Health hazards of Aflatoxin and virulent genes of *Staph aureus* enterotoxins in yoghurt

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ABSTRACT

Eighty random samples of plain and flavored yoghurt (40of each) were collected from different supermarkets in Menoufia Governorate. The collected samples were transferred immediately to the laboratory in an icebox for mycological, bacteriological examination, estimation of aflatoxins produced by certain species of moulds and enterotoxins types A and D produced by *Staph. aureus.* Mould was detected in (82.5%) of plain yoghurt samples and (97.5%) of flavored yoghurt samples. *Aspergillus* and *Pencillium* genera were frequently detected than other genera of fungi. Aspergillus spp. were the most dominant in the contaminated samples, (40%) in both plain and flavored yoghurt followed by *Pencillium spp (*30%) of plain yoghurt and (25%) of flavored yoghurt. *Aspergillus spp. A. flavus* and *A. niger* were the major isolates. *A. flavus* was isolated from plain and flavored yoghurt with apercentages of 50% and 62.5%, respectively. While, *A. niger* was (25% and 12.5%) from plain and flavored yoghurt, respectively. The four samples of plain yoghurt contaminated by *A. flavus* were analyzed for AFM1. They were positive in range from 0.049-0.472 ppb, while other four samples were free. Moreover, flavored yoghurt showed five samples contaminated with AFM1 in concentration of 0.061-0.586 ppb, while, other five samples were negative. The incidence of *Staph. aureus* in the collected plain and flavored yoghurt samples was 20% and 25%, respectively .The average count of *Staph. aureus* in the examined plain and flavored yoghurt samples was $1.20x10^3 \pm 0.15x10^3$ and $1.53x10^3 \pm 0.22x10^3$ cfu/g, respectively. The isolated *Staph. aureus* from plain and flavored yoghurt produced SEA and SED enterotoxins with a percentage of (12.5 % , 12.5 % , 10 % and 10 %), respectively.

Keywords: Public health, Aflatoxin M1, *Staph. Aureus* enterotoxins

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1. INTRODUCTION

Plain yoghurt is the traditional type of yoghurt, while flavored yoghurt is made by addition of flavoring and sweeting agents. Yoghurt is a popular dairy product because of its beneficial influence on human health and nutritional value (Tabari et al., 2011). Aflatoxins (AFS) are secondary metabolites of fungi especially *Aspergillus flavus* and *Aspergillus parasiticus*. These moulds are common contaminants of food stuffs, particularly in the tropical regions (Gourama and Bullerman, 1995).Aflatoxin is known to be human carcinogens based on sufficient evidence of carcinogenicity in human (IARC, 1987, 1993 and Yaling et al., 2008). Aflatoxin M1 (AFM1) may occur in milk and milk products resulted from the ingestion of Aflatoxin B1 in feed stuffs by dairy cow (Martins and Martins 2004). High performance liquid chromatography (HPLC), the analytical method employed for Aflatoxins determination is rapid, easily automatable and therefore useful for accurate screening of Aflatoxin M1 in yoghurt (Tabari et al., 2011). In the same manner, *Staph. aureus* is one of the most common food poisoning organism in food .The symptoms of staphylococcal food poisoning have a rapid onset (1-6 hr) and often include nausea, vomiting, diarrhea

and abdominal pain (Jablonski and Bohach, 1997). *Staph. aureus* may contaminate the dairy products even in low number and this constitutes a public health hazard. The presence of *Staph. aureus* in milk products may be due to unhygienic handling practices, inadequate heat treatment, or faulty storage and transportation. *Staph. aureus*, under unfavorable condition, may lose its viability in food but might secrete enterotoxins and cause food poisoning (Erkmen, 1995). According to the biological safety, *Staph. aureus* is classified into risk group 2 (Human Pathogens and Toxins, 2009). That require containment lab Level 2 facilities. The enterotoxins are exoprotein, which are heat stable. Initially SEA, SEB, SEC1, SEC3, SED, and SEE were characterized (Bergdoll et al., 1973). The infective dose required to induce staphylococcal food poisoning (SFP) in humans is estimated to be around 0.1 µg and it may vary with patient sensitivity (Evenson et al., 1988). The enterotoxin A is of human origin. It may contaminate milk and dairy products during different stages of production and processing or even at consumer outlet (El-Baradie, 1993). In addition, the presence of enterotoxin D can be attributed to the increased incidence of staphylococcal mastitis where strains of *Staph. aureus* were isolated from bovine mastitis and were designated as animal strains (Masud et al.,1993). Therefore, the present study was conducted to investigate the occurrence of fungi, estimation of aflatoxins produced by certain species of isolated moulds, isolation of *Staph. Aureus* and detection of virulent gene responsible for enterotoxins A and D production in plain and flavored yoghurt marketed in El-Menoufia Governorate, Egypt.

2. MATERIAL AND METHODS

2.1. Collection of samples:

Eighty random samples of plain and flavored yoghurt (40 of each) were collected in their original cups from some local supermarkets in Menoufia Governorate. Collected samples were transferred immediately to the laboratory in an ice box for mycological, bacteriological examination, estimation of aflatoxins produced by certain species of moulds, *Staph. aureus* count and detection of virulent genes responsible for enterotoxins A and D production.

2.2. Preparation of collected samples were carried out according to (APHA, 1992).

A. Mycological examination:

Isolation of mould according to APHA (1992). Identification of isolated moulds according to Koneman and Roberts (1985). Aflatoxin analysis using high performance liquid chromatography (HPLC) (Technologies, Inc. 200 Regency Forset Drive, suite 330 Cary, NC 27511, USA) at Fac. Vet. Med., Benha Univ. according to AOAC (2000).

B. Bacteriological examination:

Preparation of samples and serial dilution were done for each collected samples based on the recommendation of (APHA,1992). Enumeration and identification of *Staph aureus* according to FDA (2001).

D. Detection of genes responsible for Staph. aureus enterotoxins production:

It was made by using conventional (cPCR). PCR Master Mix used for cPCR. Oligonucleotide primers used in cPCR two pairs of primers were supplied from metabion (Germany) or Biobasic (Canada). They have specific sequence and amplify specific products. DNA Molecular weight marker. Gel Pilot 100 bp ladder (cat. no. 239035) supplied from QIAGEN. Number of bands: 6 Size range: 100-600 bp. Gel Pilot 100 bp plus ladder (cat. no. 239045) supplied from QIAGEN (USA). Number of bands: 11 Size range: 100-1500 bp. Material used for agarose gel electrophoresis. Agarose 1.5% (Sambrook *et al.,* 1989). Ethedium bromide solution 10 mg / ml (Sambrook et al., 1989). Tris borate EDTA (TBE) electrophoresis buffer

(1x) (WHO, 2002). Preparation of multiplex PCR Master Mix for *Sea, Seb, Sec* and *See* genes according to Emerald Amp GT PCR master mix (Takara) Code No. RR310A. Agarose gel electrophoreses (Sambrook *et al*., 1989) with modification.

3. Results

In this study table (2) showed the moulds in examined samples of yoghurt. They were highly recognized in 33 samples (82.5%) of plain yoghurt and 39 samples (97.5%) of flavored yoghurt, respectively. From the results obtained in Table (3), Aspergillus spp. were the most dominant in the contaminated samples (40%) in both plain and flavored yoghurt followed by pencillium spp. (30%) of plain yoghurt and (25%) of flavored yoghurt,while for table (4) showed that among isolated Aspergillus spp. *A.flavus* and *A.niger* were the major isolates. *A. flavus* was (50% and 62.5%) from plain and flavored yoghurt, respectively and *A. niger* was (25% and 12.5%) from plain and flavored yoghurt. Results presented in Table (5) indicate that four samples of plain yoghurt which contaminated by *A.flavus* were analyzed for AFM1. They were positive in range from 0.049-0.472 ppb, while other four samples were free. While flavored yoghurt indicated, five samples were contaminated with AFM1 in concentration of 0.061-0.586 ppb while other five samples were negative. The samples which contaminated by *A. flavus* were screened by HPLC and were free of aflatoxin reflect that the presence of *A. flavus* does not mean the presence of mycotoxin. The results in this study failed to comply with EOSQ (2005) which stipulated that , yoghurt should be free from Pathogenic microorganisms as *A. flavus* and their aflatoxin which recorded in this study with a percentage of 40 % for plain yoghurt and flavored yoghurt, respectively. As a rejected samples. On the other hand the incidence of *Staph. aureus* in the collected plain and flavored yoghourt samples was 20% and 25%, respectively (Table 6) and the average count was $1.20 \times 10^3 \pm 0.15 \times 10^3$ and $1.53 \times 10^3 \pm 0.22 \times 10^3$ cfu/g, respectively. The isolated *Staph. aureus* from plain and flavored yoghourt produced SEA and SED enterotoxins with a percentage of (12.5 % , 12.5 % , 10 % and 10 %), respectively (Table7). It is shown that the percentage of the enterotoxins were similar in plain and flavored yoghourt. The results in this study showed that the incidence of *Staph. aureus* in the collected plain and flavored yoghourt samples was 20 % and 25 % , respectively, which failed to comply with EOSQ (1650 / 2005) for flavored yoghurt and (1000 / 2005) for plain yoghurt stipulated that, yoghurt should be free from pathogenic microorganisms and their enterotxins.

Table (1): Oligonucleotide primers sequences.

Gene	Primer	Primer sequence (5'-3')	Length of amplified product	Reference
Sea	GSEAF-1 GSEAR-2	GGTTATCAATGTGCGGGTGG CGGCACTTTTTTCTCTTCGG	102 bp	Mehrotra*et al*., 2000
Sed	GSEDF-1 GSEDR-2	CCAATAATAGGAGAAAATAAAAG ATTGGTATTTTTTTTCGTTC	278 bp	

Table (2): prevalence of moulds in examined yoghurt samples. Samples (n=40).

Number of examined samples	Positive samples		Negative samples	
	No.	%	No.	%
40	33	82.5	7	17.5
40	39	97.5	1	2.5

Table (3): Incidence of isolated mould in the examined yoghurt. Samples (n=40).

Isolated mould spp.	Plain yoghurt		Flavored yoghurt	
	No.	%	No.	%
Aspergillus spp.	16	40	16	40
Penicillium spp.	12	30	10	25
Mucor spp.	4	10	6	15
Thamnidium spp.	6	15	4	10
Cladosporium spp.	6	15	4	10
Fusarium spp.	2	5	4	10
Sporotrichum spp.	2	5	8	20

Table (4): Incidence of examined Aspergillus spp. in relation to the positive yoghurt samples.

Aspergillusspp	Plain yoghurt (16)		Flavored yoghurt(16)	
	No.	%	No.	%
A.flavus	8	50	10	62.5
A.fumigatus	2	12.5	2	12.5
A.niger	4	25	2	12.5
A.terreus	2	12.5	2	12.5

Table (5): Incidence and levels of (AFM1) residues in examined yoghurt samples.

Types of samples	No. of examined samples	Positive samples		Range (ppb)	Negative samples	
		No	%		No	%
Plain yoghurt	8	4	50	0.049-0.472	4	50
Flavored yoghurt	10	5	50	0.061-0.586	5	50

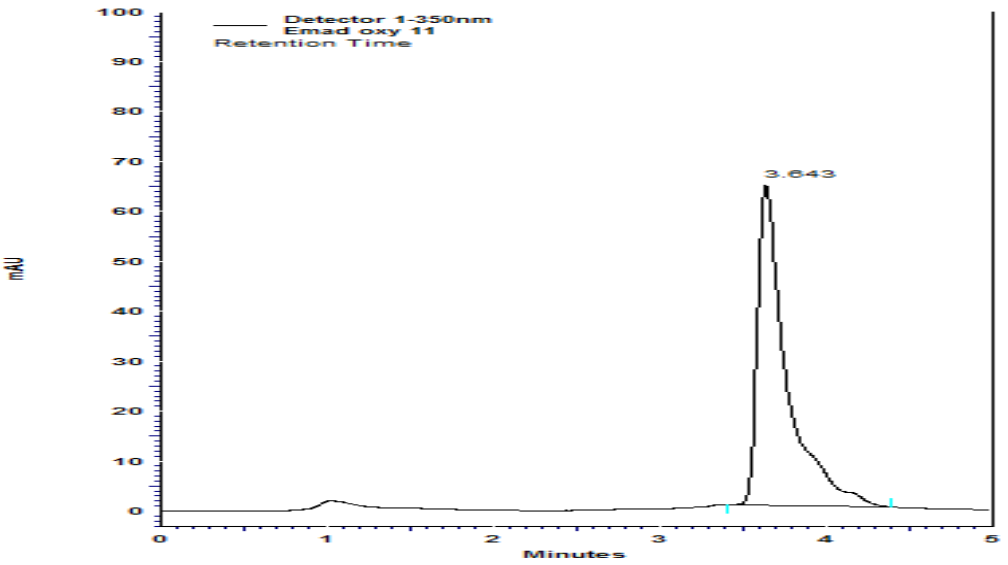


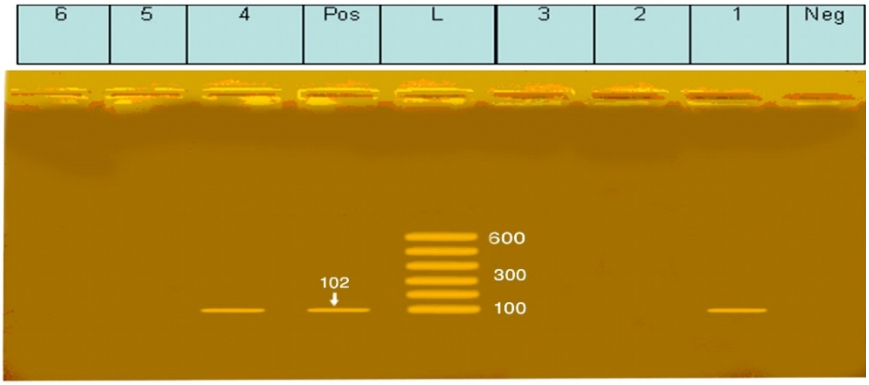
Figure (1): HPLC chromatogram of yoghurt extract for determination of aflatoxin M1 (AFM1)

Table (6): Prevalence and statistical analytical results of *Staph. aureus* count (cfu/g) of examined samples.

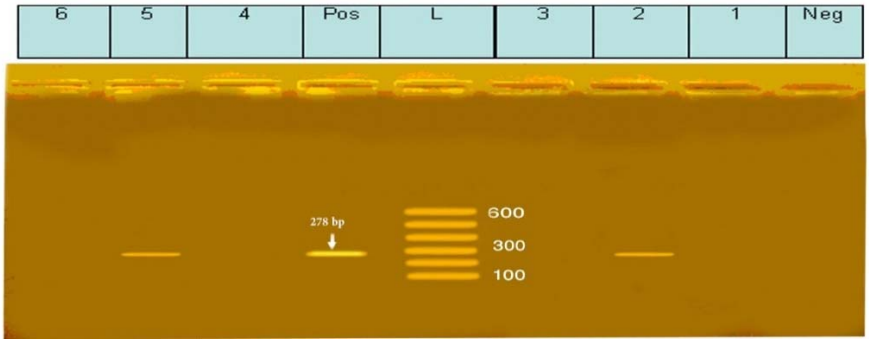
Samples type	No. of examined samples	Positive isolates		Mean ±SE
		No.	%	
Plain yoghurt	40	8	20	$1.20x10^3 \pm 0.15x10^3$
Flavored yoghurt	40	10	25	$1.53x10^3 \pm 0.22x10^3$

Table (7): Prevalence of virulent genes of isolated strains responsible for production of enterotoxins Types (A and D) in examined yoghurt samples.

Samples	No. Of tested strains	Enterotoxin A		Enterotoxin D	
		No.	%	No.	%
Plain yoghurt	8	1	12.5	1	12.5
Flavored yoghurt	10	1	10	1	10



Photograph (2): Agarose gel electrophoresis of cPCR amplified of enterotoxins SEA of *Staph. aureus*. The genomic DNA of 6 *Staph .aureus* tested using specific primers for the SEA gene was amplified in 1 (33.3%) *Staph. aureus* strains for each of plain and flavored yoghurt isolates and giving product at 102 bp. Samples (1, 2, 3): Plain yoghurt. Samples (4, 5, 6): flavored yoghurt. Lan M: 100-600 bp DNA Ladder. Pos: positive control (at 102bp). Neg: Negative control. Lane 1, 4: Staph. *aureus* toxin positive. Lane 2, 3, 5, 6: *Staph. aureus* toxin negative.



Photograph (3): Agarose gel electrophoresis of cPCR amplified of enterotoxins SED of *Staph. aureus*. The genomic DNA of 6 *Staph. Aureus* tested using specific primers for the SED gene was amplified in 1 (33.3%) *Staph. aureus* strains for each of plain and flavored yoghurt isolates and giving product at 278bp. Samples (1, 2, 3): Plain yoghurt. Samples (4, 5, 6): flavored yoghurt. Lan M: 100-600 bp DNA Ladder. Pos: positive control (at 278bp). Neg: Negative control. Lane 1, 4: *Staph. aureus* toxin positive. Lane 2, 3, 5, 6: *Staph. aureus* toxin negative.

4. DISCUSSION

Moulds yoghurt was high recognized in 33 of the examined samples plain yoghurt (82.5%) and 39 samples (97.5%) of flavored yoghurt (table 2). These, results were similar to those obtained by Saad et al., (1987), El-Badry (1998) and Bahout and Moustafa (2003). From the results obtained in Table (3). Aspergillus spp. were the most dominant in the contaminated samples (40%) in both plain and flavored yoghurt followed by pencillium spp. (30%) of plain yoghurt and (25%) of flavored yoghurt. These results are in agreement with that reported by Marwa et al., (2013) who revealed that Aspergillus, penicillium and mucor genera were frequently detected than other genera of fungi. Table (4) showed that among isolated Aspergillus spp. *A. flavus* and *A. niger* were the major isolates. *A. flavus* was (50% and 62.5%) from plain and flavored yoghurt, respectively and *A. niger* was (25% and 12.5%) from plain and flavored yoghurt. These finding were nearly similar to those obtained by Ghazal (2001), El-Asouty (2011) and Marwa et al., (2013). Results presented in Table (5) indicated that

four samples of plain yoghurt which contaminated by *A. flavus* were positive for AFM1. They were in range from 0.049-0.472 ppb, while other four samples were free. While flavored yoghurt indicated that five samples were contaminated with AFM1 in concentration of 0.061-0.586 ppb, while other five samples were negative. These results nearly similar with these findings reported by El-Diasty and Kaseh (2009) and Salwa (1999). The samples which contaminated by *A. flavus* were screened by HPLC and were free of aflatoxin reflect that the presence of *A. flavus* does not mean the presence of mycotoxin, that agreed with Paterson and Nelson (2010). The results in this study failed to comply with EOSQ (2005) which stipulated that, yoghurt should be free from Pathogenic microorganisms and their toxins as *A. flavus* and their aflatoxin which recorded in this study (8 and 10 samples)for plain yoghurt and flavored yoghurt with a percentage of 40 %, respectively. On the other hand, the incidence of *Staph. aureus* in the collected plain and flavored yoghurt samples was 20% and 25%, respectively (Table 6). The incidence of *Staph. Aureus* in yoghourt was nearly similar to those reported by Nashwa et al., (2010) and Gonui et al., (1996) but lower than those of Manal (2000), and the average count of *Staph. aureus* in the examined plain and flavored yoghurt samples was $1.20 \times 10^3 \pm 0.15 \times 10^3$and $1.53 \times 10^3 \pm 0.22 \times 10^3$ cfu/g, respectively. This result was nearly similar to Nashwa et al. (2010) lower counts than that reported by Ahmed (1999) and Manal (2000) but higher figures than that of Dereu et al., (2004) and El- Tahiri (2005). The number of samples, which failed to comply with EOSQ (1650 / 2005) for flavored yoghurt was (10 samples) and for plain yoghurt (1000/ 2005) was (8samples) as it stipulated that, yoghurt should be free from pathogenic microorganisms and their enterotxins. The contamination of yoghurt with *Staph. aureus* could be due to lower sanitation and mishandling together with increased incidence of pathogenic staphylococcus carriers among producers (Grieger et al., 1990). Some isolates of *Staph. aureus* may produce enterotoxins (SEs) that cause food poisoning if sufficient amount of SEs is ingested .The enterotoxgenic strain needs to grow to levels $>10^5$cfu/g. In addition, SE formation is influenced by some parameters such as temperature, pH, water activity, redox potential and bacterial antagonisms e.g. starter cultures used in the production of fermented milk products can prevent *Staph. aureus* growth and SEs production). The SEs contribute to bacterial virulence, cause emesis and may induce toxic shock (Dinges et al., 2000).The isolated *Staph. aureus* from plain and flavored yoghourt produced SEA and SED enterotoxins with a percentage of (12.5 % , 12.5 % , 10 % and 10 %), respectively (Table7).

5. CONCLUSION

In conclusion, the obtained results showed high contamination of examined yoghurt samples with different types of moulds, which constitute a public health hazards due to production of their aflatoxins. Sanitary precaution with handler, utensils and the surroundings during milking and processing is essential. Moreover, the validation and verification of HACCP based procedures through the use of microbiological criteria must be high –lightened.

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