fungi.	Positive Samples.	Types of examined samples.				
		Dried milk.	Raw milk.	Butter.	Cream.	Cooked (processed) cheese.
Moulds.	No.	1	11	4	5	15
	%	6.66	73.33	26.66	33.33	100
Yeasts.	No.	2	9	9	3	15
	%	13.33	60	60	20	100

(Percentages were calculated according to the number of examined samples).

Table (1) showed that the most contaminated examined samples with moulds were cooked (processed) cheese (100%) followed by raw milk (73.33%), cream (33.33%), butter (26.66%) and finally dried milk (6.66%) were the lowest contaminated samples. Moreover, the most contaminated examined sample with yeasts was cooked (processed) cheese (100 %) followed by raw milk and butter (60 % for each), cream (20 %) and dried milk (13.33 %).

These results agreed with El-Kest, *et al.* (2015) who revealed that moulds were isolated from 76.6 % of the examined raw milk samples, while no moulds were isolated from UHT milk samples. The authors could isolate moulds from 90 %, 90 %, 75%, 65%, 40% and 0% of the processed cheese, kariesh, mozzarella, akawi, roumy and yoghurt samples, respectively.

Table 2. Incidence of identified genera of isolated mould species from examined milk and some dairy products samples. (15 samples of each product were examined).

Identified genera of isolated mould spp.	Types of examined samples.									
	Dried milk.		Raw milk.		Butter.		Cream.		Cooked (processed) cheese.	
	Positive samples.									
	No.	%	No.	%	No.	%	No.	%	No.	%
Aspergillus spp.:	1	6.66	4	26.66	2	13.33	1	6.66	3	20
- A.flavus.	1	6.66	1	6.66	1	6.66	1	6.66	1	6.66
- A.niger.	0	0	1	6.66	1	6.66	0	0	1	6.66
- A.parasiticus.	0	0	1	6.66	0	0	0	0	1	6.66
- A.versicolor.	0	0	1	6.66	0	0	0	0	0	0
Pencilluim spp.:	0.	0	4	26.66	1	6.66	1	6.66	6	40
-P.chrysogenum	0	0	0	0	0	0	1	6.66	2	13.33
- P.citrinum.	0	0	1	6.66	0	0	0	0	2	13.33
- P.solitum.	0	0	0	0	0	0	0	0	2	13.33
-P.verrucosum.	0	0	3	20	1	6.66	0	0	0	0
Cladosporium spp	0	0	3	20	0	0	0	0	1	6.66
Geotrichum spp.	0	0	0	0	0	0	1	6.66	1	6.66
Mucor spp.	0	0	0	0	0	0	0	0	2	13.33
Scopulariopsis spp	0	0	0	0	1	6.66	2	13.33	2	13.33

(Percentages were calculated according to the number of examined samples).

Table (2) showed that 6 genera of moulds were recovered from the examined samples, of which *Penicillium* species were the most prevalent moulds in the examined samples (40% and 26.66%) from cooked (processed) cheese and raw milk, respectively followed by *Aspergillus* species (26.66% and 20%) from raw milk and cooked (processed) cheese, respectively. *P. verrucosum* was the most common mould, isolated from raw milk (20%); *P. chrysogenum, P. citrinum* and *P. solitum* (13.33% for each) from cooked (processed) cheese. The species of *A. flavus* was recovered (6.66 %) from all kinds of the examined samples. *A. niger, A. parasiticus* and *A. versicolor* were also recovered at different frequencies. The other mould genera were also recovered but at lower frequencies namely, *Cladosporium*, *Geotrichum, Mucor* and *Scopulariopsis* spp.

These results agreed with El-Diasty and El-Kaseh (2009) reported that 80% of the raw milk samples in Libya were contaminated with moulds, with an average quantity of 4.3×10^5 cfu/ml, whereas 50% of ready yogurt batches were contaminated with 2.1×10^4 cfu/ml, with predominant species of the *Aspergillus, Penicillium, Cladosporium, Mucor* and *Geotrichum* genera**.** El-Diasty and Salem (2009) reported that the predominant species of moulds isolated from table butter, cooking butter and kareish cheese were *Aspergillus niger , A. flavus , Geotrichum* spp. and *Mucor* spp.

were isolated from the examined samples at varying percentages ranged from (8.3-41.7). ELBagory *et al*. (2014) who revealed that, the moulds could be detected in all examined samples of Tallaga, Kareish, processed and Ras cheese (94.3, 100, 77.2 and 82.9 %), respectively. The isolated moulds were species of genera *Aspergillus, Penicillium, Cladosporium, Mucor* and *Rhizopus.*

Minervini *et al.* (2001) who reported that growth of *Penicillium, Cladosporium, Aspergillus* and *Mucor* species may responsible for discoloration, off flavor, bitterness and rancidity of cheese. *Penicillium* species may lead to softness the surface of cheese. Chipilev *et al*. (2016) who mentioned that isolated moulds from raw milk and white brined cheese were belonged to the genera *Aspergillus*, *Geotrichum*, *Mucor*, *Cladosporium* and *Penicillium*. Nilesen *et al.* (1998) who reported that some species of *Aspergillus, Cladosporium, Fusarium* and *Penicillium* were responsible for kerato-conjunctivitis in man, while *Aspergillus niger* causes otomycosis and allergic, some species of *Penicillium* causes pulmonary infections, urinary tract infections and yellow rice disease and may lead to death in man**.**

Table 3. Incidence of identified genera of isolated yeast species from examined milk and some dairy products samples. (15 samples of each product were examined).

Identified genera of isolated Yeast spp.	Types of examined samples.									
	Dried milk.		Raw milk.		Butter.		Cream.		Cooked (processed) cheese.	
	Positive samples.									
	No.	%	No.	%	No.	%	No.	%	No.	%
Candida spp.:	2	13.33	8	53.33	7	46.66	2	13.33	9	60
- C. albicans.	2	13.33	4	26.66	1	6.66	1	6.66	2	13.33
- C.lipolytica.	0	0	2	13.33	2	13.33	1	6.66	3	20
- C.parapsilosis.	0	0	1	6.66	2	13.33	0	0	3	20
- C. tropicalis.	0	0	1	6.66	2	13.33	0	0	1	6.66
Rhodoturala spp	0	0	1	6.66	2	13.33	1	6.66	4	26.66
Saccharomyces spp	0	0	0	0	0	0	0	0	2	13.33

(Percentages were calculated according to the number of examined samples).

Table (3) showed that 3 genera of yeasts were recovered from the examined samples. The most prevalent yeasts were belonged to members of genus *Candida* spp. which was recovered from cooked (processed) cheese, raw milk, butter, dried milk and cream samples at rates of (60 %, 53.33 %, 46.66 %, 13.33 % and 13.33 %) respectively. The species of *C. albicans* were recovered with (26.66 %) from raw milk, (13.33 %) from each of dried milk and cooked (processed) cheese and (6.66 %) from each of butter and cream. *C. lipolytica* was isolated (20 %) from cooked (processed) cheese, (13.33 %) for each of raw milk and butter and (6.66 %) from cream. Table

(3) also should that *C. parapsilosis* was recovered (20 %) from cooked (processed) cheese, (13.33 %) from butter, (6.66 %) from raw milk. *C. tropicalis* was recovered (13.33 %) from butter and (6.66 %) for each of raw milk and cooked (processed) cheese. Other genera of yeasts were *Rhodoturala* spp. isolated at the rates of (26.66 %, 13.33 %, 6.66% and 6.66 %) from cooked (processed) cheese, butter, cream and raw milk respectively, followed by *Saccharomyces* spp. (13.33 %) only from cooked (processed) cheese.

These results agreed with El-Diasty and Salem (2009) they found that the predominant species of yeasts isolated from table butter, cooking butter and kareish cheese were *Candida* spp., *Rhodotorula* spp., and *Saccharomyces* spp. ELBagory *et al*. (2014) revealed that, the yeasts could be detected in all examined samples of Tallaga, Kareish, processed and Ras cheese (68.6, 100, 20 and 48.6 %), respectively. The isolated yeast genera were *Candida* and *Rhodotorula.* Chipilev, *et al*. (2016) reported that the predominating yeasts in raw cow milk and white brined cheese were *Candida* spp., *Rhodotorula* spp. and *Sacharomyces* spp.

Some species of yeast especially some members of *Candida* constitute a public health hazard as they may be incriminated in case of pulmonary infection, urinary tract infection, endocarditis, eye infection, nail affection, thrush in mouth, gastrointestinal disturbance, vulvo-vaginitis, arthritis, osteomyelitis, dermatitis, meningitis and occasionally fatal systemic disease (Flee, 1990).

Table 4. Detection of AFM_1 residues (ppt) by ELISA in the examined milk and some dairy products samples.

Dried milk.			Raw milk.			Butter.			Cream.			Cooked (processed) cheese.		
No. + ve	%	AFM_1 (ppt)	No. + ve	%	AFM_1 (ppt)	No. + ve	%	AFM_1 (ppt)	No. + ve	%	AFM_1 (ppt)	No. + ve	%	AFM_1 (ppt)
1	20	81.5	2	40	85.0 82.5	1	20	79.8	1	20	79.5	2	40	82.1 81.8

(5 samples of each product were examined.) - (ppt= part per trillion.)

Table (4) illustrated that AFM_1 levels were detected in all kinds of examined milk and milk products at rates of (40 %) from each of raw milk and cooked (processed) cheese and (20 %) from each of dried milk, butter and cream. AFM_1 determined at levels of (85.0 ppt. and 82.5 ppt.) in raw milk, (82.1 ppt. and 81.8 ppt.) in cooked (processed) cheese, (81.5 ppt,) in dried milk, (79.8 ppt.) in butter and (79.5 ppt) in cream. Determination of AFM_1 residues was done using ELISA technique, which is an inexpensive, quick, reliable and highly valuable tool to monitor and ensure

food safety worldwide. AFM_1 levels exceeding the legal limits of (EU) European Union (50 ppt) (0.05 µg/L/kg) and below the legal limits of codex (US) United States (500 ppt) (0.50 µg/L/kg). In contrary, accepted by Codex Alimentarius Commission and National Agency for Food and Drug Administration (FDA) in raw milk and dairy products for human consumption (Kamkar, 2008).

These results agreed with (Hassan *et al.,* 2012) who recorded that most of detected levels of AFM_1 were over the permissible international levels. Meanwhile, 20% of yoghurt and 40% of each of kareish and Domietta cheese samples incriminated AFM_1 above FAO/WHO limits (0.05 µg/kg (50 ppt**))** (FAO, 1996), which caused them become hazardous for human health. El-Kest, M. *et al.* (2015) who found that the presence of AFM_1 was incriminated in 73% of raw milk and 75 % of processed cheese samples by average concentration of 200.25 ± 66.66 ppt, 5 samples (22.7%) which exceeded the maximum tolerance limit.

The presence of AFM_1 in milk is a major risk for humans, especially children, as it can have immunosuppressive, mutagenic, teratogenic, and carcinogenic effects (Sefidgar *et al*., 2011).

Table 5. Lipolytic activity of some fungi and *Pseudomonas* species isolates from examined milk and some dairy products samples.

Fungi and bacteria species.	No of tested isolates.	No. of +ve.	%	+++	++	+	-
*Moulds:							
A. flavus.	5	4	80	2	1	0	1
A. niger.	3	3	100	2	0	1	0
Penicillium spp.	12	8	66.66	6	0	1	1
Cladosporium spp	4	2	50	0	0	0	2
Mucor spp.	2	2	100	2	0	0	0
*Yeasts:							
Candida albicans	10	8	80	5	0	2	1
Rhodoturala spp	8	5	62.50	2	1	2	0
**Pseudomonas* spp	11	3	27.27	1	1	1	0

+++ Strong; ++ average; + weak; – no activity.

Table (5) showed that the highest lipolytic activity of moulds and yeasts isolated from milk and milk products were *A. niger* and *Mucor* spp. (100%), followed by *A, flavus* and *Candida albicans* (80 %), *Penicillium* spp. (66.66 %) and *Rhodoturala* spp. (62.50 %), while *Cladosporium* spp. presented the lowest activity (50%).

These results agreed with El-Diasty and Salem (2009) found that the strong lipolytic and proteolytic activities exhibited by the species in the genus *Aspergillus, Penicillium* and *Candida* spp. in raw milk and cheese, they added that, also *Geotrichum* spp., *Candida lipolytica* and *C. parapasillosis* had a lipolytic activity. El-

Shafei, H. (2004) stated the role played by moulds and yeasts in spoilage of foods and their ability to produce lipase enzymes which degrade fats. *Aspergillus* spp., *Penicillium* spp., *Candida albicans* and *Rhodotroula* spp. were having 100% lipolytic activity. Hassan *et al*. (2012) who reported that the lipolytic activity of fungal isolates revealed that *Candida albicans* which was isolated from milk products showed the highest lipolytic activity (100%), while, *Cladosporium* spp. presented the lowest activity (50%). Chipilev *et al*. (2016) stated that most of the isolated moulds strains exhibited pronounced lipolytic activity, which was most obvious of the genus *Geotrichum*, *Mucor* and some *Aspergillus* species. *Geotrichum* genus isolates exhibited strong lipolytic activity, followed by *Mucor* isolates, while *Cladosporium spp.* exhibited a complete lack of lipolytic activity.

Also, table (5) showed that the isolated *Pseudomonas* spp. strains were examined for lipolytic activity; three isolates had lipolytic activity (27.27%), this agreed with Dogan and Boor (2003) who found that most important lipolytic bacteria which degrade fats in cheese and milk are species of *Pseudomonas* which are psychrotrophic and produce heat stable lipases. *Pseudomonas* spp. have been implicated in the spoilage of processed milk kept under chilled condition because of their capacity to multiply under refrigeration with the production of thermostable lipases which plays an important role in milk spoilage (Rajmohan, 2002).

Table 6. Proleolytic activity of some fungi and *Pseudomonas* species isolates from examined milk and some dairy products samples.

Fungi and bacteria species.	No of tested isolates.	No. of +ve.	%	+++	++	+	-
*Moulds:							
A. flavus.	5	3	60	2	1	0	0
A. niger.	3	3	100	1	1	1	0
Penicillium spp.	12	8	66.66	2	3	2	1
Cladosporium spp	4	2	50	2	0	0	0
Mucor spp.	2	2	100	0	0	1	1
*Yeasts:							
Candida albicans	10	8	80	2	0	0	6
Rhodoturala spp	8	6	75	4	2	0	0
**Pseudomonas* spp	11	3	27.27	1	1	1	0

+++ Strong; ++ average; + weak; – no activity.

Table (6) showed that the highest proteolytic activity which was isolated from milk and milk products were *A. niger* and *Mucor* spp. (100%), followed by *Candida albicans* (80%), *Rhodoturala* spp. (75 %), *Penicillium* spp. (66.66 %) and *A.flavus* (60 %), while *Cladosporium* spp. presented the lowest activity (50%).

These results agreed with El-Shafei, H. (2004) stated the role played by moulds and yeasts in foods spoilage of and their ability to produce protease enzymes and their ability to degrade protein. *Aspergillus* spp. and *Penicillium* spp. found to have 100% proteolytic activity, while *Candida albicans* and *Rhodotroula* spp. were having 50 and 80% proteolytic activity, respectively. El-Diasty and Salem (2009) showed that most isolates of *A. flavus, A. niger, Cladosporium spp., Mucor* spp. and *Penicillium* did exhibit a proteolytic activity with different strength. Hassan *et al*. (2012) reported that the majority of the tested fungi were producer for proteases and most proteolytic fungi were *A. niger* (100%) and *A. flavus* (83.3%), followed by *A. ochraceus* (80%) and *Penicillium* spp. (83.3%).

Also, table (6) showed that the isolated *Pseudomonas* spp. strains were examined for their proteolytic activity; three isolates had proteolytic activity (27.27%), this agreed with Dogan and Boor (2003), proteolytic bacteria which degrade, digest protein and cause bitterness and putrefaction. *Pseudomonas* spp. have been implicated in the spoilage of processed milk kept under chilled condition because of their capacity to multiply under refrigeration with the production of thermostable proteases which plays an important role in milk spoilage (Rajmohan, 2002). *Pseudomonas* spp. produces extracellular toxins, which include pigments, proteolytic enzymes (Baglinière *et al.,* 2013), phospholipase and enterotoxins. Exotoxins are responsible for *Pseudomonas* spp. pathogenicity because it can produce leucopoenia, necrosis of liver, pulmonary edema, hemorrhage and kidney tubular necrosis. The enterotoxin produced is responsible for diarrhea.

Table 7. Incidence of Enteric gram negative bacteria isolated from examined milk and some dairy products samples. (15 samples of each product were examined).

Types of examined samples.	*Enterobacteriaceae*								*Pseudomonas* spp.		Total bacterial contaminants	
	E. coli		*Shigella* spp.		*Salmonella* spp.		*Klebsiella* spp.					
	No	%	No	%	No	%	No	%	No	%	No	%
Raw milk	4	26.66	1	6.66	0	0	1	6.66	5	33.33	11	73.33
Raw cream	2	13.33	0	0	0	0	0	0	1	6.66	3	20
Cooked Cheese	6	40	1	6.66	1	6.66	2	13.33	5	33.33	15	100
Butter	0	0	0	0	0	0	0	0	0	0	0	0
Dried milk	0	0	0	0	0	0	0	0	0	0	0	0
Total (75)	12	16	2	2.66	1	1.33	3	4	11	14.66	29	38.66

Results in table (7) showed that positive samples constituted 38.66% from the total samples. High percent of positive samples occur in cooked (processed) cheese and raw milk (100% & 73.33%) where no isolates in butter and dried milk. *E. coli, Shigella, Klebsiella* and *Pseudomonas* spp. could be recovered from raw milk with 26.66%, 6.66%, 6.66% and 33.33% respectively, while *Salmonella* spp. could not be detected in their products.

The obtained results are nearly similar to that obtained by Zelalem *et al.* (2006). For raw cream, in which *E. coli* and *pseudomonas* spp. could be recovered from 13.33% and 6.66% of examined samples, respectively. These results are nearly similar to those obtained by El-Kosi (2001). But in cooked (processed) cheese, *E. coli, Shigella, Salmonella, Klebsiella* and *Psudomonas* could be isolated by 40%, 6.66%, 6.66%, 13.33% and 33.33% respectively. Also table (6) showed that butter and dried milk samples were free from any organisms under test, this may be attriputed to heat treatment and processing. This high level of contamination of milk and milk products might be due to initial contamination originating from the udder surface, washed water, milking utensils and materials used for filtering the milk (Zelalem *et al.,* 2006). Provision of milk and milk products of good hygienic quality is desirable for consumer health point of view.

Table 8. Serological identification of *Salmonella* strains isolated from examined cooked cheese samples.

Salmonella serovars	Antigenic structuar		Cooked cheese	
	O	H	No.	%
S. typhimurium.	1, 4, 5, 12	i:1, 2	1	6.66

(O=Somatic.-H=Flagellar.) - (15 samples were examined.)

Table (8) showed that serological identification of *Salmonella* strain isolated by one strain from cooked cheese samples was identified as *Salmonella Typhimurium*. This organism is the most important which causes food poisoning outbreaks (WHO, 1997). The presence of *Salmonella* as enteropathogens in milk and milk products is due to unsainatory condition and unhygienic measures in all production line of milk lead to contamination of milk products.

Table 9. Serological identification of *E. coli* strains isolated from examined milk and some dairy products samples.

Strain of *E.coli*	Raw milk		Raw cream		Cooked cheese		Butter		Dried milk	
	No.	%	No.	%	No.	%	No.	%	No.	%
O_{26}	1	6.66	0	0	1	6.66	0	0	0	0
O_{55}	1	6.66	1	6.66	1	6.66	0	0	0	0
O_{111}	1	6.66	0	0	0	0	0	0	0	0
O_{157}	1	6.66	0	0	1	6.66	0	0	0	0
Untyped	0	0	1	6.66	3	20	0	0	0	0
Total	4	26.66	2	13.33	6	40	0	0	0	0

Table (9) showed the serological identification of *E. coli* isolates from milk samples which revealed that (4) different (0) Serogroups which were**,** O26 (2), O55 (3), O111 (1) and, O157 (2). While (4) strain were untyped. The presence of different strain of *E. coli* give an indication of pollution and contamination of milk and milk products which lead to gastroenteritis and food poisoning in human (Galal, 2013).

CONCLUSION AND RECOMMENDATION

1- Microbial contamination is reduced by clipping the cow, especially the flanks and udder, grooming the cow, and washing the udder with water and soap or a germicidal solution before milking.

2- Fresh cow milk has the highest nutritive values but it shouldn't be consumed in its raw state and should be boiled well before consumption to eliminate most microorganisms to avoid diseased conditions.

3- Butter should not be manufactured from raw cream or, it should be used only for cooking where it will receive adequate heat treatment.

4- It is an important to prevent mould growth to avoid toxin production indirectly via control of livestock feed hygiene during farming and crop production in farms and through preventing the natural contamination of raw materials.

5- Application of good Agricultural and Veterinary practices for pre and post harvest of dairy cow's feed.

6- Storage of food under correct conditions which prevent mould and bacterial growth and strict hygienic measures and regulations should be done during processing, good additives used, packaging and transportation.

7- Water supply should be clean, control of environmental contamination and packaging materials, good cleaning and sanitization of all food contact processing, surfaces and hygienic training of plant workers should be applied to avoid contamination. Hazard Analysis and Critical Control Points (HACCP) system application is essential to produce safe and high quality processed dairy products.

8- Products are kept at refrigeration temperature under good hygiene conditions.

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تأثير التلوث الميكروبى بالفطريات والأفلاتوكسين م١ والبكتريا المعوية سالبة الجرام على الحليب وبعض منتجات الألبان

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يعتبر اللبن الحليب ومنتجات الألبان من الأغذية عالية الجودة الممتازة، ويوفر القيم الغذائية لإحتوائه على العناصر الهامة في النظام الغذائي الصحي للإنسان. فى هذه الدراسة تم جمع عدد ٧٥ عينة لبن مجفف، حليب خام، زبدة، قشدة، جبن مطبوخ (مصنع) (١٥ عينة من كل منها) عشوائيا من محلات الألبان المختلفة والسوبر ماركت فى محافظة الجيزة. تم إختبار العينات للتلوث الميكروبى بالفطريات والبكتيريا المعوية السالبة الجرام، والكشف عن بقايا سموم الأفلاتوكسين م١ بإستخدام تقنية الإليزا. تم الكشف عن النشاط الإنزيمى المحلل للدهون والبروتينات للفطريات والبكتيريا المعوية الأكثر عزلا. تم عزل ٦ أجناس من الأعفان من العينات التى تم فحصها و ٣ أجناس من الخمائر. كانت أكثر الأعفان المعزولة سلالات من أجناس البنيسيليوم تليها الأسبريجيللس ثم الكلادوسبوريوم والجيوتريكم والميوكر والسكوبيولاريوبسيس، في حين أن الخمائر الأكثر عزلا كانت سلالات من أجناس الكانديدا تليها الرودوتوريلا ثم السكاروميسيس. تم إكتشاف تواجد الأفلاتوكسين م١ فى مختلف أنواع عينات الألبان ومنتجاتها التى تم فحصها. كان مستوى الأفلاتوكسين م١ أعلى من النسب القانونية المسموح بها حسب الإتحاد الأوروبى (٥٠ جزء فى التريليون) (٠,٠٥ ميكروجرام/كجم/لتر) وأقل من النسب القانونية الدولية المسموح بها حسب الولايات المتحدة الأمريكية ومنظمة الغذاء والدواء (٥٠٠ جزء فى التريليون) (٠,٥ ميكروجرام/كجم/لتر) باللبن الخام ومنتجات الألبان بالنسبة للإستهلاك الأدمى. تم الكشف عن أعلى نشاط إنزيمى محلل للدهون والبروتينات بالنسبة للأعفان الأسبريجيللس نيجر وسلالة الميوكر (١٠٠%) بينما كانت سلالة الكلادوسبوريوم الأقل نشاطا (٥٠%)، فى حين كانت خمائر الكانديدا ألبيكانز لها نفس النشاط الإنزيمى (٨٠%) ، وسلالة الرودوتوريلا لها نفس النشاط الإنزيمى (٦٢,٥% , ٧٥%). أما النشاط الإنزيمى للبكتيريا المعوية السالبة الجرام المعزولة من العينات التى تم فحصها كانت لسلالة السودوموناس ٣ معزولات محللة للدهون (٢٧,٢٧%) وأيضا ٣ معزولات محللة للبروتينات (٢٧,٢٧%). من ناحية أخرى كانت البكتيريا المعوية سالبة الجرام المعزولة من العينات التى تم فحصها هى إيشيريشيا كولاى ويليها سلالات السودوموناس ثم الكليبسيلا والشيجيلا والسالمونيلا. لم يتم عزل أى بكتيريا من عينات الزبدة واللبن المجفف. نوقشت الأهمية الإقتصادية والصحة العامة للنتائج الحالية وكذلك تطبيق الإشتراطات الصحية المقترحة لتقليل التلوث الميكروبى وسلامة الغذاء لصحة الإنسان.