

Determination of some virulence factors of *Candida spp.* isolated from locally produced cheese in Diyala Governorate-Iraq

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ABSTRACT

Locally produced cheese which called (Gibin Al arab) is one of the most common dairy products in Iraq, it has an economic importance and great social value. This research aimed to identify yeast species from locally produced cheese (Gibin Al Arab) in Diyala city which traditionally made and sold in markets of old town in Baquba, and study some of virulence factors (Esterase production, Phospholipase and Hemolytic production) of yeasts belong to genus of *Candida*. All cheese samples showed contamination with varying number of yeast, total 88 yeast isolates obtained from 70 cheese samples, they were *Geotrichum candidum*(20.5%), *Rhodotorela* species(19.4%), *Candida parapsilosis* (18%), *Candida albicans* (13.6%), *Candida tropicalis* (10.5%), *Candida krusei* (8%), *Saccharomyces cerevisice* (3.3%) and mixed yeast (un identified) at rate of (6.7%). Species of *Candida* formed half of the total isolates and the most prevalent isolate of *Candida spp.* was *Candida parapsilosis*. According to the results determining of (Esterase production, Phospholipase and Hemolytic production) as a virulence factors identifying *Candida spp.* these activities referred that all isolates of *Candida spp.* show one or more of these activities and that isolates of medically important species *Candida albicans* were the most virulent isolates. this referred to the importance of take attention about consuming of such types of dairy products and need for applying more hygienic measures during handling, processing of milk and form of storage and/or selling of cheese.

الخلاصة

يعد جبن العرب المصنع محليا احد اكثر منتجات الحليب شيوعا في العراق لما له من قيمة اجتماعية وثقافية واهمية اقتصادية. يهدف هذا البحث الى تشخيص انواع الخمائر المعزولة من الجبن المصنوع محليا والذي يسمى (جبن العرب) و يباع في اسواق مدينة بعقوبة القديمة ودراسة بعض عوامل الضراوة مثل (انتاج انزيم الاستريز والفوسفولابيز وقابلية تحليل الدم) لانواع الخمائر التي تعود لجنس الكانديدا المعزولة من هذا النوع من الاجبان. اظهرت هذه الدراسة ان جميع العينات من الجبن كانت ملوثة باعداد وانواع مختلفة من الخمائر، تم الحصول على (88) عزلة من فطريات الخمائر من مجموع 70 عينة جبن، شملت العزلات على

Rhodotorula (19.4%), *Candida parapsilosis* (18%), *Geotrichum candidum* (20.5%), *Candida tropicalis* (10.5%), *Candida krusei* (13.6%), *Candida albicans* (8%), *Saccharomyces cerevisice* (3.3%).

بينما عزلت الخمائر المختلطة غير (المشخصة) بنسبة (6,7%)، من ضمن انواع جنس الكانديدا التي شكلت نصف العزلات كان النوع *Candida parapsilosis* الاكثر انتشارا. بالنسبة الى نتائج تحديد انتاج الاستريز، فوسفولابيز وقابلية تحليل الدم كعوامل ضراوة خاصة بجنس الكانديدا، اشارت الدراسة الى ان جميع عزلات الكانديدا اظهرت امتلاكها عامل او اكثر من هذه العوامل وكانت عزلات المبيضات المبيضة (*Candida albicans*) (الكثير ضراوة). من الدراسة الحالية تم استنتاج ان جميع عينات الجبن تحتوي على انواع مختلفة من الخمائر والتي تتضمن جنس الكانديدا المهم طبيا، ودراسة بعض عوامل الضراوة لانواع الكانديدا المعزولة اظهرت نتيجة موجبة لواحد او اكثر من هذه العوامل ما يسترعي ضرورة الانتباه حول استهلاك هذه الانواع من منتجات الحليب وكذلك تطبيق خطوات التعقيم اثناء تحضير، خزن وبيع هذا النوع من الاجبان المصنعة محليا.

INTRODUCTION

Gibin Al-arab is considered as one of the most popular locally made cheese type in Iraq, this may be mainly attributed to its high protein content, price and its texture. The traditional method used in making of this type of cheese and selling it uncovered in non-septic conditions affords many opportunities for microbial contamination, and make it a good medium for the growth of different types of pathogenic microorganisms. This raises the possibility of dairy products being vehicles of transmission of pathogenic yeasts [1].

Yeasts play an important role in affecting the safety and quality of food products. Some yeast may pose threat to dairy products safety given their association with opportunistic infections in humans [1,2]. While there have been many reports about the bacterial composition of these products [3,4,5], little is known on the distinction of yeasts associated with chesses [1]. It is recognized that yeasts can be an important component of the microflora of many types of cheese because of the low moisture content, low pH, high salt concentration and the conditions of refrigeration for storage of these products [6,7]. The pathogenic species of *Candida* have

also been isolated from milk of animals infected by mastitis, with other genus such as *Trichosporon*, *Rhodotorula*, *Sporobolomyces* and *Geotrichum* [8,9,10,11].

The virulence of *Candida* spp. is attributed to certain factors like biofilm formation, adherence, and the production of extracellular hydrolytic enzymes leading to damage of tissues [12]. Extracellular hydrolytic enzymes like proteinase and phospholipase are important for colonization and invasion of host tissue [13, 14]. Many types of cheese made from raw milk of poor bacteriological quality and produced under unsuitable conditions. These products are sold in containers without covering, thus the risk of contamination become very high. [15,16].

In Iraq, especially in Diyala city the information about involving of (Gibin Al-arab) cheese in the human illness with pathogenic yeast species are little, because of that we aimed in this research about distribution of yeast species, also study some of virulent factors of the *Candida species* referring to the importance of isolation of these pathogenic agents in such mostly consumed types of dairy products.

MATERIALS AND METHODS

Sampling

A total of seventy (70) samples of Gibin Al arab cheese samples collected randomly from local markets in Diyala at period from May 2015 to January 2016. Every cheese sample was considered as one whole cheese (500 g) and were brought to the laboratory of Microbiology at Department of Pathological analysis, Baquba Technical Institute, Middle Technical University, under cold conditions and stored in refrigerator until analysis within 3 hours of sampling.

Isolation of yeasts

The yeast were isolated according to the method of Yarrow and Van der Walt (2009), which include taking (10) g of each cheese sample from inner parts and homogenize in 90 ml of sterile solution of 2% (w/v) sodium citrate by using a Stomacher for 30 seconds. For all samples, 10 fold serial dilutions were prepared in a sterile solution of 2% (w/v) sodium citrate, then spread on Sabouraud dextrose agar plates (SDA), after incubation at 25°C for 5 days, the numbers of yeasts were determined by surface plating.

The samples were prepared and analyzed in duplicate. Yeast colonies were defined based on their morphology (color of the colonies, smoothness of surface, consistency, regularity of border, etc.), to obtained single colonies yeast isolates streaked on yeast potato dextrose agar media (2% peptone, 1% yeast extract, 2% dextrose and 1.5% agar), incubated at 25°C for 5 days and then purity of colonies were checked [17].

Yeast species counts were calculated as number of colony forming units per gram of sample and were reported as Log₁₀ cfu g⁻¹. Yeast colonies were

preserved on malt extract agar (Merck) for being identified after purifying the colonies [1].

Identification of isolated yeast colonies

Conventional methods used for identification of yeast species, colonies which show differences in color, shape and size were selected and examined microscopically to observe typical yeast cell morphology. The suspected *Candida spp.* colonies were examined using another parameters like germ tube forming and growing on corn meal agar for the production of Chlamydospores by *Candida albicans* as recommended by Kurtzman *et al.* [18]. The biochemical reactions were performed according to Cruickshank *et al.* (1994) they were concluded by using sugar fermentation and urease tests to Complete identification of different yeast isolates [19].

Determination of virulence factors

Candida strains were tested for detection of Esterase production, Phospholipase production and Hemolytic production as virulence factors.

Esterase production

Esterase production was assayed by using Tween 80 opacity test medium with 10 g Bactopepton according to Tirunarayana and Lundbeek [20], , 0.1 g CaCl₂, 5 g NaCl and 15 g of agar in 1 ml distilled water, prepared at pH of 6.8 then autoclaved. After cooling the medium to (50 C°), 5 ml of Tween 80 was added. After inoculation of yeast colonies, the plates were incubated at 37 C° for 5 days .Detection of lipolytic activity on all substrate plates was performed by observing zones of precipitation around the colonies when viewed by transmitted light (21).

Phospholipase production

The production of phospholipase was determined by a method of *Bulhoes et al.* [22]. Sabouraud dextrose agar (20 ml) supplemented with 0.005 mol/L Calcium Chloride, 1mol/L Sodium Chloride and 8% sterile egg yolk emulsion was added to the plates of medium. A single colony of each yeast isolate was inoculated onto the surface of the medium. The plates were incubated at 37 C° for 5 days. As a positive control *Candida albicans* ATCC 10231 was used.

Hemolytic production:

Hemolytic production was assayed according to a method used by *Luo et al* [23]. A loopful of the stock culture was streaked onto SDA and incubated at 37C° for 18 h. The yeast cultures were collected and washed with sterile saline and prepared as yeast suspension with an inoculums size of 10⁸ cells/ml was, then the prepared yeast suspension inoculated on blood agar. The plates were incubated at 37C° in 5% CO₂ for 48 h. after incubation the presence of translucent halo around the colonies of inoculums indicate positive Hemolytic production.

RESULTS AND DISCUSSION

The quality and safety of food products may be affected by yeasts. Many species of yeasts have been used traditionally for the preparation of beer, wine and bread, also during ripening of cheese some yeasts have been contributed to obtain the desirable flavor and used as starter cultures [24]. Several yeasts metabolize organic acids and lead to ferment foods causing an increase in the pH, which enhance growth of pathogenic bacteria and spoilage [25].

In this study (88) isolates of yeast were cultured from (70) cheese (Gibin Al Arab) samples, All showed occurrence of varying types of yeast species, all had count > 1 Log CFU/g of tested cheese samples, the total yeast count varied between 1.5 to 2.5 Log CFU/g.

It is obvious from Table (1) that species belong to genus of *Candida* form half of the isolates 44/88 at rate of 50% (*Candida parapsilosis* 19.0%, *Candida albicans* 13.6%, *Candida tropicalis* 10.5%, *Candida krusei* 8.5%), while *Geotrichum candidum* found in the second order 18/88 (20.5%), *Rhodotorula* species 17/88 (19.4%) and *Saccharomyces cerevisiae* was 3/88 (3.3%), the rest 6/88 were isolated unidentified mixed yeasts found as contaminant in the samples at rate of 6.7%, the yeast species identified depending on conventional methods including study of morphology of colonies on Sabouraud agar and microscopic characters using Gram and Lactophenol cotton blue staining method.

Table1. Incidence of yeast species isolated from local cheese (Gibin Al-arab) in Diyala Governorate.

Yeast species	Number of isolates	Percentage (%) of isolates
<i>Geotrichum candidum</i>	18	20.5
<i>Rhodotorula</i>	17	19.4
<i>Candida parapsilosis</i>	16	18
<i>Candida albicans</i>	12	13.6
<i>Candida tropicalis</i>	9	10.5
<i>Candida krusei</i>	7	8
<i>Saccharomyces cerevisiae</i>	3	3.3
Mixed unidentified yeast	6	6.7
Total	88	100,0

These results are in accordance with Soliman and Aly (2011) [6] who isolated *Trichosporum cutaneum* and several species of *Candida* genus, *Geotrichum candidum*, *Rhodotorula* species and also *Saccharomyces cerevisiae* yeast species using conventional method from Karish cheese. The most prevalent yeast species was *Candida parapsilosis* at percentage of 18%, the incidence of the pathogenic yeast *C. albicans* from this type of cheese was in percentage of (13.6 %), this finding was higher from Neveen SM *et.al*, founding (2011) [6] who isolated *C.albicans* from cheese in percentage of 2%, while it was close in rate of *S.cerevisiae* as 3%, and are in line with Refai M *et.al*,(1996) [26], who found that the predominant identified yeasts belong to genera of

Candida and *Geotrichum candidum* also these findings are in accordance with Hakim AS *et al.* (2013) [27], who report the presence of *C.albicans* and *Geotrichum candidum* as predominant species among variety types of yeast isolates from cheese in Egypt which were at a percentage of 19.25% from total of 50 cheese samples also they isolated *Candida albicans*, *C.krusei*, *C.tropicalis* and *C. parapsilosis* in first order and other yeast species like *Cryptococcus neoformans* and *Trichosporum cutaneum* at the second order, while disagree with our results in which referred to the isolation of *Candida spp.* as a predominant yeast isolates. The incidence of such species of pathogenic yeast in dairy products like cheese may refer to poor hygienic conditions and unsuitable procedures of cleaning showing the need for improved sanitization procedures [28].

To determine the virulence factors of species belong to *Candida* genus we confirmed conventional methods including biochemical activities, Sugar assimilation, Sugar fermentation to identify *Candida* species and germ tube test to verify species of *Candida albicans* as it showed in table (2).

Table2. Biochemical characteristics of yeasts species isolated from Gibin Al-arab.

Candida species	Used tests									
	Urease	Germ Tube	Sugar assimilation				Sugar fermentation			
			Maltose	Sucrose	Lactose	Glucose	Maltose	Sucrose	Galactose	Glucose
<i>C.parapsilosis</i>	-	-	+	+	-	+	-	-	-	+
<i>C. albicans</i>	-	+	+	+	-	+	+	-	+	+
<i>C.tropicalis</i>	-	-	+	+	-	+	+	+	+	+
<i>C.krusei</i>	+	-	+	-	-	+	-	-	-	+

Many researchers have shown that prevalent yeasts typically associated with cheeses are *D. hansenii*, *K. marxianus*, *Yarrowia lipolytica* and several *Candida spp.* [29]. *Candida spp.* are the most common cause of fungal infection in immune compromised persons known as Candidiasis, predominantly caused by *C.albicans*, they may be detected in 40 to 65% of normal fecal flora, *C.albicans* can infect all areas of the skin as well as the mucous membranes [30].

Germ-tube formation is a virulence factor for *C.albicans* and also is a good tool for identification of this species [31]. High production of Germ tube among the *C.albicans* isolates in the current study refer to the pathogenicity of these isolates.

Table3. showed virulence factors as Esterase production, Phospholipase and Hemolytic production in *Candida* isolates Esterase production was higher in *C.tropicalis* 33.3% followed by *C.albicans* 16.6%, no Esterase

production was found in *C.parapsilosis* and *C.krusei* . Phospholipase production detected in *C.parapsilosis* in the first order at rate 62.5%, *C.tropicalis* produce Phospholipase in the second order at rate of 55.5% and *C.albicans* found at rate of 8.3% ,*C.krusei* isolates were negative according to produce phospholipase. *C.albicans* had the higher rate of Hemolytic production at percentage of 91.6%, half of the tested *C.parapsilosis* isolates showed positive results 50%, isolates of *C.tropicalis* had this activity at rate of 44.4% while *C.krusei* had no Hemolytic production.

Table3. Virulence factors of *Candida species* isolated from Gibin Al-arab cheese.

Virulence factors		Number of <i>Candida spp.</i> isolates			
		<i>C. parapsilosis</i> (16)	<i>C. albicans</i> (12)	<i>C. tropicalis</i> (9)	<i>C. krusei</i> (7)
Esterase production	Positive	0	2	3	0
	% :	0	16.6	33.3	0
Phospholipase production	Positive	10	1	5	0
	% :	62.5	8.3	55.5	0
Hemolytic production	Positive	8	11	4	0
	% :	50	91.6	44.4	0

Among the virulence factors analyzed in this research; Esterase, Phospholipase production and Hemolytic production we found that most of the tested *Candida* isolates, with the exception of *C.krusei* which showed absence of these factors.

Cheese have been classified as a high risk potential hazard according to the International Commission of Microbiologic Specifications for Foods in 2005. The careless in hygienic measures during production of cheese from contaminated milk and the wrong handling during processing lead to increase count of yeast in these products, also improper time and temperature during production and unsuitable storage conditions of cheese also the pH of cheese is suitable for growth of many wide distributed yeasts in the environment [32].

The precipitates found around colonies as a result of hydrolysis of the Tween compound in the medium. Subsequent to the cleavage of Ester bonds, insoluble Calcium salts formed after releasing of fatty acids which

combined with Calcium ions in the medium (21). This study revealed the activity of secretion of lipolytic enzymes such as Esterase and showed high percentage for *C.tropicalis* and for *C.albicans* at second rate 16.6% while *C.parapsilosis* and *C.krusei* had no activity of Esterase secretion, this results agree with Aktas E., (2002) [33], who studied the Esterase production of various species of *Candida* and show that all of *C.tropicalis* and most of the *C.albicans* isolates were positive to this activity. Several genes are coding virulence factors like Proteinase secretion and Phospholipase production identified in *Candida albicans* have homologues genes in other *Candida spp.* [34].

The study of virulence factor like production of extracellular enzymes is necessary to understand the pathogenic role of infecting *Candida spp.* extracellular hydrolytic enzyme production is one of the virulence factors associated with the ability of *Candida spp.* to cause infections by enhancing colonization and invasion of host tissue [14]. The activity of phospholipase has been identified in many fungal pathogens including *candida spices* [34]. Phospholipase production has been identified in this study in isolates of *C. parapsilosis* 62.5% as higher rate, then *C.tropicalis* 55.5% while isolates of *C.albicans* report lower rate 8.3%, the result of this study did not agree with Hakim et al.(2013) [27] who revealed to the detection of phspholipase production in all tested *C.albicans* isolated from cheese samples but it agree in having the other *Candida spp;* isolates to this enzymes in the same research.

The ability of *C. albicans* to produce Hemolysin and germ tube may be associated with its pathogenic potential [35], the current study refer to the ability of almost all of *C.albicans* isolates 91.6% , half of *C.parapsilosis* 50% and about 44.4% of *C.tropicalis* isolates to produce hemolysine. The data obtained by Negri et al.(2010) [35] suggest that the capacity of *C. albicans* to produce germ tubes and hemolysins may be a factor in its pathogenicity . Sachin et, al. (2012)[36] concluded that about half of the clinical isolates (51.8%) of *Candida albicans* and non- albicans *Candida species* produce extracellular hydrolytic enzymes like haemolysin, there is no studies about the ability of *Candida Spp.* isolated from cheese to produce such enzymes to compare with, since this research is one of the first studies in the field of isolation pathogenic species of *Candida* from dairy product and their ability to have extracellular enzymes activities.

CONCLUSIONS

From this research, it could be concluded that the high percentage of yeast species like *Candida albicans* and *Geotrichum candidum* also the other species of *Candida* isolated from the most consumed cheese type in Diyala which called (Gibin Al Arab) refer to the importance of having caution when dealing with the raw materials used and preparing of such types of locally produced cheese when it can be a good medium to grow such pathogenic

microorganism which may threat human life, and revealed the great need to apply more hygienic conditions during handling , storage and/or selling form in the markets.

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