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Yeasts isolated from 'Kwerionik' and some of their biological attributes of importance in milk fermentation

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Abstract

'Kwerionik' is a traditional cultured milk produced in Eastern Uganda at the slopes of Mount Elgon. Field and laboratory fermented 'Kwerionik' samples were microbiologically analysed to establish the levels of yeast strains at the various stages of fermentation. Also yeast isolates were identified to species level and their important characteristics for milk fermentation were identified. Yeast enumeration and isolation were carried out on Potato Dextrose agar under aerobic conditions fort 30°C for two days. Presence of yeasts was confirmed by colonial morphology and by microscopy. The isolates were screened into groups by polymerase chain reaction-intergenic transcribed spacer (PCR-ITS) fragment length polymorphism (18S-28S), and the various groups were identified to genus and species level by the carbohydrate assimilation tests using the ID32C kit (bioMérieux, Marcy l'Etoile, France). Other biochemical tests including ability to assimilate lactic acid and citric acid and to ferment carbohydrates (glucose, galactose, lactose, sucrose, maltose and raffinose) and enzymatic activity were also carried out. Isolates belonging to the same ITS group were distinguished by API ID32 kit and species identity was confirmed using *Candida krusei* species specific PCR . A total of 261 yeast isolates were analysed and the predominant species included *Candida krusei* (52.1%), *Candida kefyr* (26.8%). Others were *Pichia ohmeria* (5.8%), *Candida intermedia* or *Candida lusitaniae* (5%), *Candida pelliculosa* (2.7%), *Candida lambica* (3.1%), *Candida guilliermondi*i (1.5%) and *Candida holmii* (0.9%). Some of the species exhibited properties important to fermentation of milk, such as, β-galactosidase, lipase and proteolytic activity. The contribution of the yeasts to 'Kwerionik' fermentation needed to be established.

Key words: Cultured milk, yeast visolates, fermentation

Introduction

'Kwerionik' is a traditional cultured milk produced in Eastern Uganda at the slopes of Mt. Elgon. It has a mild sour flavour, a thick consistency and is primarily fermented by lactic acid bacteria (LAB), however significant levels of yeast are found. Several fermented milk products exist, where yeast adapt to a co-existence with lactic acid bacteria. Although yeasts are basically considered as contaminants, usually with undesirable effect to the fermented milk products, there is increasing evidence that some species contribute to flavour and texture development; secrete biologically active substances such as vitamins, enzymes and amino acids; increase the viability of the LAB; and have health promoting effects (1, 2, 3, 4, 5, 6).

Fermented products have been reported to exhibit the highest incidence and level of yeast contamination due to the low pH which forms a selective environment for their growth (2, 7, 8, 9). Hence, different yeast species have been isolated from fermented dairy products (10, 11, 12, 13). Their presence is due to various properties such as fermentation or assimilation of lactose, production of extracellular proteolytic and lipolytic enzymes, assimilation of lactic acid and citric acid, growth at low temperatures and tolerance to elevated salt concentrations (6, 14, 15, 16).

The yeast species frequently found in dairy products include *Debaryomyces hansenii*, *Candida famata*, *Kluyveromyces marxianus*, *Candida kefyr*, *Candida stellata*, *Saccharomycopsis lipolytica*, *Saccharomyces cerevesiae*, *Candida krusei*, *Rhodotorula glutinis* and *Rhodotorula rubra*. However, *D. hansenii* and *Kluy. marxianus*, as well as their asporagenous equivalents *C. famata* and *C. kefyr* respectively, emerge as the most prevalent ones (17). The yeast microflora in 'Kwerionik' have neither been identified nor characterized for their properties that may be of importance in milk fermentation and this was the aim of this study.

Materials and methods

'Kwerionik' samples at different stages of fermentation, 1, 2, 4, 12 and 48 weeks of age,

were obtained from the production area on the slopes of Mt. Elgon in Eastern Uganda. The samples were transported on ice in a cooler box to the laboratory for analysis in the Department of Veterinary Parasitology and Microbiology, Faculty of Veterinary Medicine, Kampala, Uganda. Laboratory trials simulating the traditional method for the fermentation were carried out and samples were withdrawn firstly, at six hour intervals for four days, then daily up to one week, then every four days until one month old and thereafter fortnightly up to one year. The pH of the samples was determined by a pH meter. Fungal isolation and counts were established onto Potato Dextrose agar (PDA, Difco) incubated at 30°C aerobically for one to two days; and the yeasts were purified for further identification. Stock cultures were kept in Malt Yeast extract Peptone Glucose (MYPG) broth (3 g malt extract, 3g yeast extract, 10g glucose, 5g peptone [all from Oxoid], 1L of distilled water, pH 6.5 –7) at –80°C.

The cells were harvested by centrifuging at 1400 × g for 5 minutes; resuspended in 200 µl of sterile distilled water and heated at 95°C for 5 min in a heating block to obtain a template for use in the PCR. The 18S – 28S ITS region was amplified using the Cy5-Y-ITS1 forward primer and Y-ITS4 reverse primer (T-A-G Copenhagen ApS, Copenhagen, Denmark), with sequences, 5'-TCC GTA GGT GAA CCT GCG G-3' and 5'-TCC TCC GCT TAT TGA TAT GC-3' respectively. Amplification was performed in a thermocycler (Trio-Thermoblok, Biometra, Gottingen, Germany) in 50 µL reaction mixtures containing 5µL of 10× PCR buffer, 0.2mmol l^{-1} of each dNTP, 25 mmol l^{-1} $MgCl_2$, 1% (v/v) formamide, 0.5µmol l^{-1} of each primer, 2.5 U of Taq polymerase (Pharmacia Biotech, Uppsala, Sweden), and 1µL of DNA template. The PCR conditions were: initial denaturation at 95°C for 5min, then 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. Final extension was carried out at 72°C for 7 min, then the amplified product was kept at 4°C and then –20°C until required.

The amplified product was electrophoresed on a 6% polyacrylamide gel using the automatic DNA sequencer, ALFexpress, (Pharmacia Biotech, Uppsala Sweden). The sizing standard was Cy5 sizer®50-500 (Pharmacia Biotech) and the electrophoresis conditions were 700V, 60mA at 55°C for 200 minutes. The amplified ITS fragment patterns were analysed by the Fragment Manager computer software (Pharmacia Biotech). Isolates with the same fragment lengths were grouped together (designated A to J, see Table 2) and representatives from each were analysed for their physiological characteristics as described below. The assimilation and fermentation activity on various carbon substrates and the enzymatic activities were investigated.The yeast inoculum for carbohydrate assimilation and organic acid assimilation tests were prepared as described by Yarrow (18).

Representative yeast strains from the various ITS groups (two from groups A, B, C, D and H, one from group E, and 10 each from groups I and J), were tested for their ability to assimilate various carbohydrates using the ID32C API system (bioMérieux, Macy l'Etoile, France) as per manufacturer's instructions. The reactions were recorded visually and the organisms were identified by the APILAB Plus software (bioMérieux). Apart from establishing the identity of the isolates, this test enabled establishment of the ability to utilize various sources of carbon by the yeasts.

Of the 136 isolates in Group J, 68 were analysed by *C. krusei* species-specific PCR to establish their identity as described by Carlotti, *et al*., (1). The presence and size of the PCR products were analysed on a horizontal submerged 1% agarose (Sigma) gel and electrophoresed at 3V/cm for 2h using 1× Tri-Acetate EDTA as the running buffer. The Gene Ruler™ 1kb DNA ladder (MBI Fermentas,Vilnius, Lithuania) was used as the sizing standard. The gel was stained in Ethidium bromide (1 mg l^{-1}) for 10 min and rinsed in distilled water, then photographed with polaroid type 667 film. Presence and size of the PCR products were manually analysed.

For groups with more than 10 isolates, at least eight strains were analysed for their ability to assimilate lactic acid and citric acid using the method described by Yarrow (18). The results were recorded as positive if they scored 2+ or 3+ by Wickerhams card after 7 days of incubation. Those with 2+ and 3+ scores were resubcultivated and the degree of growth determined.

Sterile fermentation basal medium, was supplemented with appropriate 6% (w/v) sterile filtered carbohydrate, glucose, galactose, lactose, sucrose and maltose, but for raffinose 12% (w/v) was used according to Yarrow (18). The tubes were incubated at 25°C and examined daily for 7days for acid and gas production.
The enzymatic activity of the yeast strains from some ITS groups was semi-quantitatively

investigated by use of the APIzyme kit (bioMérieux) according to the manufacturer's instructions.

Results

The pH and levels of the yeasts from the field and laboratory 'Kwerionik' samples at various stages of fermentation were as shown in Table 1 and Figure 1. The pH was almost similar ranging from 3.3 to 4.1 in both categories of samples. For the field samples, the yeast levels were fairly constant; with counts of about 10^6 cfu g^{-1} in the 14-day old field samples, but decreased thereafter. From the laboratory spontaneous fermentation trials, there was a gradual increase in the number of yeast isolates from just below 10^4 cfu g^{-1} to about 10^6 cfu g^{-1} after 28 days of fermentation; and levels fluctuated between 10^5 and 10^6 cfu g^{-1} thereafter (Fig. 1).

From the colonial morphology, two types of yeast strains, the dull and flat and the shinny and raised, were encountered in all the samples. On further identification, it was only *Candida kefyr* and *Candida krusei* that were isolated from all the samples representing 26.8% and 52.1% of the total yeast isolates respectively. *Candida lusitaniae* or *Candida intermedia* (5%), *Candida holmii* (0.8%) and *Pichia ohmeri* (5.7%) were isolated from samples obtained from one of the fermentation trials, whereas *Candida lambica* (3.1%) was also isolated from some of the two weeks old field samples. Other yeasts recovered included *C. guilliermondii* (1.5%), *C. pelliculosa* (2.7%) and the unidentified (2.2%).

The PCR-ITS fragment length analysis of the 261 yeast isolates resulted in 10 groups , A – J (Table 2) and the major PCR products (by size) are shown. Except for groups C, D, E, F and H, the others had some additional minor PCR products. . All the 68 isolates selected from group J, with various API 32D identity as *Candida norvegica*, *Candida krusei*, *Candida inconspicua*, *Candida pelliculosa* and *Candida intermedia* gave products when subjected to the *C. krusei* specific PCR, where they yielded one or more products with the size range of 1 – 1.8 kb (Fig. 2 below).

Table 3 gives a summary of the results of lactic acid and citric acid assimilation and the carbohydrate fermentation by selected yeast isolates. Most of the yeast species analysed were capable of assimilating citric acid except *P. ohmeri*, *C. holmii* and *C. kefyr*; while lactic acid was assimilated by *C. lusitaniae*, *C. holmii*, *C. lambica*, *C. kefyr* and some strains of *C. krusei*. The yeasts were quite active in fermenting the sugars, but lactose was fermented by *C. kefyr* only. There was limited activity on raffinose where acid only was produced by five groups while *Candida pelliculosa* is the only one that fermented it with gas production. From the carbohydrate assimilation patterns and in relation to the sugars found in milk, all the isolates assimilated glucose, only *Candida kefyr* utilised lactose while all fermented galactose except *Candida krusei* and *Candida lambica* (Table 4).

The enzymatic activity of the yeast isolates were as shown in Table 5. . All the yeasts showed acid and alkaline phosphatase and naphthol-AS-BI-phosphohydrolase activity. None exhibited esterase and lipase activities, but the majority had weak esterase lipase activity except *Candida kefyr* and some *C. krusei* strains. All exhibited leucine arylaminidase activity, weak valine arylaminidase activity by *C. lusitaniae*, *C. guilliermondii* and *C. kefyr*; and none had cystine activity. Some yeasts, *C. lambica*, *C. kefyr*, *C. lusitaniae* and some strains of *C. krusei*, exhibited weak endopeptidase (chymotrypsin) activity whereas alpha (α)- galactosidase activity was exhibited by a number of strains, *C. lambica*, *C. kefyr* and some strains of *C. krusei* in comparison to β-galactosidase that was seen in *C. kefyr* only. *Candida lusitaniae*, *C. kefyr* and *P. ohmerii* showed both α- and β- glucosidase activity and some strains of *C. lusitaniae* had N-acetyl-β-glucosaminidase activity.

Table 1. The pH and yeast counts (log_{10} cfu g^{-1}) of field 'Kwerionik' and laboratory samples at different stages of fermentation

	Field Samples		Laboratory samples	
Age (days)	pH	Log Yeast counts	pH	Log Yeast counts
7	4.0 ± 0.06	5.2 ± 0.75	4.1 ± 0.1	2.7 ± 2.0
14	3.7 ± 0.09	6.6 ± 0.56	3.4 ± 0.05	3.7 ± 1.7
30	3.5 ± 0.05	5.3 ± 0.45	3.3 ± 0.07	4.6 ± 1.1
90	3.6 ± 0.17	4.5 ± 0.97	3.5 ± 0.09	5.9 ± 0.6
180	3.5 ± 0.02	5.7 ±0.29	3.5 ± 0.01	4.5 ± 0.4

Table 2. Groups of yeast isolates by the ITS profile and API identity

ITS group & # of isolates	ITS fragment size*	API ID 32C identification
A (13)	380±3	*Candida lusitaniae* and *Candida intermedia*
B (15)	408±4	*Pichia ohmeri*
C (4)	603±2	*Candida guilliermondii*
D (7)	623±4	*Candida pelliculosa*
E (2)	647±0.6	*Candida holmii*
F (3)	528±8.6	ND
G (3)	443±1.3	ND
H (8)	490±3	*Candida lambica*
I (70)	704±8	*Candida kefyr*
J (136)	508±9	*Candida krusei*

* Size of the major PCR-ITS product and the standard deviation within the group

Table 3. Acid assimilation and carbohydrate fermentation by the yeasts isolated from 'Kwerionik'

Yeast identity/ITS group	Acid Assimilation		Carbohydrate Fermentation					
	Lactic	Citric	Glc	Gal	Lact	Suc	Malt	Raff
Candida lusitaniae (A)	+[1]	+	A/G[2]	A/G	-[3]	A/-[4]	A/-	-
Candida intermedia (A)	-	+	A/G	A/G	-	A/-	A/-	-
Pichia ohmeri (B)	-	-	A/G	A/-	-	A/G	A/-	A/-
Candida guilliermondii (C)	-	+	A/G	A/G	-	A/G	A/-	A/-
Candida pelliculosa (D)	-	+	A/G	A/G	-	A/G	A/G	A/G
Candida holmii (E)	+	-	A/G	A/G	-	A/G	A/-	A/-
ND (F)	-	+	A/G	A/G	-	A/G	A/G	A/-
ND (G)	-	+	A/G	A/-	-	-	-	-
Candida lambica (H)	+	+	A/G	-	-	-	-	-
Candida kefyr (I)	+	-	A/G	A/G	A/G	A/G	A/-	A/-
Candida krusei (J)	+/-	+	A/G	-	-	-	-	-

Key: 1- Positive; 2- Acid and gas production; 3- Negative; 4- Acid and no gas production; Glc- Glucose; Gal- Galactose; Lact- Lactose; Suc- Sucrose; Malt- Maltose; Raff- Raffinose

Table 4. The physiologic characteristics (carbohydrate assimilation) of selected yeasts representing various PCR-ITS groups and their tentative identification

Carbohydrate	Yeast Isolates										
	Pichia ohmeria	*Candida pelliculosa*	*Candida lusitaniae*	*Candida lambica*	*Candida krusei*	*Candida incospicua*	*Candida norvegica*	*Candida kefyr*	*Candida intermedia*	*Candida holmii*	*Candida guilliermondii*
Galactose	+	+	+	-	-	-	-	+	+	+	(+)
Actidione	-	-	-	-	-	-	-	+	-	+	(+)
Sucrose	+	+	+	-	-	-	+	+	+	+	(+)
N-acetylglucosamine	+	-	+	+	+	-	-	-	+	-	(+)
DL- Lactate	-	+	(-)	+	+	+	+	(+)	-	-	-
L-Arabinose	-	-	-	-	-	-	-	(-)	-	-	+
Cellobiose	+	-	(-)	-	-	-	-	(-)	-	-	+
Raffinose	+	+	-	-	-	-	-	+	+	+	+
Maltose	+	+	+	-	-	-	-	-	+	-	+
Trehalose	+	+	+	-	-	-	-	-	+	+	+
2-Ketogluconate	+	-	+	-	-	-	-	-	+	-	+
α-Methyl-D-glucoside	+	+	(+)	-	-	-	-	-	+	-	+
Sorbitol	+	+	+	-	-	-	-	+	+	-	+
D-Xylose	-	-	(-)	-	-	-	+	+	+	-	+
Ribose	(+)	-	-	-	(-)	-	-	(+)	+	+	-
Glycerol	+	+	+	-	+	-	+	-	+	-	+
Rhamnose	+	-	+	-	-	-	+	-	+	-	-
Palatinose	-	+	+	-	-	-	-	-	-	-	-
Erythritol	-	+	-	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-	-	-
Glucuronate	-	-	-	-	-	-	-	-	+	-	-
Melezitose	-	+	+	-	-	-	-	-	+	-	+
Gluconate	-	-	(+)	-	-	(-)	-	-	+	-	-
Levulinate	-	-	-	-	-	(-)	-	-	-	-	-
Mannitol	+	+	+	-	(-)	-	-	+	+	-	+
Lactose	-	-	-	-	-	-	-	+	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+	+	+
Sorbose	+	-	+	-	-	(-)	(+)	+	-	-	+
Glucosamine	(+)	-	+	+	-	-	-	-	+	-	(+)
Esculin	-	-	-	-	-	-	-	-	-	-	(+)

Key: + Positive reaction; (-)/(+) some negative or positive reaction; - Negative reaction

Table 5. Enzymatic activity of selected yeast strain by APIzyme

Enzyme activity	*Candida lambica* UK/801	*Candida holmii* UK/155	*Pichia ohmeri* UK/45	*Pichia ohmeri* UK/55	*Candida lusitaniae* UK/20	*Candida lusitaniae* UK/59	*Candida guilliermondii* UK/02	*Candida guilliermondii* UK/02	*Candida kefyr* UK/806	*Candida kefyr* UK/154	*Candida krusei* UK/160	*Candida krusei* UK/821	*Candida krusei* UK/803	*Candida krusei* UK/715	*Candida krusei* UK/745
Lipolysis															
• Esterase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
• Esterase-lipase	1	1	1	2	1	1	1	2	0	0	1	1	1	1	0
• Lipase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Exo (amino)peptidase															
• Leucine	3	2	5	5	4	3	3	3	2	5	2	2	2	2	5
• Valine	T	0	T	T	1	1	1	1	1	1	0	T	T	T	T
• Cystine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Endopeptidase															
• Trypsin	0	0	0	0	0	0	0	0	0	0	T	0	T	0	0
• Chymotrypsin	T	0	T	T	T	T	T	T	T	T	T	T	T	T	T
Glycosidase (oxidases)															
• β-galactosidase	0	0	0	0	0	0	0	0	4	5	0	0	T	T	T
• α-galactosidase	1	0	0	0	0	0	0	0	1	1	1	1	0	1	0

Key*: Values 0 –5: Approximate number of free nanomolecules of the hydrolysed substrate, namely: T = traces, 0 = negative, 1 = 5 nmol, 2 = 10 nmol, 3 = 20 nmol, 4 = 30 nmol, 5 = 40 nmol or more*

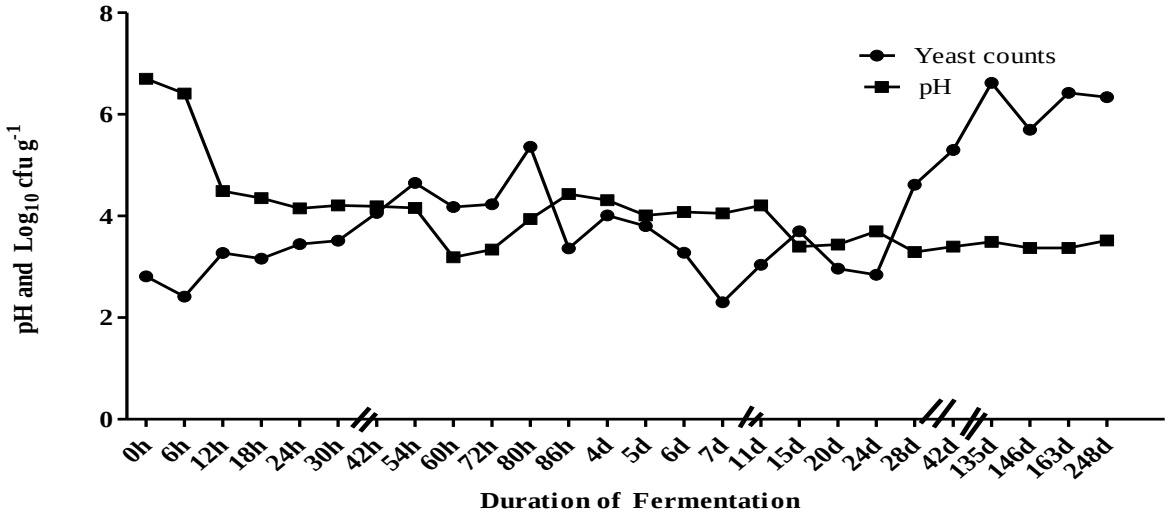

Figure 1: Changes in yeast levels (log_{10} cfu g^{-1}) and pH of 'Kwerionik' during laboratory fermentation trials(X-axis drawn not to scale; h= hours; d= days. Samples were withdrawn firstly, at six hour intervals for four days, then daily up to one week, then every four days until one month old and thereafter fortnightly up to one year)

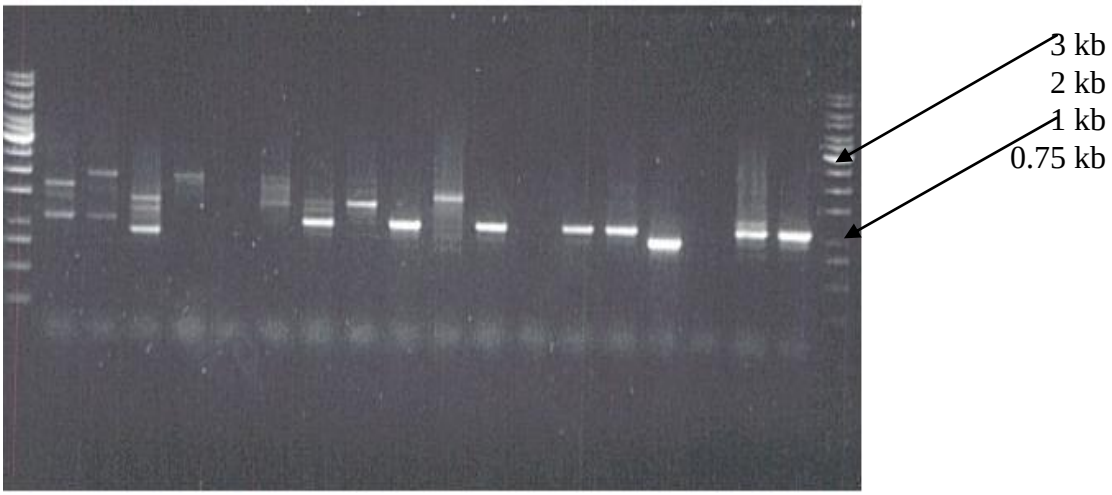

Figure 2: Candida krusei species specific PCR

From Left to Right: Lanes 1 and 20 Marker Gene Ruler^{TM} 1kb DNA Ladder (MBI Fermentas, Vilnius, Lithuania); for Lanes 2-19, the legend shows the yeast strain and the API identification. Lane2 - UK/715, *C.krusei*; Lane3 - UK/705, *C.krusei*; Lane4 - UK/64, *C.krusei*; Lane5 - UK/1691, *C.krusei*; Lane6 - UK/30, *C. intermedia*; Lane7 - UK/707, *C.krusei*; Lane8 - UK/720, *C.krusei*; Lane9 - UK/05, *C.krusei*; Lane10 - UK/840, *C.krusei*; Lane11 - UK/821, *C.krusei*; Lane12 - UK/1250, *C.krusei*; Lane13 - UK/40, *C.guilliermondii*; Lane14 - UK/379, *C.krusei*; Lane15 - UK/803, *C. norvegica*; Lane16 - UK/1265, *C.krusei*; Lane17 - UK/39, *C.kefyr*; Lane18 - UK/379, *C. inconspicua*; Lane19 - UK/7376, *C.krusei*

Discussion

The pH range of 3.3 – 4.1 found in case of 'Kwerionik' is similar to what has been reported by previous researchers (19). However the fall in pH below 4 could be attributed to the presence of the yeasts, that have been reported to have variable sugar fermentation capabilities, especially *C. kefyr* whose lactose fermentation and β-galactosidase activity might have resulted in further acidification of the medium (14, 20). The presence of the yeast is related to the fact that the lactic acid bacteria in 'Kwerionik' reduce the pH to a level that forms a conducive and selective environment for yeast growth; and the products of LAB metabolism act as a source of nutrients for the yeasts (21). Findings of this study (Table 1 & Fig. 1) are in agreement with previous reports where the levels of yeast in various fermented milk products exceed 10^3 cells ml^{-1} (19, 22, 23).

Among the yeasts isolated from 'Kwerionik', it is *Candida kefyr* (anamorph *Kluvyeromyces marxianus*) and *C. krusei* that were encountered in all the samples. These yeasts have been reported in other fermented milk products; with *C. krusei* being most predominant (10, 11, 12, 13, 20, 24, 25). The other yeast species, *Candida lusitaniae* or *Candida intermedia*, *Candida holmii*, *Pichia ohmeri*, *Candida lambica*, *C. guilliermondii* and *C. pelliculosa*, representing 21.1% of the total yeasts isolates, were only encountered in some samples and are thus likely to be environmental contaminants, however, they have been reported by other researchers too (11, 16, 17, 26, 27, 28, 29, 30).

From Table 2, it is evident that some isolates within the same ITS group were identified differently by API ID 32C. This is similar to Majoros, *et al*. (31) findings where *C. norvegensis* isolates were identified as such by the API ID32C system, although they gave restriction enzyme analysis of the PCR-amplified rDNA patterns typical for the *C. inconspicua* ATCC 16783 strain and not the one for the *C. norvegensis* ATCC 22977 reference strain. Several *Candida* spp are reported to have approximately the same ITS fragment sizes, but can be differentiated by restriction enzyme digestion due to variations in the base sequence. The size of the PCR-ITS (18S – 28S) products of some *Candida* spp have been reported to be 600 bp for *C. guilliermondii* ((32); 460 bp for *C. inconspicua*, 510 bp for *C. krusei* and 500 bp for *C. norvegensis* (33). This is in agreement with the findings of this study in case of the major PCR - ITS fragments since the same primers were employed for the amplification. However, the differences could be due to the gels used for

electrophoresis and the accuracy for determining the fragment sizes. These authors used agarose gels in contrast to the polyacrylamide gels used during this study, but included type strains as positive controls for their study. In addition, a computer software was employed in determining the fragment sizes resulting in very high precision as indicated by the standard deviation within the group.

Group J isolates represented quite a diverse number of isolates and had different identifications, *C. krusei*, *C. inconspicua* and *C. norvegica*, by API ID 32 kit. Reports of these organisms showing the same biological profile by some test kits and having almost the same ITS fragment sizes exist (31, 33, 34). This is due to existence of intraspecies variability in some physiological properties of the yeasts (35). Being the most dominant yeasts isolated from 'Kwerionik' and having been considered as opportunistic potential pathogens, a *Candida krusei* species specific PCR was carried out on Group J members to confirm their identity; and were all found to be *C. krusei* (see Fig. 2). The fingerprinting differences that were noted have been explained to be due to the polymorphisms in the non-transcribed intergenic region of rRNA genes and the presence of a variable number of tandemly repeated sequences (36). Isolates from 'Kwerionik' had amplification products of sizes ranging from 1.0 -2.0 kbp and this is in agreement with the findings of Hayford and Jakobsen (37).

In addition to being useful in identification of the yeasts, the carbohydrate assimilation and fermentation tests; lactic acid and citric acid assimilation; and the enzymatic activity provided more information on the ability of the various isolates to utilize the ingredients that are found in milk and the byproducts of lactic acid bacteria fermentation. Variable lactate assimilation has been reported by Marcellino, *et al*., (38); and from this study, about 50% of the yeast species were able to assimilate lactic acid, DL-lactate although its only *C. kefyr* that utilised lactose (Tables 3 and 4). Lactate and lactic acid utilisation is undesirable as it results in limited acidity that might allow survival of acid-sensitive bacteria, some of which could be pathogenic; and a product with a different taste and aroma. Except for *C. kefyr*, *C. holmii* and *P. ohmeri*, all the other yeast species assimilated citrate. Co-metabolism of citrate and glucose together with production of of L-lactate, results in the production of substantial amounts of fermentation products, such as diacetyl, acetoin. These products, especially diacetyl formation by some LAB strains are essential for the buttery flavour. Probably some yeast isolates such as *C.intermedia*; *C. giulliermondii* and *C. krusei* that combined both properties contributed to flavour of 'Kwerionik'. Variable carbohydrate assimilation patterns were encountered, but lactose was assimilated and also fermented by *C. kefyr* only. The isolates that were capable of utilizing lactose may be considered to be of benefit during 'Kwerionik' fermentation because they possess the lactase enzyme system that break the milk sugar into acid and gas. In addition they are capable of growing and become predominant, whereas those that assimilated lactic acid are more likely to be spoilage organisms. This is so, because assimilation of lactic acid can result in elevated pH which favours growth of some undesirable or even pathogenic and/or spoilage bacteria (39). In addition, Schwartz and Hung (40) reported diacetyl reductase activity in some *Kluyveromyces marxianus* that may remove the desirable buttery flavour imparted by diacetyl. Therefore, in this case, it is *C. kefyr* which is likely to be of significance during 'Kwerionik' fermentation; and the other yeast could be considered to be opportunistic or even spoilage organisms. However, since most of the isolates had esterase lipase activity and lipolysis of the milk fat results in flavour development, these organisms could possibly contribute to this aspect. The high peptidase activity on leucine seen in most of the analysed strains may be important in flavour development and lack of or trace proteinase activity improves the quality especially in case of cheese. *Candida kefyr* showed high β-galactosidase activity, thus it can be incorporated as an adjunct to the starter cultures for use in products for the lactose intolerant individuals.

Of the yeast species that have been reported to occur frequently in dairy products, it is *Candida kefyr* and *Candida krusei* that were isolated from 'Kwerionik'. *Candida intermedia*, *Candida lusitaniae* and *Candida holmii* were also encountered and have been reported to be frequent in retail yogurt (14). For this study, they are most likely to be contaminants as they were isolated from the fermentation trials only. The yeasts exhibited some properties that may be beneficial in milk fermentation. The predominat yeast strains that were isolated from 'Kwerionik' have been reported to occur in other fermented milk products, but their definate role in the fermentation of milk need to be established if they are to be considered for use as adjunct starter cultures.

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