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POTENTIAL PATHOGENIC YEASTS ISOLATED FROM FRESH DATE FRUITS (RUTAB)

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Fifty samples of fresh date fruits showing signs of microbial spoilage were collected from Hofuf City markets in Saudi Arabia. Yeasts involved in spoilage were counted by inoculation on PDA plates and incubation at 25°C for 2-3 days, and then isolates of the dominant strains were identified. Potentially pathogenic yeasts were involved in the spoilage of six out of the 50 samples tested (12%). The yeast counts in these samples were in the range 10^4 to 10^7 CFU/g. The pathogenic yeasts constituted 12 to 100% of these counts, i.e. some samples were spoiled by the pathogenic yeasts only. The dominant pathogen was *Candida tropicalis* found in three of the six spoiled samples representing 12, 75 and 100% of the yeast population involved in spoilage. *C. krusei, Issatchenkia orientalis* and *C. albicans* were each found in one sample making 14.7, 17 and 25.3% of the yeast population detected in the spoiled samples, respectively.
Keywords: Date fruits, spoilage microflora, pathogenic yeasts, candidiasis

INTRODUCTION

Date palms (*Phoenix dactylifera* L.) are by far the most important food crop produced in the Kingdom of Saudi Arabia. The country is ranked as the second largest producer of date fruits in the world with **1,122,820** metric tons annual production (FAO, 201**6**). The fruit becomes ripe and edible in two stages, namely the *Rutab* and the *Tamr* stages. *Rutab* contains 35 to 40% moisture and 45 to 48% sugars (dry matter basis) while *Tamr* contains 10 to 15% moisture and 60 to 88% sugars (dry matter basis) (Barreveld, 1993). Hence *Tamr* is generally regarded as stable while *Rutab* is highly susceptible to microbial spoilage. Studies on the microbiological contamination and spoilage of date fruits are limited. Reports on microbial contamination of *Tamr* describe aerobic mesophilic bacteria, yeasts, molds, coliforms, *Staphylococcus aureus, Aspergillus flavus* and lactic acid bacteria as the main contaminants (Bolin *et al.,* 1972; Abu-Zinada and Ali, 1982; El-Sherbeeny *et al.,* 1985; Nussinovitch *et al.,* 1989; Aidoo *et al.,* 1996; Shenasi *et al.,* 2002). Little work is done on the microbial spoilage of date fruits, which is mainly caused by molds, yeasts and lactic acid bacteria (Hamad, 2012; Hamad, 2008; Kader, 2007).

Though yeasts are described as important spoilage agents of date *Rutab*, no reports about pathogenic yeasts associated with this fruit are found in the literature cited. Candidiasis, caused by yeasts, usually occurs in persons with impaired immune response or when the normal body microflora is suppressed (e.g. by antibiotics). The disease affects mucosa, outer skin and sometimes inner organs of the human body such as the mouth and vagina (Kayser *et al.,* 2005; Singleton and Sainsbury, 1997). *Candida albicans* infects human bodies, especially the skin and the gastrointestinal and genitourinary tracts, causing the majority of *Candida* bloodstream infections (candidemia) (Pfaller *et al.,* 2006). *Candida krusei* is related to diarrhea in young children and occasionally to systemic diseases. It is reported to colonize gastrointestinal, respiratory and urinary tracts of humans (Rippon, 1988), and was also isolated from foods like beer, milk products, pickle brine, and from skins and feces of animals and birds (Kreger-Va, 1984).

This study was carried out to investigate the presence of potential pathogenic yeasts among the spoilage microflora of date fruits at the *Rutab* stage.

MATERIALS AND METHODS

***Samples collection*:** Fifty fresh date fruit (*Rutab* stage) samples from different varieties showing signs of microbial spoilage were collected in sterile bottles from 10 outlets in Hofuf City markets in Saudi Arabia. The samples were transported within one hour to the laboratory and analyzed for microbiological content on the same day.

***Microbiological analysis*:** Samples of *Rutab* (10 g) were weighed into sterile stomacher bags, 90 ml sterile peptone water (CM0009, Oxoid, Basingstoke, UK) added and homogenized in a stomacher (Lab-Blender 400, Seward Medical, Worthington, UK) for 45 second. Aliquots (1.0 or

0.1 ml) were plated in duplicate as 10-fold dilutions in peptone water. Yeasts were grown on potato dextrose agar plates (PDA; CM0139, Oxoid) at 30°C for 3 days, and molds on PDA plates at 20 to 30°C in 3 to 7 days. Lactic acid bacteria (LAB) were enumerated on deMan Rogosa Sharpe agar for lactobacilli (CM0361, Oxoid) and on M17 agar for streptococci and lactococci (CM0785, Oxoid). The plates were incubated in anaerobic jars with anaerobic gas generating kits (BR0038, Oxoid) for 2 to 3 days at 30°C. For identification of yeasts, colonies of different forms were counted, and then 5 isolates were made from each colony form. The isolates were finally purified by successive streaking on PDA plates and the pure cultures kept in the refrigerator (4±1°C) in PDA test tubes for identification.

***Identification of yeasts*:** The yeast isolates (30 isolates) were identified according to Barnett et al. (2000). First, microscopic examination of the appearance of non-filamentous vegetative cells grown in shake flasks in malt extract broth (CM0057) for 2 days at 25°C, and microscopic examination for filamentous growth using the slide culture technique was performed. The isolates were then examined for glucose fermentation in Durham tubes and for sporulation on; malt-yeast-glucose-peptone (YM agar), Gorodkowa agar, McClary acetate agar, Malt extract agar. The plates incubated at 25°C and examined after 3 days for up to 6 weeks. After that, the appropriate subsequent tests according to Barnett identification keys 1, 2, 3, and 4 (among 14 fermentation, 47 aerobic growth (auxanograms), 10 nitrogen source, 10 vitamin requirement, 7 growth temperature, 2 cycloheximide concentration, urea hydrolysis, 2 NaCl concentration and 2 glucose concentration tests) were performed (all chemicals used were from Sigma). Using the results of the above mentioned tests, the isolates were then identified according to Barnett identification keys.

RESULTS

Potential pathogenic yeasts were found in 6 out of 50 spoiled tested *Rutab* samples (Table 1), representing 12% of total samples. In sample 4, the yeast population involved in spoilage was $2.4x10^5$ CFU/g, of which the potential pathogenic yeasts formed about 75% ($1.8x10^5$ CFU/g). Molds at a load of $8.8x10^5$ CFU/g were also involved in the spoilage of this sample. Since the microbial load causing food spoilage is about 10^6 cells/g.ml.cm^2 (Ray, 2004), this sample can be regarded as spoiled. The relatively high load of the potential pathogenic yeasts ($1.8x10^5$ CFU/g) indicates their substantial involvement in sample spoilage. Sample 14 was spoiled by mixed population of lactic acid bacteria at a load of $4.2x10^5$ CFU/g, and yeasts at $3.8x10^6$ CFU /g (Table 1). The load of the pathogenic yeasts was $9.6x10^5$ CFU/g making about 25.3% of the total yeast load. This is a relatively high load and indicates that these pathogenic yeasts significantly contributed to the spoilage of the sample. The spoilage of sample 17 was caused by molds at a load of $5.7x10^5$ CFU/g and yeasts at $4.7x10^5$ CFU /g. The pathogenic yeasts constituted about 17.0% of the yeast population with a load of $8.0x10^4$ CFU/g, which is a relatively low load indicating that the contribution of the pathogenic yeasts to the spoilage of the sample was relatively weak. Sample 20 was spoiled by yeasts only, with a load of $6.2x10^7$ CFU/g. The pathogenic yeasts represented about 12% of the spoilage yeasts population with a load of $7.5x10^6$ CFU/g, meaning that they significantly contribute to the sample spoilage. Sample 41 was ~~also~~ spoiled by yeasts only, with a load of $5.3x10^6$ CFU/g for total yeast count and $7.8x10^5$ CFU/g for the potential pathogenic yeasts. The pathogenic yeasts made about 14.7% of the total yeast population, indicating their moderate contribution to the sample spoilage. The spoilage of sample 48 was caused by a mixed population of molds and lactic acid bacteria at loads of $2.6x10^5$ and $5.6x10^6$ CFU/g, respectively, and yeasts at $8.3x10^4$ CFU/g. All yeast strains were pathogenic with their low count designates there relative weak contribution.

Table 1. Presence of pathogenic yeasts together with other spoilage microbes in 6 of the 50 spoiled *Rutab* fruit samples.

Sample No.	Microbial load (CFU/g)				
	Total yeasts	Patho-genic yeasts	% pathogen ic yeasts	Lactic acid bacteria	Molds
4	$2.4x10^5$	$1.8x10^5$	75%	n.d.	$8.8x10^5$
14	$3.8x10^6$	$9.6x10^5$	25.3%	$4.2x10^5$	n.d.
17	$4.7x10^5$	$8.0x10^4$	17.0%	n.d.	$5.7x10^5$
20	$6.2x10^7$	$7.5x10^6$	12.0%	n.d.	n.d.
41	$5.3x10^6$	$7.8x10^5$	14.7%	n.d.	n.d.
48	$8.3x10^4$	$8.3x10^4$	100%	$5.6x10^6$	$2.6x10^5$

***Identification of yeast isolates*:** Thirty yeast isolates were obtained from six spoiled date fruit samples in the *Rutab* stage (5 isolates from each sample, showing identical colony form) were identified according to Barnett *et al.* (2000) using morphological and physiological tests. The 5 isolates from each sample were found to represent one yeast strain with identical biochemical profiles. The isolates from samples 4, 20 and 48 (isolates 4, 20 and 48, Tables 2 and 3) were identified as *Candida tropicalis.* They represented three different strains as shown by differences in their biochemical profiles (Table 3). The isolates from samples 14, 17, and 41 (isolates 14, 17 and 41, Tables 2, 3) were identified as *Candida albicans*, *Issatchenkia orientalis* and *Candida krusei* (asexual state of *I. orientalis*, Barnett *et al*. 2000), respectively. All of these yeast strains were osmotolerant showing growth at 50 and 60% glucose concentration (Table 3), and were sufficiently thermotolerant growing at temperatures up to 45°C (results not shown). They are therefore well adapted to growth on date fruits with their high sugar content and in the hot environment of the Kingdom of

Table 2. Morphological characteristics of yeasts isolated from spoiled *Rutab* samples.

Yeast isolates	Morphological characteristics			
	Colony	Vegetative cells	Filaments	Sexual reproduction
Isolates 4 Identified as *C. tropicalis*	Butyrous and cream in color	Budding, pseudohyphae	+[a]	-[b]
Isolates 14 Identified as *C. albicans*	Butyrous and white in color	Budding	-	-
Isolates 17 Identified as *Issatchenkia orientalis*	Butyrous and white in color	Budding, pseudohyphae	+	2 to 3 round ascospores
Isolates 20 Identified as *C. tropicalis*	Butyrous and cream in color	Budding, septate hyphae	+	-
Isolates 41 Identified as *C. krusei*	Butyrous and cream in color	Budding, long chains	-	-
Isolates 48 Identified as *C. tropicalis*	Butyrous and cream in color	Budding, pseudohyphae	+	-

[a]Positive results, [b]Negative results

Table 3. Biochemical profiles of the isolates (all of them ferment glucose) compared to relevant yeasts.

Organism	Maltose	D-Galactose	D-Xylose	L-Rhamnose	Sucrose	Trehalose	Citrate	Lactose	Raffinose	DL lactate	Starch	Erythritol	Methanol	2-keto-gluconate	5-keto-gluconate	50% Glucose	60% Glucose	Ethylamine	Glucosamine (N)	Nitrate	0.1% CHI	10% NaCl	16% NaCl
Isolates 4 Identified as *C. tropicalis*	+	+	+	-	+	+	+	-	-	-	+	-	-	-	+	+	-	+	-	-	+	-	-
Isolates 14 Identified as *C. albicans*	+	+	-	-	+	+	-	-	-	+	-	-	-	-	+	+	+	-	-	-	+	+	-
Isolates 17 Identified as *Issatchenkia orientalis*	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	+	-
Isolates 20 Identified as *C. tropicalis*	+	+	+	-	+	+	+	-	-	-	-	-	-	+	-	+	+	+	-	-	+	-	-
Isolates 41 Identified as *C. krusei*	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	+	-	-	-	+	-
Isolates 48 Identified as *C. tropicalis*	+	+	+	-	-	+	+	-	-	+	+	-	-	+	-	-	-	+	-	-	+	-	-

+ = positive growth , - = negative growth, D = delayed growth, C. = *Candida*, I. = *Issatchenkia*, CHI = Cycloheximide

Saudi Arabia. Furthermore, all of these yeast species are described as pathogenic causing different forms of candidiasis (Bourgeois *et al*., 2010; Kothavade *et al*., 2010; Kayser *et al.*, 2005; Kockova-Kratochvilova, 1990). Candidiasis usually occurs in persons with impaired immune response or when the normal body microflora is suppressed (e.g. by antibiotics). The disease affects mucosa, outer skin and sometimes inner organs of the human body such as the mouth, vagina etc. (Kayser *et al.*, 2005; Singleton and Sainsbury, 1997).

Potential pathogenic yeasts were isolated from 6 out of 50 spoiled *Rutab* samples. They represent 12 to 100% of the detected yeast population in the samples and contributed in varying degrees to their spoilage. The dominant pathogen was *Candida tropicalis* found in three of six spoiled samples. *C. albicans, Issatchenkia orientalis* and its asexual state *C. krusei* were both found in one sample. All of these yeast strains were thermotolerant and osmotolerant, hence well adapted to growth on dates and in the hot environment of the Kingdom of Saudi Arabia. It could, therefore, be said that these potential pathogens are spreading widely in date fruits initiating a significant human health hazard. Development of rutab fruits handling processes capable in reducing the extent of contamination with these pathogenic yeasts is urgently needed. According to the Saudi

DISCUSSION

specifications for microbial levels in foods, no more than 10 to 100 yeast cells/g should be detected in 2 out of 5 replicates tested for a sample of dates (SASO, 1999). Aleid

et al. (2012) evaluated the utilization of modified atmosphere packaging to extend the shelf life of Khalas fresh dates. They observed that the washed khalal samples exhibited low yeast and mold counts (<10 CFU / g). Special care should be taken during harvest which occurs in the windy dry season in Saudi Arabia. Moreover, preventive measures should be implemented in traditional fresh dates markets in Saudi Arabia to overcome poor hygienic conditions.

Candida tropicalis and *Issatchenkia orientalis* were isolated from wine and Korean grape wine pomace (Seo *et al.*, 2007). One of the characteristics of these microbes is acid and ethanol tolerance (Okuma *et al*., 1986; Kim *et al.*, 2008). According to Basu *et al*. (2011) *Candida tropicalis* was isolated from blood and urine of patients admitted to intensive care units of different hospitals. *Candida albicans* is a constituent of human flora, coexisting on skin and the gastrointestinal and genitourinary tracts, causing the majority of *Candida* bloodstream infections (candidemia) (Pfaller *et al.*, 2006). *Candida krusei* is related to diarrhea in young children and occasionally associated with systemic disease. Also, it is reported to be colonizing gastrointestinal, respiratory and urinary tracts in patients (Rippon, 1988.). *Candida krusei* was isolated from foods like beer, milk products and pickle brine as well as from skins and feces of animals and birds (Kreger-Va, 1984). *Issatchenkia orie*ntalis was isolated from camel's milk (Rezki *et al.*, 2013). Some date fruit varieties contain antimicrobial components such as tannins which can inhibit the growth of many fungi and bacteria strains on fruits (Nelson *et al.*, 1997; Ishida *et al*., 2006).

***Conclusion*:** Potential pathogenic yeasts are widely spread in harvested date fruits in Saudi Arabia, hence presenting a significant health hazard. They also contribute to spoilage of date fruit and may represents up to 100% of the yeast population involved in spoilage. The dominant pathogen is *Candida tropicalis* followed by *C. albicans, Issatchenkia orientalis* and its asexual state *C. krusei*. These yeasts cause varying forms of illness to humans. Improved Rutab fruits handling methods that help in reducing the extent of contamination with these pathogenic yeasts is urgently needed.

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