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Characterization of Yeast Isolated from *Berkeep*, a Traditional Fermented Milk Product of Darfur State, Western Sudan

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Abstract

Berkeep is a naturally fermented yoghurt-like milk product consumed as common food in parts of the Darfur State, Western Sudan. Its production and consumption derive much food security and economic benefits to the rural people in the region. The objective of this study was to isolate and characterize yeasts of Berkeep by both conventional and molecular-based methods. A total of 220 yeast isolates, were obtained from 75 samples of Berkeep from three production sites namely, Elkoumah, Manwashy and Kass in Darfur state, were purified then characterized to species level by molecular tools. Based on ITS-PCR using the forward primer Cy5-Y-ITS1 and reverse primer Y-ITS4, the isolates were screened into five groups. Representative of each group was selected for sequence analysis of ITS rRNA gene. The gene sequence was subjected to nucleotide BLAST. The yeast microbiota of Berkeep was dominated by *Kluyveromyces marxianus* (YG4, 85 isolates) which showed 100% homology towards *Kluyveromyces marxianus* isolate QKM4. Sixty-eight (Group YG5) identified as *Saccharomyces cerevisiae* showed 97.22% homology towards *Saccharomyces cerevisiae* isolate JKSP39. Thirty-two isolates Group YG3 revealed 100% homology towards *Pichia kudriavzevii* isolate Sugarcane. Eleven isolates, (YG2) showed 99.37% homology towards *Pichia kudriavzevii* strain YS179. The twenty-four isolates of group, (YG1) recorded 99.70% similarity towards *Candida intermedia* isolate B-NC-12-f 16i. Very minute variation was observed within the three production sites concerning the yeast count and type of yeast species.

Keywords: Berkeep fermentation, Yeasts, ITS Gene

Introduction

Traditional fermentation is well known to serves many purposes. It enrich the diet through development of a diversity of flavors, aromas, and textures in food substrates hence improving the taste giving rise to bland food, enhance the digestibility of a food that is difficult to assimilate, preserve substantial amounts of food from degradation by noxious organisms through lactic acid, alcohol, acetic acid, and alkaline fermentation(Sarkar and Nout,2014) , enrich food substrates with protein, essential amino acids, and vitamins and to reduce cooking time and the associated use of fuel (Steinkraus, 1995).

Only a very limited recent research was conducted using the present state-of-the-art technology for identifying of the fermenting microbial flora of local products (Bacha *et al*., 2010, Anteneh *et al*., 2011).

A wide variety of fermented foods and beverages are consumed in Sudan being prepared from a wide range of raw materials using traditional techniques. Fermented milks have been acclaimed both by popular wisdom and by some research findings for being more nutritious and health-promoting than fresh milk (Abdelgadir *et al*., 1998). In Sudan many of these fermented milk products are available including *Rob, Gariss, old milk, zabadi, biruni* and *Mish* (Dirar, 1992). Some of these products are common and well known all over the country but others are confined to some areas like Berkeep.

Microbiological researches performed in fermented dairy products, revealed that the fermentation microbiota consisted of both lactic acid bacteria and yeasts. Due to their tolerance of low pH, low water activity, and high salt concentrations, low storage temperatures, yeasts can grow well during the fermentation of dairy products (Roostita and Fleet, 1996; Ferreira and Viljoen, 2003; Banjara *et al*., 2015).Yeasts are particularly promising because they are not affected by antibacterial agents (Caselli *et al*., 2013), and this property is relevant since some therapies combine the administration of probiotics with antibiotics in the treatment of gastrointestinal infections. The use of yeasts is also advantageous because their genetic material cannot be transferred to commensal bacteria (Czerucka *et al*., 2007), have long been considered safe for applications (Diosma *et al*., 2014), and their beneficial effects include competitiveness for nutrients, better cell adhesion ability (in consequence of its size), production of antagonistic compounds, immunomodulation, cholesterol assimilation, toxin elimination and then neutralization of pathogenic bacteria (Ceugniez *et a*l., 2015; Moslehi *et al*., 2016).

One of the most popular and widely consumed fermented milk product confined to Darfur, Western Sudan, is Berkeep. This product is characterized by its nice flavor, pleasant aroma and taste and smooth texture. Lactic acid bacteria responsible for its fermentation was identified by Musa *et al*. (2023). The aim of this study is to isolate and characterize the yeast flora that share the fermentation process of Berkeep.

Materials and Methods

Sampling

A total of 75 samples of Berkeep were collected randomly from traditional production sites in Kass, Elkoumah, and Manwashy (25 samples each). All samples were collected in sterile containers and transported immediately in an ice chest to the Microbiology Laboratory, Faculty of Agriculture, University of Khartoum and were analysed upon arrived.

Yeast count and isolation

From original Berkeep fermented milk, 10 ml of each sample was sterilized diluted with peptone water, plated on potato dextrose agar (PDA) (Himedia, India) containing sterile 10% tartaric acid and incubated aerobically at 28°C±2° C for 72 h. Colonies were counted by using a colony counter and the results were presented as $\log_{10}$ cfu/ml^{-1}.Yeast colonies were white, Creamy, circular, convex and elevated. Representative colonies were selected based on colony morphology aiming at selecting colonies of varying morphology, then examined by microscopy, purified by successive streaking on PDA and stored at 4 C° for short term storage and in potato dextrose broth (PDB) with 15% (v/v) glycerol at −80°C for long term storage.

Identification of yeast isolates

Two hundred and twenty purified yeast isolates were identified phenotypically according to their (a) colony morphology where color, texture and other features were observed(b) yeast cell microscopy, where isolates were stained using methylene blue and viewed under high power microscope (100× magnification) (Schillinger and Lucke 1987;Iranmanesh *et al.*, 2014) (c)biochemical test, catalase and oxidase reactions, acid and CO_2 production from Glucose and Lipolytic activity were carried out (Harrigan, 1998).

Molecular characterization of yeast

Genomic DNA Extraction: Genomic DNA was extracted by the method as described by (Doyle and Doyle, 1987).

Amplification of ITS rRNA of yeast isolates

Yeasts isolates (220 isolates) were screened into groups by polymerase chain reaction (PCR) with primers that amplified the intergenic transcribed spacer (ITS) region of the rRNA gene. The 18S-28S ITS region was amplified using the fluorescein labeled Cy5-Y-ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) forward primer and Y-ITS4 (5´TCCTCCGCTTATTGATATGC-3) reverse primer (T-A-G Copenhagen APS, Denmark) (Abdelgadir *et al.*, 2001). Maxime PCR PreMix Kit (i-Tag) for 25 µl, run was used to achieve the PCR process with a slight modification of extra addition of 2.5µl of $MgCl_2$.

The PCR cocktails (25 µl) consisted of 1 µl of the primers, 5µl of DNA, 2.5 U i-Tag DNA polymerase, 2.5 mM of each dNTP, 1 X reaction buffer, 1 X gel loading buffer and 2.5 µl of $MgCl_2$.Amplification conditions were initial denaturation at 95° C for 5 minutes, 35 cycles of denaturation at 95° C for 30 seconds, annealing at 55° C for 30 seconds and extension at 72° C for 30 seconds, followed by a final extension at 72° C for 7 minutes. The PCR products were visualized by running in 1.5% agarose gel electrophoresis with 100 bp DNA ladder (Sigma, Saint Louis, USA). The electrophoresis conditions were 100 V, 60 mA, for 30 minutes with 1 X TAE (Tris, Acetate EDTA buffer) as the running buffer.

Cloning and sequencing of ITS1-4 rRNA

One representative of each group was selected for sequencing of the ITS-rRNA region using an automated DNA sequencer with specific primers using the facility at Macrogen Inc (Macrogen Inc., Seoul, Korea).

Identification of isolates

The search in database of ITS rRNA genes sequences was performed in GenBank NCBI (http://www.ncbi.nlm.nih.gov/) using the BLAST program of references yeasts.

Statistical analysis

Data were analyzed by One Way ANOVA, using SPSS (2008) version 16 and reported as mean ± SD. The levels will be considered significantly different at ($P < 0.05$).

Results

Phenotypic Characteristics of isolates

From 75 samples of Berkeep from the three different regions in Darfur State, 220 yeast isolates, were obtained and purified. Table 1. Shows that the yeast counts were 7.71±0.57, 7.37±0.36 and 7.40±0.48 in Elkoumah, Manwashy and Kass, respectively. Although Elkoumah has the highest ($P<0.01$) yeast count, but generally the level of yeasts was high suggesting that they play a role in Berkeep fermentation.

Based upon colony morphology on PDA, biochemical and physical characteristics, different yeasts were observed showing several morphotypes i.e. white, creamy, orange, smooth and flat colonies with varying edges, ovoid, spherical and different sizes and different reactions were observed. All isolates were catalase positive, oxidase positive, produced acid from glucose but showed different lipolytic activity and gas production from glucose (Table 2). Based on the above reactions yeast isolates were divided into 4 groups. GroupYG1 10.91% (24 isolates) were ovoid, elongated which appeared alone when seen under the microscope. These isolates were found to belong to the genus *Candida*. Forty-three isolates (19.54%) were ovoid, elongated which appeared alone, pairs and heap. These isolates belonged to the genus *Pichia*. While, 38.64% (85 isolates) of the isolates were ovoid appeared alone, pairs small chain and heap. These isolates were assigned to the genus *Kluyveromyces*. Finally, 68 isolates (30.91) were Ovoid, spherical which appeared alone, pairs and heap. These isolates were belonged to the genus *Saccharomyces.*

Table 1. Yeast counts $\log_{10}$ cfu/mL^{-1} and pH of Berkeep from different production sites

Source of samples	Yeasts spp.	pH
Elkoumah	$7.71\pm.57^{a}$	3.59 ± 0.07^{c}
Manwashy	$7.37\pm.36^{b}$	4.06 ± 0.45^{a}
Kass	$7.40\pm.48^{b}$	3.78 ± 0.19^{b}
Sig.	(0.024) **	(0.000) **

Sig.: Significant different at ($P<0.05$)

**: Highly significant different at ($P<0.01$)

a,b,c: Within the same column followed by different superscript are significantly different at ($P<0.05$).

ITS-PCR results

In this study, two hundred and twenty yeast isolates from Berkeep were ascribed to 5 different species by ITS-PCR profile using Cy5-Y-ITS1 forward primer and Y-ITS4 reverse primer (Table 3, partly shown in Fig. 1). The isolates of each group showed identical fragment sizes.

Cloning and Sequencing of ITS rRNA

Similarity analysis was used to study the relationships between our isolates by comparing their ITS rRNA gene sequences with NCBI sequences available in these databases by BLAST program. The ITS rRNA gene sequence of these isolates exhibited 97.22%−100% (NCBI) similarity to the sequences available in these databases (Table 3).

As observed in Table 3, it is noticed that the genotypic traits appeared to separate Pichia spp. into two strains. GY2, (11 isolates) showed two different ITS bands and assigned as *Pichia kudriavzevii* strain YS179 while the remaining 32 isolates showed only one band and assigned as *Pichia kudriavzevii* isolate sugarcane (Table 3). Accordingly, the ITS rRNA sequencing of the selected yeasts clearly screened into five groups.(Group 1Y)100% similarity towards *Candida* intermedia isolate B-NC-12-OM10, (Group 2Y) showed 99.58% homology towards *Pichia kudriavzevii strain YS179*, (Group 3Y) revealed 100% homology towards *Pichia kudriavzevii* isolate Sugarcane, whereas the majority of isolates (Group 4Y) showed 100% homology towards *Kluyveromyces marxianus* isolate *QKM4* and (Group 5Y) showed 97.22% homology towards *Saccharomyces cerevisiae* isolate *JKSP39*. Thus, these yeasts could be affiliated as *Candida intermedia, Pichia kudriavzevii, Kluyveromyces marxianus and Saccharomyces cerevisiae* according to their similarity.

Sequence accession numbers

The sequences determined in current study have been deposited under Genbank NCBI (http://www.ncbi.nlm.nih.gov/) with accession numbers (YG1: MZRH0200013; YG2: MZRU1NPN016; YG3: MZRYNBYX016; YG4: MZS18WXU016; YG5: MZS4KG3V013.

Discussion

This is the first report on isolation, identification and characterization of yeast from fermented milk Berkeep of Darfur, Western Sudan. Berkeep manufacture is based on the old empirical knowledge of several Darfur indigenous tribes, among which the traditional methods of preparation have little unnoticeable changes. These processing methods involve many microorganisms, which are important sources for the production of bioactive substances during the fermentation. Certain yeasts are used as probiotic or biocatalysts producing compounds of interest (aromas, vitamins, antibiotics) in fermentations.

Berkeep, from the different production sites, contained yeast species, dominated by the *K. marxianus*, *S. cerevisiae*, and *Pichia kudriavzevii* with a small number of *Candida intermedia*. The relatively high yeast counts found during the present study indicate that yeast contribute to the fermentation of milk to produce Berkeep. Besides a direct biochemical action mediated by their proteolytic and lipolytic activities, yeasts could also contribute towards maturation of some milk products (e.g. cheeses) by promoting the growth of LAB species through the release of amino acids and vitamins (Roostita and Fleet, 1996).

Table 2: Characteristics of yeast isolated from Berkeep of Western Sudan

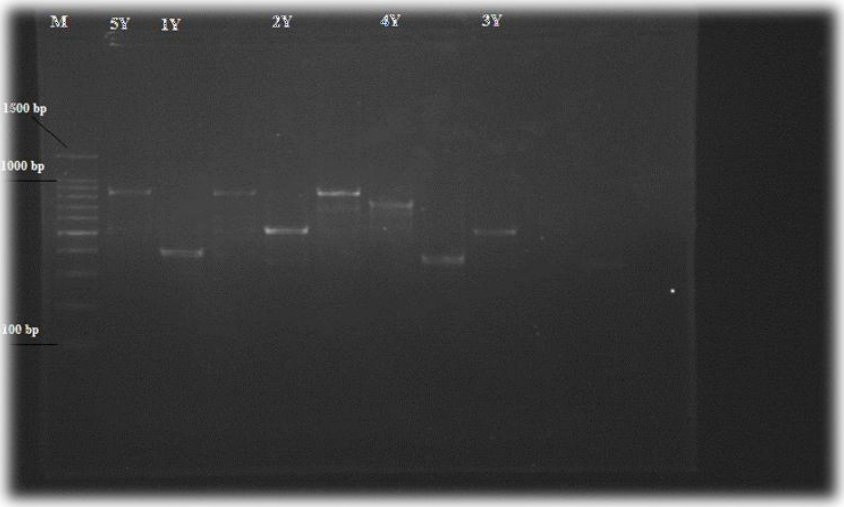
Groups	Number of isolates	Colony characteristics Color	Shape	Texture	catalase	Oxidase	Co_2 production from glucose	Acid production from glucose	Lipolytic activity
YG1	24	Creamy	Ovoid, elongated and alone	Flat, Smooth	+	+	+/-	+	_
YG2	43	Creamy whitish	Ovoid, elongated, alone, pairs and heap	Flat	+	+	+++	+	+/-
YG3	85	Creamy whitish, Orang	Ovoid, alone, pairs small chain and heap	Raised, smooth	+	+	+++	+++	+/-
YG4	68	White creamy	Ovoid, spherical, alone, pairs and heap	Oblong	+	+	++	++	_

Legend: YG1 to YG4: Group of yeast isolates, (+++): Strong Positive reaction, (++): Medium Positive reaction, (+): weak reaction , (-): Negative reaction, +/-: Different reactions.

Table 3: Identification of yeasts isolated from Berkeep

ITS groups	Number of isolates	ITS-PCR fragment size (bp)	Identification	ITS rRNA gene sequencing
YG1	24	400	*Candida* intermedia (100 %)	*Candida intermedia isolate B-NC-12-f 16 i*
YG2	11	510, 700	*Pichia kudriavzevii* (99.58 %)	*Pichia kudriavzevii strain YS179*
YG3	32	510	*Pichia kudriavzevii* (100 %)	*Pichia kudriavzevii* isolate Sugarcane
YG4	85	750	*Kluyveromyces marxianus* (100 %)	*Kluyveromyces marxianus* isolate *QKM4*
YG5	68	890	*Saccharomyces cerevisiae* (97.22 %)	*Saccharomyces cerevisiae* isolate *JKSP39*

(%): Similarity percentage.



Figs. 1: Profile of fingerprinting of yeasts isolated from Berkeep by ITS-PCR

Legend: M: DNA molecular marker, 1Y to 5Y: isolates groups

The ability of *Kluyveromyces* spp. to utilize lactose is one of the major reasons for its being pursued as a host for the biotechnological processes. Although *S.* cerevisiae cannot metabolize lactose, *Kluyveromyces* spp. transport it using an inducible lactose permease. The *K. marxianus* has lactose permeases that are proton symports. These can also transport galactose. The lactose permeases are induced by lactose or galactose. Introduction of this lactose transporter (LAC12) along with a β-galactosidase into *S. cerevisiae* renders this yeast able to utilize lactose (Batt and Tortorello, 2014). This fact could justify and explain the coexistence of these two yeasts in Berkeep. The inhibitory role of the Exopolysaccharides (EPSs) of *Kluyveromyces marxianus* and *Pichia kudriavzevii* on different colon cancer cell lines was studied by Saadat *et al*. (2020). The authors found that both EPSs can induce apoptosis and are able to reduce the expression levels of AKT1, JAK1 and mTOR mRNA which have been over-activated in colon cancer. Therefore, the probiotic yeast EPSs present beneficial effects and may provide as novel molecular-targeted therapeutics for combating colorectal cancer (CRC)

S. cerevisiae is well-known as the oldest industrial microorganism used as yeast culture during food processing. Several strains of this yeast were studied to identify their probiotic properties. Investigations on their survival under simulated gastrointestinal conditions and the antimicrobial effects on some pathogenic organisms and survival at low pH were well confirmed (Martins *et al*., 2005; van der Aa Kühle *et al*., 2005).

By conventional culture and molecular biology methods, Watanabe *et al*. (2008); Miyamoto *et al*. (2010); sun *et al*. (2014) reported alcoholic-fermenting yeasts such as *Kluyveromyces* marxianus and *Saccharomyces cerevisiae* to be the predominant microbial species in Tarag, airag, undaa, and hoormog, naturally fermented cow milk products in Mongolia and the northwest of China. They are expected to play important roles in processes such as hydrolyzing milk proteins and fat, assimilating lactic acid, and producing ethanol (Sudun *et al*., 2013). *Kluyveromyces marxianus, Saccharomyces cerevisiae and Candida* intermedia were among the yeast isolated from Serro Minas, a traditional Brazilian cheese by Cardoso *et al*. (2015). Abdelgadir *et al*. (2001) isolated *Saccharomyces cerevisiae* and *Candida kefyr* isolated from Rob, a Sudanese traditionally fermented product. Forty percent of the

yeasts isolated by Yang *et al*. (2014) from yak milk dreg of Tibet in China was identified as *Pichia kudriavzevii*. *Kluyveromyces marxianus* is found by Rahman *et al*. (2009) to be the dominant yeast isolate in shubat, a special fermented product prepared from unheated two-humped camel milk of China. Abdelgadir *et al*. (2008) identified *S. cerevisiae*, and *Issatchenkia orientalis* (lately, *Pichia kudriavzevii*), as dominant yeast flora of Gariss, a fermented camel milk of Sudan.

As compared to the results of the present study, a larger species diversity has been found on traditional milk products elsewhere. Gadaga *et al*. (2000) identified 20 different species from Amasi, a South African fermented cow milk, with the most predominant being. *S. cerevisiae*, *Candida lusitaniae*, *C. colliculosa*, *S. dairensis*). *Saccharomyces cerevisiae, Saccharomyces pastorianus, Candida kefyr, Yarrowia lipolytica, Candida stellata, Kluyveromyces marxianus, Zygosaccharomyces bisporus, Zygosaccharomyces rouxii* were isolated by Akabanda *et al*. (2010) from Nunu, a spontaneously fermented yoghurt-like milk product consumed as a staple food product in parts of the Saharan West Africa. In Sameel, a traditional fermented milk product consumed mostly in nomadic areas of Saudi Arabia and made of cow, goat, camel or sheep milk, Al-Otaibi (2012) isolated 36 different species, with the most prominent being *Candida lusitania*, *Cryptococcus laurentii*, *S. cerevisiae*).*Candida intermedia, Kluyveromyces marxianus*, and *Pichia kudriavzevii* were the most frequently isolated yeast species in white-brined cheese and have been characterized as typical dairy spoilers at the Danish Dairy (Geronikou *et al*., 2022).In Chal, a fermented camel milk of Golestan province of Iran, Yam et al. (2015) reported *Kluyveromyces lactis* and *K. marxianus* as being the dominant yeast. This species difference with in the various fermented milk products probably due to differences in raw material, environmental factors (temperature, humidity etc.) and processing environment (the microflora of the people handling the fermentations, cleaning procedures, etc.).

Conclusion

The results of these study revealed that the traditional fermented milk (Berkeep) of Darfur, Western Sudan was confirmed to be rich in yeast comparable with other fermented milks in Sudan and elsewhere. *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* were the most yeast species. The high yeast counts confirm its role in fermentation and aroma compound production. The role of yeasts for aroma formation and their use as starter culture in combination with selected lactic acid bacteria should be investigated.

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