A Survey on Selected Quality Parameters of Buffalo Milk Samples Collected from Consumer Markets of Three Different Central Governorates in Egypt

F.M.F. Elshaghabee[1], M.I. Abdel-Hamid[1], H.-G. Walte[2]

[1] Dairy Science Department, Faculty of Agriculture, Cairo University, 12613 Giza, Egypt
[2] Department of Safety and Quality of Milk and Fish, Max Rubner-Institut (Federal Research Institute of Nutrition and Food), Kiel, Germany

Date submitted: 09/04/2017 *Date accepted: 02/11/2017* *Volume/Page(s): 70/25-29*

Abstract

One hundred and twenty samples of raw buffalo milk were collected at consumer markets in central Cairo, Giza and Qualubya, Egypt. All samples were analysed chemically using Lactoscan, and microbiologically using the pour plate technique. Also, levels of aflatoxin (AF) M1 were assessed by using a commercial ELISA kit. The mean values were - 0.476±0.07 °C, 5.89±0.79 %, 4.13±0.53 %, 4.25±0.53 %, 0.69±0.14 %, and 14.59±1.39 % for freezing point, fat, protein, lactose, ash and total solids respectively. Bacteriological enumeration of total mesophilic aerobic bacteria, coliform, spore forming bacteria and psychrotrophic bacteria were 5.37±0.78, 3.77±0.90, 2.78±0.36 and 2.84±0.39 Log CFU/ml respectively. The median concentration of AF M1 in all samples was 44.31 ng/L. Our results indicate that there is an essential need for improving the hygienic conditions in the production of raw milk.

Key Words: Milk quality, safety, aflatoxin M1, codex, HACCP, ISO 22000

Introduction

Nutritionally, milk has been defined as "the most nearly perfect food", it is part of the daily diet and is important for growing children [1]. Moreover, the consumer demand for high quality milk and dairy products has a significant impact on all aspects of the dairy chain [2]. Buffalo milk receives an increasing research interest and investment in various countries because of its higher nutrient content compared to cow milk [3,4].

Freezing point, chemical composition (mainly fat, protein, lactose and total solids) and microbiological analysis of raw milk samples are important indicators of its quality. Measurement of the freezing point is used to detect any milk adulteration with water [5]. In Egypt, the average of the freezing point of buffalo milk samples ranges from -0.552 to -0.558 °C [6] and the regulatory standard ranges from -0.530 to -0.560 °C [7]. A freezing point of - 0.528 °C for raw buffalo milk has been found in Italy [8] and Poland [9].

As with any type of raw milk, different microbial groups (lactic acid bacteria, coliform, *Staphylococcus spp.*, *Listeria spp.*, yeast and moulds, etc.) can rapidly multiply because of its high nutrient content. At cold storage temperatures, an increase in growth and metabolic activities of psychrotrophic bacteria can be noticed. Some of them are able to produce heat resistant proteolytic and lipolytic enzymes that can survive during UHT processing [10-12] resulting in the deterioration of UHT milk.

Aflatoxins (AF) are toxic metabolites produced by two major fungi; *Aspergillus* (*A.*) *flavus* and *A. parasiticus* [13]. AFM1 is the type of aflatoxin that is present in milk and dairy products. Hydroxylation of AFB1 results in the formation of AFM1 which has been categorized as a class 1 human carcinogen [14]. The regulatory limits for AFM 1 vary in different countries; according to the regulations of the USA, levels of AFM1 in milk should not be higher than 500 ng/kg [15]. The ministry of health in Egypt established that milk should be free from AFM1 [16]. However, the European commission has stipulated that the maximum limit of AFM1 in liquid milk is 50 ng/kg, which is lower than the limit set by the Codex Alimentarius Commission [17] with 500 ng/kg [18].

In Egypt, Buffalo milk is produced at small-holder farms (unorganised) and mostly marketed without bactofugation, thermization or pasteurization treatment resulting in a significant decrease in the quality of raw market milk. Furthermore, there are few studies conducted on the quality of raw market Buffalo milk in the different regions of Egypt. Therefore, this study was undertaken to examine selected parameters of chemical and microbiological qualities as well as levels of AFM1 in raw milk samples taken at farmers' markets of three different central governorates (Cairo, Giza and Qualubya). The samples were collected from bulk tanks, in which the temperature of milk varied from 3 to 7 °C.

Considering the economic potential of buffalo milk and the growing awareness of Egyptian consumers regarding the importance of food safety, the results obtained will be useful in future studies dealing with the development of hygienic conditions in the production and marketing of buffalo milk.

Material and Methods

Sampling:

One hundred and twenty samples of raw buffalo milk were collected from markets at central locations of three different governorates (Cairo, Giza and Qualubya, 40 samples for each governorate) from November 2015 to February 2016 and stored at 4±1 °C until further analysis.

Physico-chemical analyses:

Raw milk samples were analysed for freezing point, total solids (T.S.), fat, protein and lactose using ultrasonic milk analyser (Lactoscan, Milkotronic Co., Nova Zagora, Bulgaria) in order to give us a quick analysis of the samples. Ash was measured according to Ling (1963) [19].

Microbiological analyses:

The pour plate technique with subsequent decimal dilutions in peptone saline (0.1 % neutral peptone and 0.9 % NaCl) as well as duplicate counting plates of appropriate dilutions were used for each sample. Standard plate count (32 °C /48 hours) was used to enumerate total bacterial count (TBC), MacConkey agar (37 °C /24 hours) was applied to enumerate coliform bacteria. Penicillin and pimaricin agar (PPA, 25 °C/48 hours) was used to enumerate psychrotrophic bacteria. All microbiological media were prepared according to the Oxoid manual [20], however, the PPA medium was purchased from Biolife Co., Milan, Italy.

Determination of aflatoxin M1 levels:

Twenty milliliters (20 ml) of liquid milk were centrifuged at 3500 xg / 7 °C. The fatty layer was removed and 100 µl of the skim milk was applied directly to the ELISA kit for AFM1 determination (R-Biopharm Co., RIDASCREEN® aflatoxin M1, Cat. No. R1121, Darmstadt, Germany). The limit of detection for milk samples was 5 ng/Kg.

Statistical analysis:

Microsoft Excel 2010 was used for data processing. Data are presented as mean ± standard deviation (SD) of two replicates for each sample. Results were subjected to one-way analysis of variance (ANOVA) through the general linear model (GLM) and multiple comparisons were performed using the Tukey test; the statistical significance was set at $P < 0.05$. All analyses were performed using SAS software version 9.4.

Results and Discussion

Physical and chemical analyses:

Results of the physical and chemical analyses of raw milk samples are presented in Table (1). The range, showing the minimum and maximum values, of the freezing point in raw market buffalo milk samples collected from central Cairo, Giza and Qualubya was - 0.25 to - 0.55, - 0.39 to - 0.55 and - 0.25 to - 0.55 °C respectively. The differences between the mean values of Giza and Qualubya were significant, but the decrease of mean values of the freezing point in all raw milk samples (average of 120 samples; -0.47 °C±0.07) of three different areas indicates that some of the raw milk samples were adulterated with water [5].

Data of the chemical composition (Table 1) of all samples were studied by variance analysis. A range of 4.18-7.20, 4.75-6.95 and 4.18-7.15% was found for fat, 2.85-4.93, 3.30-5.02 and 2.85-4.87 for protein, 2.55-4.87, 3.70-4.98 and 2.56-4.80 for lactose, 0.35-0.88, 0.49-0.89 and 0.37-0.84 for ash, 11.36-16.41, 12.18-16.44 and 11.38-16.41 for total solids contents in central of Cairo, Giza and Qualubya respectively. These results indicate that approx. 58% of the samples were not in accordance with Egyptian standards [7] for Buffalo milk (fat content is not less than 5.50 % and solids not fat (SNF) content is not less than 8.75 %), despite of the fact that the mean value or the average of all parameters were in accordance with the national standard as shown in the same Table. Our results are not in accordance with Najdenova *et al.* (2003) [21] and Supino *et al.*(2004) [22], because the average of fat content in buffalo milk was 7.5 %. Also, the mean concentrations from one hundred buffalo milk samples were 7.59±1.31 % for fat, 4.86±0.44 % for crude protein, 0.85±0.05 % for ash and 18.44 ±1.56 % for total solids [23]. There are several factors such as type of breed, lactation period, forage, feeding system, seasonal changes, milking frequency and milking methods which can impact the physicochemical parameters as described by Suman *et al.* (1998) [24].

Microbiological analyses:

The total bacteria counts (TBC), coliform, spore forming bacteria and psychrotrophic are presented in Table (2). A difference in the mean of the tested bacterial groups in all milk samples was noticed, which might be due to differences in the sanitation process of the different farmers' markets. Also, 36 % of samples had higher TBC than 6 Log CFU/mL. However, the average of TBC for all samples (5.37±0.78) was in accor-

Table 1: Physical and chemical properties of samples collected from local markets of three different central governorates in Egypt

Area	Parameter	FP °C	Fat	Protein	Lactose	Ash	Total Solids
Central Cairo (n=40)	Range	0.25-0.55	4.18-7.20	2.85-4.93	2.55-4.87	0.35-0.88	11.36-16.41
	Mean	0.46±0.01^{b}	5.83±0.90^{b}	4.10±0.59^{b}	4.21±0.61^{b}	0.67 ±0.15^{b}	14.54±1.56^{b}
Central Giza (n=40)	Range	0.39-0.55	4.75-6.95	3.30-5.02	3.70-4.98	0.49-0.89	12.82-16.44
	Mean	0.49 ±0.05^{a}	6.11±0.61^{a}	4.28±0.44^{a}	4.39±0.38^{a}	0.73±0.11^{a}	14.96±1.10^{a}
Central Qualubya (n=40)	Range	0.25-0.55	4.18-7.15	2.85-4.87	2.56-4.80	0.37-0.84	11.38-16.41
	Mean	0.45 ±0.08^{b}	5.74±0.82^{b}	4.01±0.53^{c}	4.16±0.56^{b}	0.66±0.14^{b}	14.29±1.43^{c}
Average (n=120)	Range	0.25-0.55	4.18-7.20	2.85-5.02	2.55-4.98	0.35-0.89	11.36-16.44
	Mean	0.47±0.07	5.89±0.79	4.13±0.53	4.25±0.53	0.69±0.14	14.59±1.39

Data are expressed as the mean ± standard deviation (SD), samples were analysed in duplicate. Means with different superscript letters differ significantly (p<0.05).

Table 2: Microbiological properties of samples collected from local markets of three different central governorates in Egypt

Area	Parameter	Total bacterial count	Coliform	Spore forming bacteria	Psychrotrophic bacteria
Central Cairo (n=40)	Range	4.15-6.59	2.15-4.68	2.25-3.43	2.14-3.51
	Mean	5.41±0.83[a]	3.85±0.86[a]	2.81±0.36[a]	2.88±0.40[a]
Central Giza (n=40)	Range	4.21-6.35	2.10-4.68	2.25-3.45	2.14-3.37
	Mean	5.22±0.10[b]	3.54±0.91[b]	2.71±0.33[b]	2.74±0.35[b]
Central Qualubya (n=40)	Range	4.15-6.59	2.15-4.68	2.25-3.43	2.12-3.48
	Mean	5.50±0.80[a]	3.92±0.88[a]	2.84±0.39[a]	2.89±0.41[a]
Average (n=120)	Range	4.15-6.59	2.10-4.68	2.25-3.45	2.12-3.51
	Mean	5.37±0.78	3.77±0.90	2.78±0.36	2.84±0.39

Data are expressed as the mean ± standard deviation (SD), samples were analysed in duplicate. Means with different superscript letters differ significantly (p<0.05).

dance with the Egyptian standards [7] for raw buffalo milk, being 5.70 Log CFU/mL. Results of TBC in raw buffalo milk samples indicate that there are inadequate sanitary conditions during production and marketing of raw buffalo milk. In Italy, the TBC of raw buffalo milk was 5.23 Log CFU/mL [22] and it was 5.59 Log CFU/mL in China [23]. However, the TBC average of buffalo milk samples in the Alexandria Governorate was 6.70 Log CFU/mL [25] and in the Menoufia Governorate, Egypt, 7.60 Log CFU/mL [26].

The occurrence of the Coliform group and *E. coli* in milk indicates poor hygiene or fecal contamination. Other enteric pathogens may also originate from the external surface during manual milking [27]. Results in Table (2) show that the levels of the coliform group in the three different governorates were higher than the acceptable levels in both the Egyptian standards [7] and the EU specification [28] where the average coliform count was 3.77 Log CFU/mL. Furthermore, 46 % of the samples had a coliform count of more than 4 Log CFU/mL. Our results are not in accordance with results obtained by Han *et al.* (2007) [23] who reported that the average coliform count in buffalo milk samples was 2.42 Log CFU/mL. Results obtained by Ombarak and ELbagory (2015) [26] showed higher levels of coliform bacteria (with ca. 5.90 Log CFU/mL) in raw buffalo milk samples collected from local markets in Menoufia.

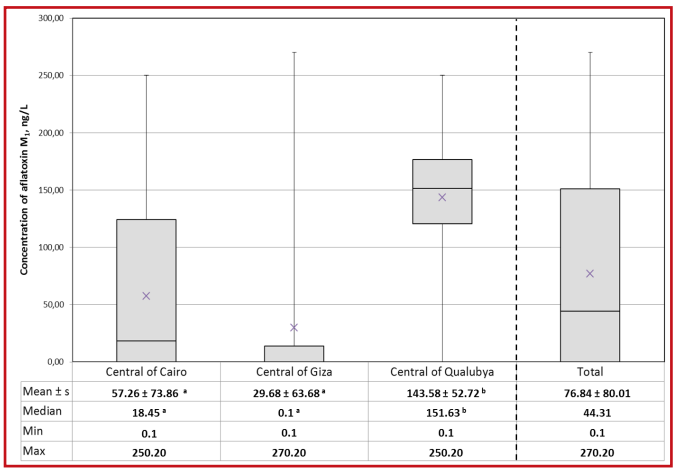

Figure 1. Aflatoxin M1 content of samples collected from local markets of three different central governorates in Egypt. The box and whiskers plot displays the entire range of values, IQR (interquartile range; 25 % - 75 %, median(-), mean(x)), samples were analysed in duplicate. Means with different superscript letters within row differ significantly (p<0.05).

Spore forming bacteria (such as *Bacillus* sp. and *Clostridium* sp.) derive from the farm environment. They can well survive during the pasteurization of milk [29] and grow during refrigerated storage [30] causing milk and its products to spoil and limiting their shelf life [31]. Although there are some regional, seasonal and methodological differences, the average counts of aerobic spore forming bacteria (mostly predominance *Bacillus licheniformis*) in raw milk are 10 to 10^2 CFU mL^{-1} [32-34]. The range of aerobic spore forming bacteria between the different tested regions was relatively the same (Table 2). The relative stability of the spore forming bacteria counts in the three different regions might be due to the similarity of milk chain productions from farmers located in Giza and Qualubya to retailers. Here we also analysed samples of bulk milk (collected from different sources) at farmers' markets and were unable to find significant variations in the viable count of spore forming bacteria between tested regions. Results of the analysis of one hundred and twelve buffalo milk samples in China showed that the average of bacterial endospores was 2.31 Log CFU mL^{-1} [23]. In the end improving management practices and forage quality could be a useful solution in decreasing the contamination of milk with spore forming bacteria [35].

Psychrotrophic bacteria are defined as those growing at 7 °C. They represent the dominant microflora during the cold storage period and produce heat resistant lipases and proteases causing different dairy products to spoil [36]. Our data in Table (2) show that there was no big variation in the range of psychrotrophic bacteria between central Cairo (2.14 to 3.51), Giza (2.14-3.37) and Qualubya (2.12-3.48). The average psychrotrophic bacteria count for all samples was 2.84 Log CFU/mL, indicating the absence of proper standardization of cold storage systems during production and marketing or raw buffalo milk. Our results are slightly in accordance with Cempírková (2002) [37] and Cempírková *et al.* (2009) [38] who reported the average of psycotrophic bacteria in samples of cow milk at 3.00 and 3.41 Log CFU/mL. In another study, the range of psychrotrophic bacteria in goat milk samples of twelve farms was 2.9 to 5.0 Log CFU/mL [39].

Levels of AFM1 in raw milk samples:

Results (Figure 1) show that levels of AFM 1 in central Qualubya (143.58 ng/L) are significantly (P < 0.05) higher than in central Cairo (57.26 ng/L) and central Giza (29.68 ng/L). This result may be due to the contamination of buffalo feed with higher levels of AFB1 in Qualubya

compared to the other two governorates. The median concentration of AFM 1 in all samples was 44.31 ng/L. This level of AFM1 is near to the maximum permissible limit of 50 ng/L set by EU regulations [40] and higher than the limit of zero ng/L set by Egyptian regulations [16]. Finally, concentrations of AFM 1 in 27 % of the milk samples were less than the limit of detection (5 ng /L). In the Alexandria governorate, the mean level of AFM1 in fifty samples of raw cow milk was 49.74 ng/L [41]. However, the average level of AFM1 in thirty samples of raw buffalo milk in the Sohag and Assiut governorates was 64.49 and 130.60 ng/L respectively [42].

Conclusion

We analysed one hundred and twenty samples of market buffalo milk for selected physico-chemical and microbiological parameters as well as levels of AFM1. Our results indicate that there is a serious hygiene problems under the Egyptian regulations. Therefore, we recommend that good hygienic practices and regulations, such as improving farm management practices and implementing the ISO 22000 food safety management system including HACCP to facilitate the production of high quality and safe buffalo milk. Indeed, extensive research is still needed regarding the characterization of predominant spore forming bacteria and other microorganisms causing spoilage in Egyptian buffalo milk.

Acknowledgment

Authors thank Ms Abdel Hadi, Eman, Ms Abdel Maksood, Noura, Ms Said, Nagla, and Ms Mahmoud, Eman for their help in collecting milk samples from central Giza.

References

1. Adesiyun A, Webb L, Rahman S. Microbiological quality of raw cow's milk at collection center of Trindad. J Food Protect 1995; 58: 139-146.
2. Javaid SB, Gadahi JA, Khaskeli M, Bhutto MB, Kumbher S, Panhwar AH. Physical and chemical quality of market milk sold at Tandojam. Pakistan. Pak. Vet. J. 2009; 29: 27-31.
3. Fundora O, Gonzalez ME, Lezcano O, Montejo A, Pompa N, Enriquez AV. A comparative study of milk composition and stability of Murrah river buffaloes and Holstein cows grazing star grass. Cuban Journal of Agricultural Science, 2009; 35: 219–222.
4. Amarjit SN, Toshihiko N. Role of buffalo in the socioeconomic development of rural Asia: Current status and future prospectus. Anim Sci J, 2003; 74: 443–445.
5. Fox PF, McSweeney PLH. Dairy Chemistry and Biochemistry. Blackie Academic & Professional, London: 1998; 443-47.
6. Hofi AA, Rifaat, ID, Khorshid, M A. Studies on some physicochemical properties of Egyptian buffaloes and cows' milk. Indian J. dairy sci, 1966; 19: 113-117.
7. Egyptian Standards. Milk and milk products, part 1: Raw milk. Egyptian Organization for Standardization and Quality, 2005, Egypt.
8. Coni E, Di Pasquale M, Fava L, Bocca A. A pilot survey on the freezing point of raw and heat-treated Italian milk. Ital J Food Sci, 1997; 9: 239-248.
9. Kuczaj M. Inter-relations between year season and raw milk hygienic quality indices. Electronic Journal of Polish Agricultural Universities, Animal Husbandry, 2001; 4: 1-10.
10. Mayerhof H J, Marshall CH, White M. Characterization of a heat-stable protease of *Pseudomonas fluorescens* P 26. Appl Microbiol, 1973; 25: 44-50.
11. Boor K J, Brown DP, Murphy SC, Kozlowski SM, Bandler DK. Microbiological and Chemical Quality of Raw Milk in New York State. J Dairy Sci.1998; 81: 1743–1748.
12. Boycheva S, Dimitrov T, Tsankova M, Iliev T. Investigation on microflora of buffalo milk. Bulg J Agric Sci, 2009; 8: 279–282.
13. Creppy EE. Update of survey, regulation and toxic effects of mycotoxins in Europe. Toxicol Lett. 2002; 127: 19-28.
14. International Agency for Research on Cancer (IARC). Some naturally occurring substances: food items and constituents heterocyclic aromatic amines and mycotoxins, France: Lyon., 1993; 56:245-395.
15. Stoloff L, Van Egmond HP, Park DL. Rationales for establishment of limits and regulations for mycotoxins. Food Addit Contam, 1991; 8: 213-222.
16. Egyptian Regulations. Maximum Limits for Mycotoxin in Foods. Part L Aflatoxins E.S. Egyptian Organization for Standardization and Quality Control, 1990; 1875-1900.
17. Codex Alimentarius Commission . General Standard for Contaminants and Toxins in Food and Feed (CODEX STAN 193-1995), Amended 2016.
18. Walte H-G, Schwake-Anduschus C, Geisen R, Fritsche J. Aflatoxin: food chain transfer from feed to milk. J Consum Prot Food Saf., 2016; 11: 295-297.
19. Ling ER. A text book of dairy chemistry. 1963, Vol. 2, 3rd ed. Chapman and hall Ltd.37. Esser Str., London, W.C.Z.
20. Oxoid Manual. The Oxoid manual of culture media, ingredients and other laboratory services. 1982, Oxide limited, Basingstoke, Hampshire, England.
21. Najdenova N, Dimitrov T. Technological qualities of buffalo milk from the Bulgarian Murrah breed for production of Bulgarian yoghurt. J Anim Sci, 2003; 40: 33–35.
22. Supino MT, Gallo M, Capo G, Morena C, Durante G, Galiero G. Buffalo milk produced in the province of Salerno: Evaluation of sanitary and product parameters. Bubalus Bubalis, 2004; 10: 22–26.
23. Han B-Z, Meng Y, Li M, Yang Y-X, Ren Fa-Z, Zeng Q-K, Nout MJR. A survey on the microbiological and chemical composition of buffalo milk in China. Food Control, 2007; 18: 742–746.
24. Suman CL, Saxena, M M, Pandey HS, Dubey PC, Singh, R., Sanyal MK, Yadav, P. L.. Some factors affecting milk constituents yield of Murrah buffalo. Indian Vet J, 1998; 75: 176–177.
25. Gomaa NF, Hashish M, Kassem MM. Quality assessment of milk in Alexandria. Bulletin of High Institute of Public Health, 2008; 38: 291-305.
26. Ombarak R A, ELbagory AM. Bacteriological Quality and Safety of Raw Cow's and Buffalo's Milk Sold in Menoufia Governorate, Egypt. Minufiya Vet J, 2015; 9:101:113.
27. Fook, YC, Aminah, A, Mohd, KA. Bacteriological quality and safety of raw milk in Malaysia. Food Microbiol, 2004; 21: 535–541.
28. EU specification 92/46/EEC. Laying down the health rules for the production and placing on the market of raw milk, heat-treated milk and milk-based products. Council of the European Union, 1995; 1-39.
29. Collins, EB. Heat resistant psychotrophic microorganisms. J Dairy Sci, 1981; 64: 157-160.
30. Ivy, RA, Ranieri M L, Martin, N H, den Bakker HC, Xavier BM, Wiedmann M, Boor KJ. Identification and characterization of psychrotolerant sporeformers associated with fluid milk production and processing. Appl Environ Microbiol, 2012; 78: 1853-1864.

31. Durak, MZ, Fromm HI, Huck JR, Zadoks RN, Boor KJ. Development of molecular typing methods for *Bacillus* spp. and *Paenibacillus* spp. isolated from fluid milk products. J. Food Sci., 2004; 71: M50-M56.
32. Waes G. Aerobic mesophilic spores in raw milk. Milchwissenschaft, 1976; 31: 521–525.
33. Phillips JD, Griffiths MW. Factors contributing to the seasonal variation of bacillus spp. in pasteurized dairy products. J Appl Bacteriol, 1986; 61: 275-285.
34. Te Giffel MC, Wagendorp A, Herrewegh A, Driehuis F. Bacterial spores in silage and raw milk. Antonie Van Leeuwenhoek., 2002; 81: 625-630.
35. Zucali M, Bava L, Colombini S, Brasca M, Decimo M, Morandi S, Tamburini A, Crovetto GM. Management practices and forage quality affecting the contamination of milk with anaerobic spore-forming bacteria. J. Sci. Food Agric., 2015; 95: 1294-1302.
36. Craven HM, Macauley BJ. Microorganisms in pasteurized milk after refrigerated storage. 1. Identification of types. Aust. J. Dairy Technol., 2002; 47: 38–45
37. Cempírková R. Contamination of cow's raw milk by psychrotrophic and mesophilic microflora in relation to selected factors. Czech J Anim Sci, 2002; 52: 387–393.
38. Cempírková R, Mikulová M, Trávníček J. Counts of psychrotrophic lipolytic bacteria in cow's raw milk samples from the aspect of technological quality. Journal of Agrobiology, 2009; 26: 113-121.
39. Yamazi A, Moreira T, Cavicchioli V, Burin R, Nero L. Long cold storage influences the microbiological quality of raw goat milk. Small Ruminant Res, 2013; 113: 205-210.
40. European Commission Regulation (2010). No. 165 , of 26 February 2010, amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. Official Journal of the European Commission L50 8-12.
41. Amer AA, Ibrahim E M A . Determination of aflatoxin M1 in raw milk and traditional cheeses retailed in Egyptian markets. J Toxicol Environ Health Sci, 2010; 2: 50-53.
42. Shakar E, Elsharkawy E. Occurrence and the level of contamination of aflatoxin M1 in raw, pasteurized, and ultra-heat treated buffalo milk consumed in Sohag and Assiut, upper Egypt. J Environ Occup Sci, 2014; 3: 136-140.