Isolation, identification and detection of some virulence factors in yeasts from local cheese in Mosul city

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Abstract

Fifty samples of local cheese were purchased from Mosul city markets during the period from April 2012 to November 2013 to identify and characterize yeast species in these samples. Fifty-eight yeast isolates were identified and confirmed biochemically. They were *Candida albicans* (13.8%), *Candida krusei* (9.6%), *Candida tropicalis* (10.3%), *Candida parapsiloosis* (25.8%), *Geotrichum candidum* (20.6%), *Rodotorella* spp. (10.3%) and mixed yeasts (12.9%). Virulence factors (Hemolytic, Phospholipase, Aspartylprotinase and Estrase activities) of Candida isolates were determined. All isolates show one or more of these activities except *Candida krusei*. The presence of Candida isolates in this type of cheese refer to the importance of this genus to the health of consumers was discussed.

Keywords: Isolation, Virulence factors, Yeasts, Cheese
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عزل وتوصيف وتحديد بعض عوامل الفوعة للخمائر من عينات الجبن المحلي في مدينة الموصل

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الخلاصة

تم فحص خمسون عينة من عينات الجبن محلية الصنع والتي تم جمعها من اسواق مدينة الموصل خلال الفترة من نيسان ٢٠١٢ ولغاية تشرين الثاني ٢٠١٣ لتحديد وتوصيف أنواع الخمائر في هذه العينات. ثمانية وخمسون عزلة من الخمائر تم عزلها وأكدت بالفحوص الكيميائية. شكلت *Candida albicans* (١٣,٨%)، *Candida krusei* (٩,٦%)، *Candida tropicalis* (١٠,٣%)، *Candida parapsiloosis* (٢٥,٨%)، *Geotrichum candidum* (٢٠,٦%)، *Rodotorella* spp. (١٠,٣%)، والخمائر المختلطة (١٢,٩%). تم تحديد عوامل الفوعة (Hemolytic, Phospholipase, Aspartyl and Estrase) من عزلات المبيضات. اظهرت كل العزلات فعالية لواحد أو أكثر من هذه العوامل ماعدا *Candida krusei*. تمت مناقشة أهمية وجود جنس المبيضات في هذا النوع من الاجبان على صحة المستهلكين.

Introduction

Cheese is the name given to a group of fermented milk products produced throughout the world in great variety of flavors, textures and forms. Fox *et al*. (1) suggested that there are more than 1000 varieties of cheese worldwide. Walter and Hargrove (2) described about 400 varieties and list the names of a further 400, while Burkhalter classify 510 varieties (3). Cheese manufacturing in Mosul city is one of the traditional preservation method for milk in rural areas. Dairy products are extremely popular and the people are incorporating more dairy products in their daily diets due to its durability in hot weather compared with milk as well as for its convenience and nutritive value. The increasing demand by Iranian consumers is mainly attributed to its high protein content and low price. Soft white cheese locally known as Jiben-Al-Arab is the major natural locally produced cheese type during spring season

from sheep milk in Mosul city. Jiben-Al-Arab is a pickled cheese to which unknown percentage of salt is added, depending on the procedure of the manufacturer. Jiben-Al-Arab is usually packed in tins filled up with salted whey and stored at room temperature. No known amount of this type of cheese is usually produced in Mosul governorate and no known complete bacteriological picture of this product is available except some studies referred to (4,5).

The local soft cheese as one of the demand food is not a high stable product, since it is a fresh product, with very high moisture (>55%) content and low (may range between 1.4-1.6%) salt concentration. These characteristics easily allow the microbial growth in the cheese (6). Milk and milk products serves as an excellent growth medium for a wide range of microorganisms (7).

Yeasts and molds can cause various degrees of food decomposition. Their ability to proliferate in a wide range of pH and temperatures, and in foods of various water contents, make it necessary to detect fungal spoilage at the early stages. The presence of yeasts is the major microbiological economical and sensory problems (8). They usually present in raw milk do not survive pasteurization and their presence in cheese considered as one of the indicatives of low processing temperature and re-infection during manufacturing (9) especially if free moisture is available at the surface like walls and shelves of ripening rooms, air, equipment, water, milk, brine, etc. (10). The number and types of microorganisms in milk immediately after milking are affected by factors such as animal and equipment cleanliness, season, feed and animal health (11). In the last years, a considerable increase of illness caused by infection with *Candida* species, these yeasts have been related to animal variation species, due to the hazard use of different of antibiotics. There were several species of *Candida* speciessuch as *C. albicans, C. tropicalis, C. pseudotropicalis C. krusi.* and*C. albicans*is considered the most important pathogen responsible for infectious diseases in animals (12).

So, the objective of this study is to isolate yeasts from locally produced cheese and evaluate some virulence factors of the pathogenic isolates of Candida.

Materials and methods

Sampling

A total of 50 soft cheese samples were collected from local Mosul city markets in their marketing plastic press lid containers during the period from April 2012 to November 2013. Each cheese sample was represented by one whole cheese (450 g) and transferred in an ice box to the laboratory of microbiology, college of veterinary medicine within an hour for enumeration and identification of yeasts.

Enumeration and isolation of yeast strains

The method of Hakim and *et al.* (13) was employed for Enumeration and isolation of yeast strains. The method is briefly as follows: Ten grams of cheese samples were placed in 90 ml of sterile 2% (w/v) sodium citrate preheated to 45°C and were homogenized using a stomacher blender for 90 s at 250 rpm (22). Serial dilutions in 2% sodium citrate solution were applied and spread on Sabourauds dextrose agar plates (SDA). The plates in duplicates were incubated at 25°C for 3 days. Counts were calculated as the number of colony forming units per gram of sample and were reported as log10 CFU g^{-1}. Three to five colonies were selected from each dilution sample, and streaked to single colonies on SDA and were further purified and maintained on slants of SDA. The isolated yeast strains were characterized with classical methods, and identified to species level by morphological and physiological standard methods as recommended by Kurtzman *et al*. (14)**.**

Biochemical activities of yeasts

The biochemical reactions were concluded by using sugar fermentation and urease test. Complete identification of different yeast isolates was performed according to Cruickshank *et al.* (15).

Virulence factors

Thirty three of the isolates (Candida isolates), were tested for hemolytic activity, phospholipase production, extracellular proteolytic activity and esterase activity.

Detection of hemolytic activity

Hemolytic activity was assayed using a method described by Luo *et al*. (16). A loopful of the stock culture was streaked onto SDA and incubated at 37°C for 18 h. The resultant cultures were harvested and washed with sterile saline, and a yeast suspension with an inoculums size of 10^8 cells/ml was prepared. The plates were incubated at 37°C in 5% CO_2 for 48 h. The presence of distinct translucent halo around the inoculums site, viewed with transmitted light, indicated positive hemolytic activity.

Detection of secreted aspartylprotinase

Proteolytic activity was measured by the ratio of the diameter of the colony and the total diameter of the colony plus precipitation zone as described by Price *et al*. (17). The halo around the colony produced by proteolytic activity of the *candida* strain will be referred to as the "Precipitation zone"**.** *C. parapsilosis* ATCC 22019 served as a negative control.

Detection of phospholipase activity

Phospholipase production was assayed according to a method described by (17). Sabouraud dextrose agar (20 ml) supplemented with 1mol/l sodium chloride, 0.005 mol/l

Calcium chloride, and 8% sterile egg yolk emulsion was poured onto 90 mm diameter plates. A single colony (~ 6 mm) of each yeast isolate was inoculated onto the surface of the medium. The plates were incubated at 37 C^o for 5 days. *C. albicans* ATCC 10231 served as a positive control. Phospholipase activity was measured according to proteinase testing.

Detection of estrase activity

Esterase activity was determined using the Tween 80 opacity test medium, prepared with 10 g Bactopepton, 5 g of Nacl, 0.1 g $Cacl_2$, and 15 g agar in 1 l of distilled water, adjusted to a pH of 6.8 and autoclaved. When the medium cooled (50°C), 5 ml of Tween 80 was added. Lipolytic enzymes were added to hydrolyze the medium, librating fatty acids that bind with calcium and form a precipitation halo around the inoculation site. Detection of lipolytic activity on all substrate plates was performed by observing zones of precipitation around the colonies when viewed by transmitted light. These precipitates are found as a result of hydrolysis of the Tween compound. Subsequent to the cleavage of ester bonds, the fatty acids released combine with calcium ions in the medium to form insoluble calcium salts (18).

Results

Total yeast count of local cheese samples

From table 1, it is evident that all local cheese samples were contaminated with varying number of yeasts and all had count ≥ 1LogCFU/g of tested cheese samples. More than half of the tested cheese samples (60%) had total yeast count of 1.5-2 Log CFU /g. In the second order (32%) were the cheese samples with total yeast count between 2-2.5 Log CFU/g of. The least yeast percentage (8%) of the cheese samples had count of 1-1.5 Log CFU/g.

Table 1: Log CFU/gm of total yeasts count of fresh local cheese samples in Mosul city

Log CFU/gm.	Samples No.	(%)
Not detected	0	0
< 1	0	0
1-1.5	4	8
1.5-2	30	60
2-2.5	16	32
Total samples	50	100

Identification of yeast species

Yeast species from local cheese samples confirmed by biochemical activities are illustrated in table 2 and 3. There are evident that more than half 33/58 of the yeast species were belonged to the genus *Candida* with a percentage of (56.9%). In the second order 12/58 (20.69) were belonged to the genus Geotrichum (*Geotrichum candidum)*. In the third order 6/58 (10.34), were the yeasts belonging to genus *Rodotorella* [unidentified species]. Still 7/58 (12%) of the yeasts contaminated local cheese samples were mixed (unidentified).

Table 2: Yeast species isolated from fresh local cheese

Yeast species	Number	Percentage (%)
Candida albicans	8	13.80
Candida krusei	4	6.90
Candida tropicalis	6	10.34
Candida parapsiloosis	15	25.86
Geotrichum candidum	12	20.69
Rodotorella spp.	6	10.34
Mixed unidentified yeasts	7	12.07
Total	58	100.0

Table 3: Biochemical activities of the *Candida* spp. Isolated from local cheese samples

Candida species	Sugar fermentation				Sugar assimilation				Germ tube	Urase
	Glucose	Galactose	Sucrose	Maltose	Glucose	Lactose	Sucrose	Maltose		
Candida albicans	+	+	--	+	+	--	+	+	+	--
Candida krusei	+	--	--	--	+	--	--	+	--	+
Candida tropicalis	+	+	+	+	+	--	+	+	--	--
Candida parapsiloosis	+	--	--	--	+	--	+	+	--	--

The virulence factors of Candida isolates from local cheese samples are shown in table 4. Hemolytic activity of these isolates show variance in their activity among species, being higher (87.5%) in *C. albicans*, followed by *C. tropicalis* and *C.parapsiloosis* with percentages of 60% and 59% respectively. No hemolytic activity was found with *C.krusei*. Phospholipase activity was found in *C. parapsiloosis* and *C. tropicalis* with 73.3 % and 50% respectively. No Phospholipase activity was found in *C. albicans* and *C. krusei*. All candida species had Aspartyl activity, in percentages of 66.6%, 50% and 33.3% for *C. tropicalis*, *C. albicans* and *C. parapsiloosis* respectively, except *C. krusei*, which show no activity.

Table 4: Virulence factors of the Candida species isolated from local cheese samples

Candida species	Virulence factors											
	Hemolytic activity			Phospholipase activity			Aspartyl activity			Estrase		
	No.	Positive	%	No.	Positive	%	No.	Positive	%	No.	Positive	%
Candida albicans	8	7	87.5	8	0	0	8	4	50	8	0	0
Candida krusei	4	0	0	4	0	0	4	0	0	4	0	0
Candida tropicalis	6	3	50	6	3	50	6	4	66.6	6	1	16.66
Candida parapsiloosis	15	9	60	15	11	73.3	15	5	33.3	15	0	0

Discussion

It is widely recognized that yeasts can be an important component of the microflora of many cheese varieties because of the low pH, low moisture content, high salt concentration and refrigerated storage of these products (19). Nevertheless, yeasts play a dual role depending on the cheese. In fact, in some cheese types they make a positive contribution to the development of flavor and texture during the stage of maturation, while in other varieties, yeasts can be regarded as spoilage organisms. Yeast spoilage is recognized as a problem primarily in fermented milk and cheese (20,21).

The most prevalent yeast genera identified in this study were *Candida, Geotrichum* and *Rodotorella* (Table 2). This finding is in line of many results obtained by (22), who found that the predominant yeast genera identified were *Candida, Geotrichum* in addition to *Cryptococcus neoformance* and *Trichosporum cutaneum*, and also to the results of (23), who reported *Candida,Rodotorella* and *Trichosporum.* Very close percentage of yeast genera identification for *Candida, Geotrichum and Rodotorella* was reported by (24) to ours of the same yeast genera (90% vs. 88%).

Carbohydrate fermentation and assimilation give an aid in the identification of yeasts, and this is also the idea of wang (25) and Zaini *et al.* (26), who reported that carbohydrate assimilation is necessary for definitive identification of Candida species, but Finegold and Baron (27) go further when they stressed that biochemical identification is of great importance of laboratory identification of *C. albicans*.

The main mechanisms by which yeast growth influences the final quality of cheese are: fermentation of lactose, utilization of lactic acid and lipolytic and proteolytic activities (28).

Candida speciesnow rank as the fourth most common cause of bloodstream infections and attributable mortality rate is 35%. (29) to it is of interest to determine whether virulence factors are present in *C.albicans* isolates in our study. The answer came from the various percentages of different virulence activities in different *C. albicans* isolates (Table 4).

Hemolysin, a putative virulence factor thought to contribute to Candidal pathogenesis. In particular, the secretion of hemolysis, followed by iron acquisition, facilitate hyphal invasion in disseminated candidiasis (30). In this study, it is well evident that *C. albicans* isolates were the highest in hemolytic activity, since 7/8 of *C.albicans* isolates (87.5%), were positive to hemolysin activity. This finding goes in the line of (31), who thought that although other Candida species than *C.albicans* are frequently reported, but *C. albicans* still among the most pathogenic species of fungal pathogens.

Phospholipases activity has been identified in many fungal pathogens including Candida species, *Cryptococcus neoformance* and *Aspergillus fumigates* (32).

In this study *C.parapsiloosis* isolates were the highest in Phospholipase activity, but not *C. albicans* which show no Phospholipase activity (73.3% vs.0% respectively). Phospholipase are believed to be involved in the disruption of host membranes and it has been implicated in *C. albicans* virulence in several systems, but their role in *C.parapsiloosis* is not clear (33). No explanation for the absence of Phospholipase activity in *C. albicans* could be drawn, but the sample size and the biological differences between tested strains may give an exit to this inconsistency with others.

Proteinase activity is an important virulence factor because this activity facilitates the establishment of the infection (34). refering to (35) protease activity is an isolate- specific activity and therefore depends on the strain used. Available information about the higher virulent secretary aspartic proteinase (sapIp to Sap 10p) in *C.albicans* compared to less Sap activity in *C.parapsiloosis* is referred to (23), which in turn occur in the line of our results of 50% vs.33.3% respectively.

lipolytic activity has been correlated with the virulence of certain organisms (36). Various species of Candida have been reported to have lipolytic activity through secreation of lipolytic enzymes such as esterases and phospholipases. Lipolytic activity here was recorded in the side of *C.tropicalis* in a percentage of 16.66%. This finding is agreed with the results found by Aktas (37), who observed Estrase activity only in *C.tropicalis* and *C.guilliermondii,* but not other Candida species.

From all Candida species examined for their virulence activity, table 4 shows that *C. krusei* did not exhibit any of the fore mentioned virulence factors. The reason for that could be referred its low adherent capability to both epithelial and prosthetic surfaces, to its lower proteolytic potential and production of phospholipases, to its difference in its structural and metabolic features and behavior patterns towards host defenses in compare to other Candida species, which impress an idea of re-assigned its taxonomy (38).

Changes in the host of these cheese may lead candida opportunistic yeasts from harmless commensally microorganisms to fetal consumer pathogens. Therefore, a new technologies and process need to be considered in local cheese production, and care in cleaning and sanitizing equipment such as vats, knives, paddles or rakes, and containers will aid greatly in the control of these pathogenic organisms.

References

1. Fox PF, Timothy MG, Mcsweeney PLH. Fundamentals of cheese science an aspen publication. Aspen Publishers, Inc Gaithersburg, Maszaryland. 2000; 253.
2. Walter HE, Hargrove RC. Cheese of the world. New York: Dover. 1972;pp:78.
3. Burkhalter G. Catalogae of cheese (Bulletin 141). Russels: International Dairy Federation. 1981.
4. Ali HH. Salmonella serotypes isolated from local cheese in Mosul city. Iraq J Vet. Sci 2005;19:27-30.
5. ArifE D. Isolation and identifiation of salmonella species from local cheese in Sulaimania province. Al-anbar J Vet Sci. 2012;5:82-84.
6. Carvalho JDG. Avaliação da Qualidade de QueijosTipo Minas Frescal Elaboradospor Diferentes ProcessosTecnológicos e Comercializadosem Campinas-SP.[MSc thesis] Campinas, São Paulo: State University of Campinas. 2005.
7. Varga, J, Peteri Z, Tabori K, Teren J, Vagvlgyi C. Degradation of ochratoxin A and other mycotoxins by Rhizopus isolates. Internat J Food Protec. 2005; 99: 321-328.
8. Marth HE and Steele LJ. Cheese. Applied Dairy Microbiology. 2nd edition. Marcel Dekker, Inc., New York, 2001; pp:354-358.
9. Zamora A, Ferragut V, Jaramillo PD, Guamis B and Trujillo A J. Effects of ultra-high pressure homogenization on the cheese-making properties of milk. J Dairy Sci. 2007;90:13-23.
10. Jay JM. Modern food microbiology (5th ed.). New York, Chapman&Hall, 1996; pp:137–141, 328–342, 347–352.
11. Rogelj I M. In: Mikrobiologijaživilživalskegaizvora (Eds.: Bem, Z./ Adamič, J./ Žlender, B./ SmoleMožina,S./ Gašperlin, L.). Ljubljana, Biotehniškafakulteta, Oddelekzaživilstvo, 2003;515–538.
12. Brito EHS, Fontenelle ROS, Brilhante RSN, A, Cordeiro JJC, Rocha MFG. Candidosis on veterinary medicine: a mycological,clinical and therapeutic approach. J Ciência Rural. 2009;39(9):2655-2664.
13. Hakim AS, AbuelnagaASM, Sayed EL, Ahl RMH. Isolation, biochemical identification and molecular detection of yeasts from Kariesh cheese. Internat J Microbiol Res. 2013;4(1):95-100.
14. Kurtzman P. Molecular taxonomy of the yeast. Yeast.1994;10:1727-1720.
15. Cruickshank, R, Duguid JP, Marmion BP, Swain RHA. Medical microbiology. 12 edit. th Vol. 2 Churchill Livingstone, Edinburgh, London and New York.22-Refai, M., A.H. Gobba and H. Rieth, 1969. Monograph on yeast diagnosis, disease and treatment. Egypt Vet Med J. 1975;16: 255-315.
16. Luo G, Samaranayake LP, Yan JYC. Candida species exhibit differential in vitro hemolytic activities. J Clin. Microbiol. 2000;39:2971-2974.
17. Price MF, Wilkson ID, Gentry LO. Plate method for detection of phospholipase activity in *Candida albicans*. Sabouradia. 1982;20:7-14.
18. Tirunarayanan, MO, Lundbeek, H. Investigations on the enzymes and toxins of staphylococci: assay of lipase using Tween as the substrate. Acta pathol Microbiol Scand. 1968;72:263-433.
19. Devoyod JJ. Yeasts in cheese making. In: Spencer, F.J., Spencer, D.M. Eds.., and Yeast Technology. Springer-Verlag, Berlin. 2008;6:228–245
20. Brocklehurst TF, Lund BM. Microbiological changes in cottage cheese varieties during storage at 7 C. Food Microbiol. 1985;2:207–233.
21. Fleet GH. Yeasts in dairy products: a review. J Appl Bacteriol.1990;68:199–211.
22. Refai, M., A.H. Gobba and H. Rieth. Monograph on yeast diagnosis, disease and treatment. Egypt Vet Med J.1969;16: 255-315
23. Krukowski HM, Tietze T M, Ro Anski P. Survey of yeast mastitis in dairy herds of small-type farms in the Lubin region, Poland. Mycopathol. 2001;150(1):5-7.
24. Bourtzi-Hatzopoulou E, Zdragas A, Petridou E, Filiousis G. Yeasts as a causative agent of bovine mastitis in Greece. J.Hellenic. Vet Med Society.2003;54(2):105-110.
25. Wang BY. The laboratory examination of *C. albicans*. Zhonghua Kou Qiang Yi XueZaZhi. Chinese. 1990;25(5): 294-296.
26. Zaini F, Gerami SM, Kordbacheh P, Khedmati E, Safara M, Gharaeian N. Performance of five phenotypical methods for identification of Candida isolates from clinical materials. Iranian J Publ Health. 2006;35(1):25-32.
27. Finegold SM, Baron EJ. Diagnostic microbiology,7th ed. C.V. Mosby company St. Louis, Toronto, Princetown, 1986.
28. Tudor DA, Board RG. Food spoilage yeasts. In: The Yeast. Yeasts Technology (Rose, A.H., Harrison, J.S. Eds). 2nd ed. Academic Press, London. 2010;5: 436–451.
29. Wenzel RP. Nosocomial candidemia: risk factors and attributable mortality. Clin Infec Dis.1995:20:1531-1534.
30. Odds FC. Candida and Candidiosis: a review and bibliography. London, Baillieere Tindall.1998
31. Yang YL. Virulence factors of Candida species. J Microbiol Immunol Inf. 2008; 36:223- 228.
32. Trofa D, Gacser A, Nosanchuk JD. *Candida parapsilosis*, an emerging fungal pathogene: A Review. Clin Microbiol Rev. 2008;21(4):606-525.
33. Yildirim Z, Kilic N, KalkanciA. Fluorometric determination of acid proteinase activity in Candida albicans strains from diabetic patients with vulvovaginal candidiasis Mycosis. 2011;54:463-467.
34. Kalkanci A, Bozdayi G, Biri A, Kustimur S. Distribution of secreatedaspartyl proteinases using a polymerase chain reaction assay with SAP specific c primers in *candida albicans* isolates. Folia Microbiol. 2005;50: 409-413.
35. Rodrate MP, Diass DR, Vilela DM Schwan RF. Proteolytic activities of bacteria, yeasts and filamentous fungi isolated from coffee fruit (Coffee Arabica L). ActaScientiarum Agronomy. 2011;33(3):457-464.
36. Chattaway FW Od ds FC, Barlow AJE. An examination of the production of hydrolytic enzymes and toxins by pathogenic strains of *Candida albicans*. J Gen Microbiol. 1971;20:7-14.
37. Aktas E, Yigit N, Ayyildiz A. Estrase activity in various candida species. J. Int Med Res. 2002;30:322-324.
38. Samaranayake, Y.H, Samaranayake, L.P. *Candida krusei*: biology, epidemiology, pathogenicity and clinical manifestations of an emerging pathogen, review article: clinical mycology. J Med Microbiol. 1994;41:295-310.