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 researchaimed 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%), *Rhodotorula* species (19.4%), *Candida parapsilosis* (18%), *Candida albicans* (13.6%), *Candida tropicalis* (10.5%), *Candida krusei* (8%), *Saccharomyces cerevisiae* (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.
have been many reports about the bacterial composition of these products [3,4,5]. little is known on the distinction of yeasts associated with cheeses [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 refrigerater 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 Balhoes 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 37°C for 18 h. The yeast cultures were collected and washed with sterile saline and prepared as yeast suspension with an inoculums size of 10⁷ cells/ml, then the prepared yeast suspension inoculated on blood agar. The plates
were incubated at 37°C 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 affected by yeasts. Many species of yeasts have been used traditionally for the preparation of beer, wine and bread, also during ripening of 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% (Candia parapsilosis 19.0%, Candida albicans 13.6%, Candia tropicalis 10.5%, Candia krusei 85%), 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 Sabouraudagar and microscopic characters using Gram and Lactophenolcotton blue staining method.

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

| Yeast species          | Number of isolates | Percentage (%) |
|------------------------|--------------------|----------------|
| Geotrichum candidum    | 18                 | 20.5           |
| Rhodotorula            | 17                 | 19.4           |
| Candia parapsilosis    | 16                 | 18             |
| Candida albicans       | 12                 | 13.6           |
| Candida tropicalis     | 9                  | 10.5           |
| Candia krusei          | 7                  | 8              |
| Saccharomyces cerevisae| 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 Candia parapsilosis at percentage of 18%, the incidence of the pathogenic yeast C. albicans from this type of cheese wasin 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 on 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 founding are in accordant 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 Trchosporum 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 | Sugar assimilation | Sugar fermentation |
|-----------------|------------|--------------------|--------------------|
|                 | Urease     | Ge rm Tube         | Maltose Sucrose    | Glucose Maltose Sucrose Galactose Glucose |
| C.parapsilosis  | -          | -                  | + + +              | - - + + + + + |
| C. albicans     | -          | +                  | + + +              | - - + + + + |
| C.tropicalis    | -          | +                  | + + +              | + + + + + + |
| C.krusei        | +          | -                  | - - +              | - - - - + + |

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 this species [31]. High production of Germ tube among the *C. albicans* isolates in the current study refer to the pathogenicity of these isolates.

Table 3 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 a rate of 62.5%. *C. tropicalis* produce Phospholipase in the second order at rate of 55.5% and *C. albicans* at rate of 8.3%. *C. krusei* isolates were negative according to producing 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 50.0%, isolates of *C. tropicalis* had this activity at rate of 44.4% while *C. krusei* had no Hemolytic production.

### Table 3. Virulence factors of *Candida* species isolated from Gibin Al-arab cheese.

| Virulence factors          | Number of *Candida* spp. isolates |
|----------------------------|-----------------------------------|
|                           | *C. parapsilosis* (16) | *C. albicans* (12) | *C. tropicalis* (9) | *C. krusei* (7) |
| Esterase production       | Positive 0: 0 | 2 | 3 | 0 |
|                           | Percentage 0: 0% | 16.6 | 33.3 | 0 |
| Phospholipase production  | Positive 10: 62.5 | 1 | 5 | 0 |
|                           | Percentage 62.5% | 8.3 | 55.5 | 0 |
| Hemolytic production      | Positive 8: 50 | 11 | 4 | 0 |
|                           | Percentage 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 of 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 sp*.[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 phospholipase 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 hemolsins 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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