Incidence of lipolytic and proteolytic fungi in some milk products and their public health significance

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Eman M. El-Diasty and R.M. Salem
Faculty of Veterinary Medicine ,Omar El-Mokhtar University- Libya.

ABSTRACT

A total of sixty random samples of dairy products; 20 of either table butter, cooking butter or kareish cheese; were collected and subjected to mycological and mycotoxicological evaluation. The mean total mould counts were 7.3X 10^{1} ± 6X$10^{1.1.8}$ X10^{3} ± 5.4X 10^{2} and 4.1X 10^{3} ±3.1 X 10^{3} cfu ml^{-1} for table butter, cooking butter and kareish cheese samples respectively. Respective yeast counts were 1 X10^{3} ± 6.1 X 10^{2}, 3.3 X 10^{4} ±3 X 10^{4} and 1.5 X 10^{4} ± 1.3 X 10^{4} cfu ml^{-1}. The highest frequency distribution of all examined samples for mould and yeast counts lies within the range 10 - 100 / gm . Aspergillus niger , A. flavus , Geotrichum spp. and Mucor spp. were isolated from the examined samples at varying percentages of 8.3- 41.7 . The predominant species of yeasts isolated from table butter, cooking butter and kareish cheese were Candida spp., Rhodotorula spp., and Saccharomyces spp. The isolated moulds and yeasts were evaluated for proteolytic and lipolytic activities on Tributyrin .Aflatoxin M_1 was detected in 4 kareish cheese samples in a variable levels ranging from 5 to 35 ppb. The economic and public health significance of isolated moulds and yeasts as well as the sanitary precautions were discussed.

Keywords: Proteolytic , lipolytic, fungi , milk products .

INTRODUCTION

Butter is that food product, which is made exclusively from milk or cream or both.Since butter is one of the popular varieties of dairy products and of high nutritive value, it could, if contaminated, constitutes a public health hazard besides economic losses throughout its deterioration Cheese, also, is a milk concentrate, that consists mainly of protein (casein) and fat and considered one of the most important consumed foods in Egypt and other developing countries.

The presence of moulds and yeasts in butter and cheese are objectionable, as they grow at a wide range of temperature and pH values, resulting in spoilage of the product (Pitt and Hocking, 1997).Their counts are used as index of storability and sanitary quality of the products .Such 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 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 by *Aspergillus versicolor*, *A. nidulans* and others (Van Egmond *et al.*, 1997). The carcinogenic

mycotoxins ochratoxin A is on the other hand not considered a problem in cow milk since it is cleaved in the rumen (Engel , 2000). However, ochratoxin A and citrinin might be produced on the surface of cheeses by penicillia during the ripening (Tornadijo *et al.*, 1998).

Aflatoxin M_1 causes DNA damage in cultured rodent cells and gene mutation in bacteria. Naturally occurring aflatoxins are carcinogenic to humans (classified as group 1) , and aflatoxin M_1 is possibly carcinogenic to humans (classified as group 2) and was reported as causing liver damage and thymus aplasia in mammals (Pier , 1987) .

This study was carried out to throw some light on the fungal contamination of butter and Kareish cheese with quantitative evaluation of the Aflatoxin M_1 existing in such products and measuring the proteolytic and lipolytic activities of isolated moulds and yeasts .

MATERIALS AND METHODS

Collection of samples

A total of 60 random samples representing table butter (20), cooking butter (20) and locally manufactured Kareish cheese(20) were collected and immediately subjected to mycological and mycotoxicological nalysis.

Isolation and identification of isolates

For isolation of moulds and yeasts, the technique described by Bailey and Scott (1998) was adopted.

Preparation of serial dilutions

Kareish cheese samples:

Eleven grams of each were removed aseptically and transferred into sterile homogenizer flask containing 99 ml of sodium citrate (2%). The content was homogenized at 1400 rpm for 2.5 min..One ml from cheese homogenate was transferred to a separate sterile test tube containing 9 ml of sterile peptone water (1%) from which ten fold serial dilution up to 10^{-6} were prepared .

Butter samples:

Eleven grams of the prepared samples were transferred into a sterile flask containing 99 ml of warm sterile peptone water 1% (40 ± 1 ^{o} C) to prepare decimal dilutions up to 10^{-6}.

Yeast and Mould counts:

Duplicate plates of Sabouraud dextrose agar medium (containing 0.05 mg of chloramphenicol per ml) were inoculated each with 1 ml from the prepared serial dilutions. Inoculated plates were incubated at 25 ^{o}C for 5 days. The first examination of plates was done after 3 days incubation to determinate the degree of yeast growth, and if large numbers are visible, a count was made and repeated on the 5 th day .Developed yeast and mould colonies were counted.

Suspected mould isolates were identified according to Pitt and Hocking (1997). Isolated moulds were cultured onto malt extract plates for 3- 5 days at 25 ^{o}C, then identified macroscopically and microscopically .Suspected isolates of yeasts were identified according to Lodder and Krger-Van Rij, (1970). Yeast colonies were identified by using the following tests: growth on Sabouraud dextrose agar, Ascospore formation, vegetative reproduction, sugar fermentation, sugar assimilation, nitrate assimilation and urea hydrolysis.

Proteolytic activity of mould and yeast was measured according to (O'reilly and Day, 1983). Either mould or yeast isolate was inoculated on the surface of skim milk agar in which skim milk was added just before pouring the medium into the Petri- plates. The plates were incubated at 28^{o}C for 7 days. After the incubation period, the clear zones of hydrolysis were measured and recorded.

Lipolytic activity of moulds and yeasts was determined using Tributyrin Agar Medium according to Technique recommended by(Koburger and Jacger, 1987) each mould or yeast isolate was inoculated on the surface of Tributyrin agar plates. The plates were incubated at 30°C for 3 days, the medium appeared opaque but lipolytic colonies were surrounded by a clear zone.

Detection of mycotoxin (Aflatoxin M_1) from the examined samples was carried out according to Roberts and Batterson (1975): Extraction and purification of mycotoxin AFM was done according to Roberts and Batterson(1975) . Quantitative estimation of the examined mycotoxin was performed using thin layer chromatography (T.L.C.). Standard toxin (AFM_1) was obtained from Sigma Chemical Company, USA.

RESULTS AND DISCUSSION

Results in Table (1) revealed that mould counts of table butter, cooking butter and kareish cheese samples ranged between $1X10^1$ to 2×10^3, 7×10^1 to 6×10^4 and 9×10^1 to 5x104 cfu g^{-1} with mean value of $7.3 \times 10^1 \pm 6.0 \times 10^1$, $1.8x\ 10^3 \pm 5.4 \times 10^2$ and $4.1 \times 10^3 \pm 3.1x10^3$ cfu g^{-1} respectively. Comparatively, lower counts were reported by Ubach Turull (1985) and Fleet and Mian (1987). Nearly similar results were reported by Bahout (2001) and Aiad (2002) in butter and Kareish cheese, while higher counts were reported by Khair Allah.(2000) who reported that the mean count in Kareish cheese was $5.92 \times 10^6 \pm 8.31 \times 10^5$ cfu g^{-1}. The high counts of moulds do not agree with the Egyptian Standards of1998 and 2000 of butter and Kareish cheese which recommended that total mould counts should not exceed 10 /g in case of kareish cheese but in butter must be free from obvious mould growth.

It could be seen from Table (2) that 25 % of table butter samples were positive for yeast with a total mean counts of $1 \times 10^3 \pm 6.1 \times 10^2$ cfu g^{-1} and 85% of cooking butter were positive with a mean count of $3.3 \times 10^4 \pm 3 \times 10^4$ cfu g^{-1} while in case of kareish cheese all samples were found to be contaminated by yeast with a mean count value of $1.5 \times 10^4 \pm 1.3 \times 10^4$ cfu g^{-1}. These results are in accordance with those obtained by Fleet and Mian (1987) and Bahout (2001) .The high counts of yeasts did not agree with the result of Khair Allah (2000) and Egyptian Standards (1998) for butter and kareish cheese which recommended that total yeast counts not should be more than $> 4 \times 10^2$ cfu g^{-1} and in case of butter must be free from yeasts. Also, mould and yeast counts are used as index for the proper sanitation and quality control of certain dairy products (Jay, 1986).

Regarding the results recorded in Table (3), it is evident that the highest frequency distribution of all examined samples for mould and yeast counts lies within the range 10 – 100. These results contradict those obtained by Seham et al. (1983) who reported that the highest frequency distribution of examined cheese samples (72.5%) lies within the range 400 – 700. Atherton and Newlander (1982) , stated that butter of good quality must not contain total mould and yeast counts more than 50 cfu ml, while fair, poor and very poor quality butter usually contain mould and yeast counts in the range of 51 – 100, 101- 500 and 500 cfu ml respectively.

Table (1): Results of mould count (cfu gm) of the examined butter and Kareish cheese Samples.

Product	No. of examined samples	No. of positive samples		Min.	Max.	Mean ± SE
		+ve	%			
Table butter	20	3	15	$1X10^1$	$2 X 10^2$	$7.3 X 10^1 \pm 6 X 10^1$
Cooking butter	20	15	75	$7 X 10^1$	$6 X 10^4$	$1.8 X 10^3 \pm 5.4 X10^2$
Kareish cheese	20	16	80	$9 X10^1$	$5 X 10^4$	$4.1 x10^3 \pm 3.1X 10^3$

Table (2): Results of yeast counts (cfu gm)of the examined butter and Kareish cheese amples .

Product	No. of examined samples	No. of positive sample		Min.	Max.	Mean ± SE
		+ve	%			
Table butter	20	5	25	$1 X10^1$	$3 X10^3$	$1 X10^3 \pm 6.1X 10^2$
Cooking butter	20	17	85	$1 X10^2$	$5.1 X 10^5$	$3.3 X 10^4 \pm 3 x10^4$
Kareish cheese	20	20	100	$1 X10^2$	$2.6 X 10^5$	$1.5 X10^4 \pm 1.3 X 10^4$

Table (3): Frequency distribution of moulds and yeasts of e butter and kareish cheese samples.

Frequency	Table butter				Cooking butter				Kareish cheese			
	Mould		Yeast		Mould		Yeast		Mould		Yeast	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
> 10	3	15	5	25	15	75	17	85	16	80	20	100
$>10^2$	1	5	3	15	14	70	16	80	15	75	19	95
$>10^3$	0	0	1	5	5	25	7	35	6	30	6	30
$>10^4$	0	0	0	0	1	5	2	10	1	5	2	10

Results given in Table (4) show that *A. niger, A.flavus, Geotrichum spp., Mucor spp.* could be isolated from examined table butter, cooking butter and kareish cheese samples at varying percentages ranged from 8.3 to 41.7.While *Cladosporium spp.*was isolated from table butter and cooking butter samples were at percentage of 8.3 to 8.8. But *Penicillium spp.* was isolated only from Kareish cheese at the percentage of 18.6 .On the other hand, the predominating species of yeasts isolated from table butter , cooking butter and kareish cheese were *Candida lipolytica* (50, 25 and 11.8%), *Candida parapsillosis* (30, 30 and 23.5%), *Candida tropicalis* (10, 10 and 3%), followed by *Rodotorula spp.* isolated in varying percentages of 10 to 44.1, while *Saccharomyces spp.* isolated only from Kareish cheese samples .Similar results were obtained by Azza *et al.* (1997), Ismail and Sabreen (2001). and Aiad (2002). The presence of such moulds and yeasts may cause spoilage of butter and cheese by breaking down their components and liberating different acids and gases with subsequent changes of their odour and flavour. Moreover, mould growth on butter and cheese causes economic losses encompassing discolouration, poor appearance and off flavours.

Results in Table (5) showed that most isolates of *A.flavus, A .niger, Cladosporium*

spp. ,Mucor spp. and Penicillium did exhibit a proteolytic activity with different strength. *Geotrichum spp.* Had a lipolytic activity. Also most isolates of *Candida lipolytica, C. parapasillosis* showed lipolytic activity. These results agrees with Sayed (1999) , Nasser (2002) and El-Diasty (2004) , who found that both *Aspergillus* and *Penicillium* species as well as *Candida spp.* possessed of proteolytic and lipolytic activities. In addition, some moulds are capable of producing toxic metabolites known as mycotoxins such as aflatoxins which are to be carcinogenic (Pitt and Hocking, 1997).

Table (4): Frequency distribution of moulds and yeasts isolated from butter and Kareish cheese samples.

Isolates	Table butter		Cooking butter		Kareish cheese	
	No. of isolates	%	No. of isolates	%	No. of isolates	%
Moulds						
Aspergillus genera	2	16.7	8	23.5	10	23.3
- Aspergillus nigar	1	8.3	6.0	17.6	6.0	13.9
-Aspergillus flavus	3	25.0	5	14.7	9.0	20.9
Muocr spp.						
Geotrichum spp.	5	41.7	12	35.2	10.0	23.3
Cladosporium spp.	1	8.3	3	8.8	0	0
Penicillium spp.	0	0	0	0	8	18.6
Total	12	100	34	100	43	100
Yeasts						
Candida genera	5	50.0	5	25.0	4	11.8
- Candida lipolytica	3	30.0	6	30.0	8	23.5
- Candida parapasillosis	1	10.0	2	10.0	1	3.0
- Candida tropicalis	1	10.0	7	35.0	15	44.1
Rhodotorula spp.	0	0	0	0	6	17.6
Saccharomyces spp.	10	100	20	100	34	100
Total						

Table (5): Proteolytic and lipolytic activities of isolated moulds and yeasts from examined samples .

Isolates	No. of isolates	Proteolytic activity				Lipolytic activity			
		+++	++	+	-	+++	++	+	-
Moulds									
Aspergillus genera									
- Aspergillus nigar	20	4	7	4	5	10	0	3	7
-Aspergillus flavus	13	8	5	0	0	5	3	0	5
Muocr spp.	17	2	3	5	7	17	0	0	0
Geotrichum spp.	27	0	0	0	27	20	7	0	0
Cladosporium spp.	4	4	0	0	0	0	0	0	4
Penicillium spp.	8	2	3	2	1	4	0	1	3
Yeasts									
Candida genera									
- Candida lipolytica	14	0	0	0	14	14	0	0	0
- Candida parapasillosis	17	0	0	0	17	0	0	0	0
- Candida tropicalis	4	4	0	0	0	0	1	0	3
Rhodotorula spp.	23	20	3	0	0	5	4	10	4
Saccharomyces spp.	6	3	3	0	0	0	6	0	0

Table (6): Incidence and levels of mycotoxins (ppb) determined in examined samples.

Product	Type of toxin	No. of examined samples	Positive samples		Range (ppb)	Mean ±SE (ppb)
			No.	%		
Table butter	AFM_1	20	ND	ND	ND	ND
Cooking butter	AFM_1	20	ND	ND	ND	ND
Kareish cheese	AFM_1	20	4	20	5-35	17.5 ±6.61

Table (6) revealed that aflatoxin M_1 was detected only in the examined samples of kareish cheese. Aflatoxin M_1 was detected in 4 samples (20%) in a variable levels ranging from 5 to 35 (ppb) with mean value of 17.5 ± 6.61 (ppb). The obtained results are similar to those obtained by Abouzeid *et al.*(1996) ,while Azza *et al.* (1997) recorded lower levels.

In conclusion, the obtained results showed high contamination of butter and Kareish cheese with different types of yeasts and moulds and their toxins which constitute a public health hazard. Obviously, it is of an important to prevent mould growth to avoid toxin production through preventing the natural contamination of raw materials.

Storage of food under conditions which prevent mould growth and strict hygienic measures and regulations should be imposed during processing, packing and transportation.

REFERENCES

Abouzeid, A.M.; Hassan ,A.A. And Ragheb ,R.R.(1996). Mycological studies on hared (Roume) and skim milk soft cheese (Kareish) with quantitative evaluation of the existing mycotoxins.4th Sci Cong . Proc ., April (1996) Vet. Med. J., Giza .44(2): 113-121.

Aiad, A.S.E. (2002). Criteria for evaluation of locally manufactured dairy products. Ph.D. Thesis ,Fac. Vet. Med., Alex. Univ., Egypt.

Atherton, H.V. and Newlander, J.A.(1982). Chemistry and Testing of Dairy Products 4th Ed. AVI Publishing Company I.H.C. Westport, Connecticut.

Azza, H.; Zeinab, N.; Abd-El- aziz, A.S. and Tolba, K.S.(1997). Mycological aspects of cheese and meat products with their relation to aflatoxins. Beni-Suef Vet. Med. Res.,7(1): 265- 284.

Bahout, A.A.(2001). Occurrence of fungi in butter sold in Sharkia Province.1st Cong. of Food Hygiene & Human Health, 6-8 February 2001 Dept. of food Hygiene, Fac. Vet. Med. Assiut, Egypt,145- 164.

Bailey, W.R. and Scott, E.G.(1998). Diagnostic Microbiology. A Textbook for the isolation and identification of pathogenic microorganisms. The C.V. Mosby Company Saint Louis, USA.

Egyptian Standards (1998). Milk and Dairy Products. Part V: Natural cow's butter. Egyptian Organization for Standardization and Quality Control.EOS, 154.

Egyptian standards (2000). Soft Cheese. Part 4: Kareish Cheese. Egyptian Organization for Standardization and Quality Control.EOS, 1008- 2000.

El-Diasty, E.M.(2004). Study on mycological quality of fish. Ph.D. Thesis, Fac. Vet. Med., Cairo Univ., Beni-Suef branch., Egypt.

Engel, G.(2000). Ochratoxin A in sweets ,oil seeds and dairy products. Arch. Lebensmittethygiene, 51: 98 -101.

Fleet, G.H. and Mian, M.A.(1987). The occurrence and growth of yeasts in dairy products. International Journal of Food Microbiology 4: 145-155.

Ismail, M.A. and Sabreen, M.S.(2001). Associated mycobiota of some types of cheese and cooking butter. Assiut Vet. Med. J. 44 (88): 176- 197.

Jay, J.M.(1986). Modern Food Microbiological, 3rd Ed. Van Nostrand Reinhold Co., New York. **Khair Allah,H.M.A.(2000).** Safety and quality of some dairy products through microbial criteria. M.V.Sc., Fac. Vet. Med., Alex. Univ., Egypt.

Koburger, J.A. and Jacger, K.E.(1987). " Specific and sensitive plate assay for bacterial lipases". Appl. Environ. Microbiol., 53: 211.

Lodder, J. and Krger-Van Rij, N.J.W. (1970). The Yeasts: A toxonomic Amestrdam. North Holland Publishing.

Nasser, L.A.(2002). "Mycological status of imported canned fish consumed in Saudia Arabia with special reference to proteolytic activity" .Assiut Vet. Med. J., 47: 125- 130.

O'reilly, T. and Day, D.F.(1983). "Effects of culture conditions on protease production by *Aeromonas hydrophila*." Appl. Environ., 45: 1132 .

Pier,A. C. (1987). Aflatoxicosis and immunosuppression in mammalian animals, Appl. Environ. Microbiol., 58-65.

Pitt,J.I. and Hocking, A.D.(1997). "Fungi and Food Spoilage Book " 3rd Ed., Academic Press, New York, London, Toronto, Montreal, Tokyo.

Roberts, B.A. and Batterson, D.S.p.(1975). Detection of twelve mycotoxins in mixed animal feedstuffs. J. AOAC. , 58: 1178.

Sayed, A.A.K.(1999). "Proteolytic fungi in some meat products: Their incidence and methods for inhibition". M.V.Sc. Thesis, Fac. Vet. Med., Assiut Univ., Egypt.

Seham, M.M.; Abd El-Rahman, H.A.; Morgan, S.D. and Hafez, R.S.(1983). Mycological studies on Egyptian soft cheese and cooking butter. Assiut Vet. Med. J., 11(21): 149- 155.

Tornadijo , M.E.; Fresno, J.M.; Sarmiento , R.M. and Carballo, J.(1998). *Penicillium* species during the manufacturing and ripening of Armada raw goat's milk cheese: Identification, characteristics in vitro potential toxins production. Lait., 78: 661-672.

Ubach Turull, M. (1985). Microbiological quality of Spanish butter and margarine. Anal. Bromatol. , Xxx V 11-2, 307 – 317.

Van Egmond, H.P., Svensson, U.K. and Fremy, J.M. (1997). Mycotoxins. Int. Dairy Fed. S.I., 79 – 88.

Viljoen ,B.C. and Greyling , T. (1995) . Yeasts associated with cheddar and Gouda making . Int. J. Food Microbiol., 28:79-88.

الملخص العربي

مدى تواجد الفطريات المحللة للدهون والبروتينات في بعض منتجات الألبان والأهمية الصحية لها

إيمان محمود الدياسطي – رمضان مصطفي تاج الدين
كلية الطب البيطرى –جامعة عمر المختار -ليبيا

تم فحص عدد 60 عينة عشوائية من منتجات الألبان من المحلات والسوبر ماركت، (20 عينة من كل من زبد المائدة , زبد الطهي والجبن القريش) حيث تم تجميع تلك العينات وأخضعَت للفحص الفطري والتقييم للسموم الفطرية . وكان متوسط العد الفطري الكلى / جرام هو $7.3 \times 10^{3} \pm 1.8, 1 \times 10^{3}$ و $5.4 \times 10^{2} \pm 4.1 \times 10^{3}$ و 3.1×10^{3} لكل من زبد المائدة , زبد الطهي والجبن القريش على التوالي . وقد وجد أن التوزيع التكراري الأعلى لكل العينات المفحوصة لإحصاء الخميرة والعفن يقعان ضمن المدى 10- 100 / جرام . أنواع الأسبرجيلس نيجر، والأسبرجيلس فلافس والجيوترايكم الميوكور تم عزلهم من العينات تحت الفحص بنسب مئوية مختلفة ، تراوحت من 8.3 - 41.7. وكانت الأنواع السائدة للخمائر التي تم عزلها من زبد المنضدة , زبد الطهي والجبن القريش هي خمائر الكانديدا والرودتيرلا والسكارو ميسيس .الفطريات والخمائر المعزولة من العينات التي تم فحصها اختبرت لنشاط الإنزيمات وقيمت للنشاطات المحللة للبروتين وقدرتها على تحلل الدهون باستخدام الوسط المخصص لذلك (الترابيتورين) . وقد تم في هذه الدراسة الكشف عن تواجد سموم الافلاتوكسين ب1 في أربعة عينات فقط من عيناتِ الجبن القريش عند مستويات متغيرة تتراوح من 5 - 35 أجزاء من البليون. وقد تم في هذه الدراسة الإشارة إلي الأهمية الاقتصادية و الصحية للفطريات والخمائر المعزولة بالإضافة إلى ذكر الإجراءات الوقائية الصحية .