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Molecular identification and proteinase activity of yeasts isolated from fermented milk

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Abstract

The aim of this study was to identify yeasts from traditional Tanzanian fermented milk, "mtindi" and industrial fermented milk, "yoghurt" and to determine their *in vitro* proteolytic activities. A total of twenty five yeast isolates were studied. Identification was done by ribosomal DNA - Polymerase Chain Reaction (PCR) amplification and sequencing of the domains D1/D2 of the 26S rRNA gene. The identified yeasts were *Candida tropicalis*, *C. pararugosa*, *Clavispora lusitaniae*, *Issatchenkia orientalis/Pichia kudriavzevii*, *Pichia guilliermondii/Meyerozyma guilliermondii*, *Galactomyces geotricum*, *Debaryomyces* sp and *Yarrowia lipolytica*. However, *Y. lipolytica* and *D. hansenii* were detected from yoghurt samples only, while *G. geotrichum* was found in both mtindi and yoghurt samples. *Candida* sp. and *Pichia* sp. were detected in mtindi samples only. The highest yeast load was of *C. paragurosa* (over log 6 CFU/ml) and *P. guilliermondii* was the least isolated strain in numbers of slightly over log 2CFU/ml ($p<0001$). Proteolytic activity was analysed by plate assay using nutrient agar media supplemented with milk casein. Over 80% of the isolates were protease positive. The highest activity was detected on *C. pararugosa* isolated from mtindi with the diameter of clear zone 36.667+5.4mm, ($p<0.01$). Our results showed the potential of dairy yeasts as a source for further exploitation of the production of proteolytic substances with either potential health benefits or spoilage abilities.

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Keywords (4 to 6) L Yeasts, molecular identification, proteinase, fermented milk

Introduction

Fermented milk is an important addition to the local diet and it provides essential building blocks for good health. Yeasts are some of the commonly found microorganisms in both raw and processed dairy products. The acid environment created by Lactic acid bacteria (LAB), creates a selective environment for a broad diversity of yeast growth [1, 2, 3, 4]. The fact that some yeasts may be associated with opportunistic infections and adverse conditions in humans and hence food safety threat, there is a growing interest in the biodiversity and ecology of yeast associated with different foods [5]. The realization that yeasts are able to interact with themselves and other microbial species in various ecosystems with outcome possibly affecting their roles they have in foods, increases curiosity to identify yeasts associated with different foods [6].

Moreira and others [7] reported the richness of yeasts in yoghurt from Brazil, up to 2723 CFU g^{-1}, with the most abundant yeasts being *Deboryomyces hansenii*, *Deboryomycescastelii*,*Candidaparapsilosis Saccharomyces cerevisiae*, *Mrakia frigida*, *Hansenula* spp., *and Candida maltosa*. Similar studies from Turkey reported *Candida sp.*, *Kluyveromycese sp.*, *Saccharomyces sp.*, *Trichosporon sp.*, *Geotricum sp.*, *Pichia sp.*, *Yarrowiwa sp.*, and *Rhodotorula sp.* [3] with more than 10^5 CFU/ of yeast counts being exhibited in retail marketed yoghurt [8]. Up to log 6 CFU/g of yeasts count was reported in yoghurt samples from retail outlets in Karachi, Pakistan [9]. The diversity and richness of yeasts in yoghurt have further been reported in Egypt (10^4 to 10^5 CFU/ml) [10] and in Pakistan (10^7 CFU/ml) [9]. Yoghurt from Pakistan was reported to be rich in *Candida sp.*, *Cryptococcus sp.*, *Debaryomyces sp.* and *Pichia sp.* [10].

Mtindi is a spontaneously fermented bovine milk beverage consumed in Tanzania. Like many other African countries such as Burkina Faso and Kenya, more or less similar methods of preparation of *mtindi* are practiced across Tanzania with slightly variation in fermentation time due to local indigenous microflora and also the climatic conditions from one region to the other [4, 11, 12]. In rural Tanzania, raw milk is left to spontaneously ferment in gourds at

ambient temperature varying from 25°C to 34°C from 24 to 36 hours, where the cream is skimmed away and the remaining is the protein rich fermented milk which is called *mtindi*. In urban settings, mtindi is made by addition of a portion of remnant of previous fermentation on to pre-heated milk (back-slopping) and left for 12 hours, the cream and the protein rich part are all mixed up together to form *mtindi* as well.

Like many other spontaneously fermented foods, the nature of *mtindi* is different from one region to another and the acidic environment found in fermented milk provides favourable environment for growth of yeasts [13, 14]. The presence of yeast in fermented milk indicates their ability to proliferate and positively interact with LAB and hence playing both beneficial and detrimental role in fermented milk safety and quality [5,15,16,17]. Some yeasts like *Saccharomyces sp*. which have been isolated from Koumiss ([18] have shown to contribute to the development of desirable flavour in fermented milk products like cheese, koumiss and kefir [19 12]. The improved flavour in traditional fermented milk is attributed by the presence of yeasts and thus, formation of volatile compounds like acetaldehydes [2].

Extracellular proteases can affect the quality of milk products in various ways, but largely by producing bitter peptides. Large numbers of somatic cells in raw milk affect the shelf life of pasteurized milk. Increased somatic cell numbers are reported to have a positive correlation with the concentration of plasmin, a heat stable protease and of lipoprotein lipase in freshly produced milk [20]. The activity of these proteolytic enzymes can supplement those of bacterial hydrolases hence fasten spoilage. The initial numbers of cells, their generation times, their ability to produce specific enzymes and the time and temperature at which the milk is stored are the major determinants of quantities of these enzymes in milk products.

Yeasts like *Candida lipolytica* are important in rendering longer shelf life of certain foods due to its ability of producing extracellular protease with antimicrobial activities [12, 21, 23]. There are studies reporting *Candida* species to be effective in enhancing longevity and activity of LAB and are now being used with some dairy starter cultures [31].

The diversity and richness of yeasts with different activities like probiotics [5] flavour and aroma of foods like egg odour in cheese [22]; and bio-control like the inhibition of growth of spoilage bacteria [23] calls for more studies on yeasts from milk from different settings and preparations around the globe. This is because, each country prefers indigenous fermented milk products according to their own palatal satisfaction which the aroma may be contributed by yeast strain present in fermented milk. Therefore, the main objective of this study was to isolate yeasts from selected samples of fermented milk, genetically identify them and examine their ability to hydrolyze casein.

Materials and methods

Sample collection, preparation and pH determination

A total of 25 yeasts were isolated from *mtindi* and yoghurt samples from local markets in four regions of Tanzania between the months of April to June in 2009 - North-west Tanzania (Mwanza), Eastern Zone (Dar es Salaam and Morogoro) and Northern zone (Kilimanjaro) were studied. The samples included 4 samples from Mwanza rural, coded as TFMMw1, 2, 3 and 4 and were prepared by natural fermentation of raw milk at room temperature for 24 to 36 hours in earthenware which is not sanitized after previous fermentation. Three samples from Morogoro peri-urban with codes TFMM1, 2 and 3 (a portion of mtindi from previous fermentation which was used as starter culture [back-slopping] in pre-boiled and cooled milk); 1 sample from Kilimanjaro (TFMK) and 7 samples from Dar es Salaam with codes TFMD 1 – 7 in which a portion of yoghurt was added in pre-boiled and cooled milk in to a well sanitized and covered plastic containers for 12 hours. Samples were collected in sterile 250 mL glass containers and immediately placed in ice box and transported to University of Dar es Salaam, Molecular Biology and Biotechnology laboratory and preserved on refrigerator at 4°C to 7°C for analysis within 6 hours of sampling. The pH of the samples was determined by direct insertion of the electrode into the sterilized jar containing 25mL of fermented milk at 30°C using a pH metre (pH 209 Hanna Instruments, USA).

Enumeration and Isolation of yeasts

One gram of each yoghurt and *mtindi* sample was diluted in 9mL sterile 0.1% Ringer's solution (0.9% NaCl, 0.042% KCl, 0.48% $CaCl_2.6H_2O$, 0.02% $NaHCO_3$, and 1% Glucose) followed by decimal dilution up to 10^{-6} and 1ml was used for pour plating in duplicate on malt extract agar, MEA (HIMEDIA, India) at 30°C for 24 hours. About 0.1mL of 10^{-3}, 10^{-5} and 10^{-6} dilutions each was spread on Malt extract agar supplemented with Chloromphenicol (100mg/L) to inhibit bacterial growth then incubated at 30°C for 24 to 48 hours. The average sum of colonies formed was used to determine the total yeasts populations. Yeast isolates per plate were selected based on their microscopic appearance of the cells grown, morphological differences, colour, margin and elevation of the colonies [3] further sub-cultured overnight at 30°C on Malt extract broth supplemented with Chloromphenicol (100mg/L). Overnight broth cultures were then streaked on malt extract agar plates supplemented with Chloromphenicol (100mg/L) for purification. The strains were then cultured on malt extract slants at 30°C for 24 to 48 hours and kept at 4°C.

DNA isolation

A modified technique for yeast DNA extraction by [24] was applied. Briefly, an aliquot of 1 mL overnight Malt extract broth cultures was centrifuged at 10,000 rpm for 1min. A pellet was collected and suspended in 500 µL lysis buffer (50 $mmolL^{-1}$ Tris; 250 $mmolL^{-1}$ NaCl; 50 $mmolL^{-1}$ EDTA; 0.3% w/v SDS; pH 8). Glass beads equivalent to a volume of 200 µL 425-600 µm was added to remove/crush cell membranes (Sartorius Mikro dismembrator U) for 1.5 min, and then the tubes were heated in water bath for 20 min at 99°C. The suspensions were then centrifuged for 30 min at 4°C. Supernatant was collected and diluted 1: 750; 5 µL was used for polymerase chain reaction (PCR).

PCR assay

The amplification of the 26S rDNA D1/D2 region was done by NL1 (5' GCATATCAATAAGCGGAGGAAAAG

-3) and NL4 (5' GGTCCGTGTTTCAAGACGG-3') [26], under 25 µL reaction mixture. The latter was prepared by 1.5U taq polymerase, 0.5 µM of each primer, 0.2 mM of each dNTP, 10 mM Tris- HCl_2, 50 mM KCl (buffer) and 5-15 ng yeast DNA. The mixture was subjected to 30 PCR cycles; 1 minute of denaturation at 94°C; annealing at 60°C for 1 minute; extension at 72°C for 2 minutes and then final extension at 72°C for 7 minutes.

Analysis of PCR products and sequencing

PCR products were analyzed electrophoretically with 1.5% agarose gel containing 0.5 µg/mL ethidium bromide and visualized with transilluminator. Gene Ruler DNA ladder mix (100-1000bp) (Invitrogen**)** was run as a molecular-size marker in one of the lanes for size comparison. PCR products (600bp – 650bp) were sequenced at a commercial facility in South Africa (INQABA Biotechnology Ltd). Identity and similarity of the sequences were tested using BLAST search (*http://blast.ncbi.nml.nih.gov/Blast.cgi*). The sequence data were then assembled and submitted at the NCBI GenBank for acquisition of accession numbers.

Screening of proteolytic activity

The ability of yeasts to produce extracellular proteolytic enzymes was examined by means of agar plates containing milk-agar [25]. About 5 µL of an overnight yeast suspension (2.0 x 10^5 cells) was added into bore holes previously made in milk agar plates, pH 5. The inoculated plates were incubated at 30°C for 5 days. The plates were observed for an increasing opacity around the holes caused by growing fungi. Clearing of opacity by hydrolysis of precipitated casein was affected by protease of the fungi. The millimetric zone measurements were evaluated as the level for proteolytic enzymes as: no proteolytic activity (0); low proteolytic activity (d<10mm); medium proteolytic activity (10mm < d<20mm) and high proteolytic activity (d > 20mm).

Statistical analysis

The significance of the yeasts load in *mtindi* and yoghurt samples was evaluated by comparing means using one-way ANOVA, followed by Tukey-Kramer Multiple Comparisons Test. Results were expressed as means + SEM and differences were considered significant when $p < 0.05$. Statistics were performed with Graphpad Instat version 3 for Windows (Graphpad Software, San Diego CA, USA).

Results

Yeast total counts, pH and diversity in yoghurt and *mtindi* samples

Based on [26], thirteen and sixteen yeast cultures were isolated from yoghurt and *mtindi*, respectively. The total yeast counts for *mtindi* varied from 10^3 + 0.92 to 10^5 + 0.34 CFU/mL with pH values ranging from 4.16 + 0.08 to 4.8 + 0.14 (Fig. 1) while yeast counts in yoghurt samples were generally higher than Log 3.9 but slightly lower than log 5 CFU/mL while the pH values ranged from 4.20 + 0.10 to 4.40 + 0.13 (Fig.2),

Identification of yeast isolates

Sequences data obtained from the three isolates TFMMw1B, TFMMw2B and TFMMw4B showed a 99% sequence identity to *Candida pararugosa.* The BLAST results for DNA sequence data for isolate TFMMw1A showed 99% sequence similarity to that of *Candida tropicalis* (GenBank accession number DQ099738 a Chinese botanical sample). TFMM3, TFMD6, and TFMD7 showed 99% homology similarity with that of *Pichia guilliermondii* FJ468466 in GenBank database (Chinese marine yeast) and *Meyerozyma guilliermondii* HM988686, native Italian wine yeast. The Blast results for sequence data for isolates A4 and B3 showed 99% homology to *Debaryomyces hansenii* JF 781387 isolated from material and environment in process of Chinese liquor fermentation and *D. hansenii* HM988696 isolated from Italian wine production materials. Isolates TFMMw2A exhibited 99% homology to that of *Pichia kudriavzevii* EU394711 isolated from Chinese lignocelluloses hydrolysate while TFMMw3B showed 87% homology to *Issatchenkia* sp. JF781389 isolated from materials of Chinese liquor fermentation. Isolate TFMMw3A had 99% homology to *Clavispora lusitaniae* FJ627984 isolated from Egyptian traditional fermented dairy products. The results for blast search for sequences of isolate B2 showed 99% sequence identity to *Yarrowia lipolytica* GQ121611 isolated from Tibetian natural fermented milk.The isolates A2, A3, TFMKA, TFMKB and TFMD3 and TFMD4 showed 99% sequence homology to *Galactomyces geotrichum*. Isolate A1 showed 99% sequence similarity to that of *Geotrichum* sp. However, the BLAST sequence data for Isolate TFMD2 showed 97% sequence identity to that of *G. geotrichum*.

Phylogenetic reconstruction based on D1/D2 26S rDNA gene sequences grouped these isolates into 8 groups (Fig. 3). Group one is of *G. geotrichum* comprising of isolates TFMD1, TFMF3, TFMKA,TFMKB, A1, A2 and A3; Second group is *Y. lipolytica* group comprising of isolates B1, B2 and C3; *D. Hansenii* is the third group comprising of isolates A4 and B3; *C. tropicalis* comprising of isolate TFMMw1A made the fourth group while isolates TFMM3, TFMD6 and TFMD7 form group fifth of *P. guilliermondii* (*M. guilliermondii*). *C. pararugosai* is the sixth group made by isolates TFMMw1B and TFMMw4B. *P. kudriavzevii* (*I. orientalis*) group made by isolates TFMMw2A and TFMD5 while TFMMw3A formed *C. lusitaniae* group are the seventh and eighth groups respectively.

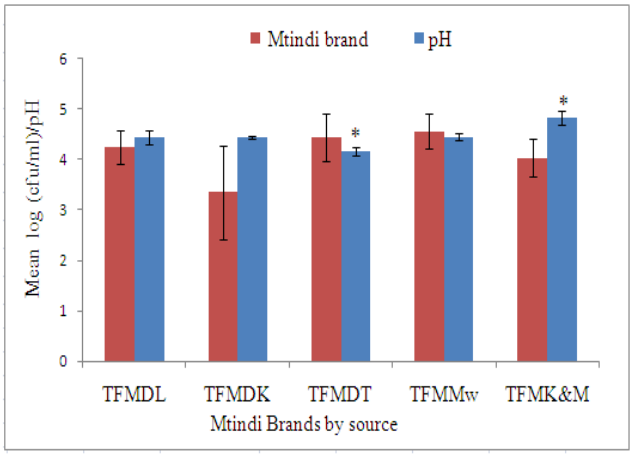


Figure 1: Mean yeast counts and pH isolated from Traditional fermented milk (TFM) *mtindi* bought from Dar es Salaam (DSM; Ilala (I), Kinondoni (K), Temeke (T) municipalities), Kilimanjaro and Morogoro (K&M)

Proteolytic activity

Protease activity was revealed as a clearance zone around the hole filled 5ul of 18 hours yeast culture on Milk agar medium (Table 1). Of 25 yeast isolates tested 21(84%) were protease positive. Of the yeast isolates examined in this study, the highest proteolytic extracellular enzymes were revealed by the strains belonging to among the *Candida* isolates tested (Fig.5), *C. pararugosa* (TFMMw2B) produced the strongest protease activity (37+5.487) as well as *C. tropicalis* (TFMMw1A) with (27+1.202).

Table 1: Some yeasts found with strong proteolytic activity

Name	***P.guilliermondii***	***P. kudriavzevii***	***Cl. lusitaniae***	***G. geotrichum***
Reference No.	**TFMM3**	**TFMMw2A**	**TFMMw3A**	**TFMD2**
Inhibitory zone (mm)	**17.667 +0.333**	**19.667+1.856**	**10.667+0.333**	**29.333+2.333**
Photo				

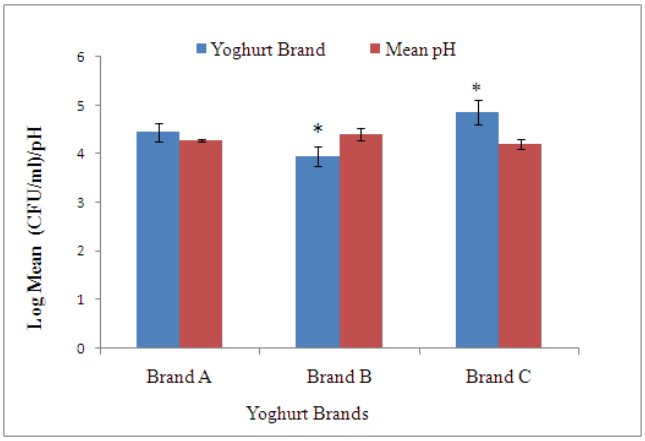


Figure.2: Mean yeast counts isolated from major Tanzanian yoghurt brands; A, B and C bought from Dar es Salaam City.

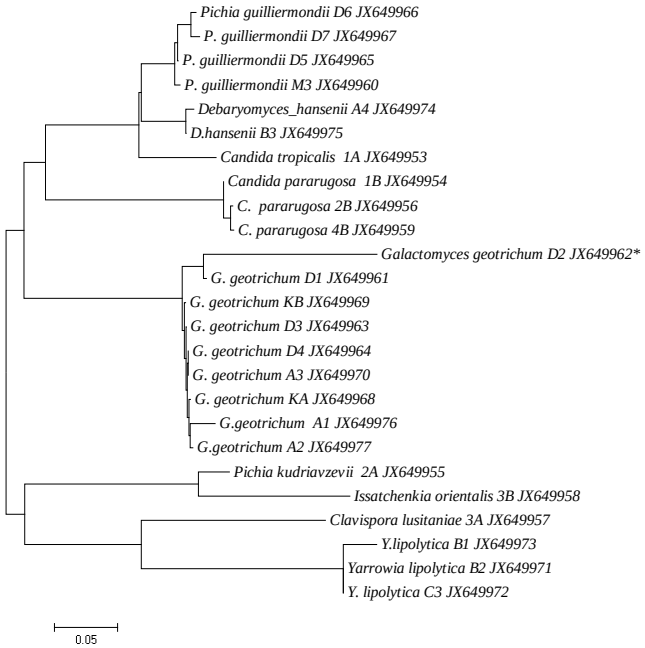


Figure 3: The evolutionary history inferred by using the Maximum Likelihood method. The analysis involved 25 nucleotide sequences. Evolutionary analyses were conducted in MEGA5. *Isolate TFMD2 diverging from the rest of the *G. geotrichum* group.

Discussion

The mean yeast counts in *mtindi* ranged from 10^3 to 10^5 CFU/ml with a pH ranging from 4.16 to 4.82 and selective environment for yeast proliferation which compliments [30] findings. *Mtindi* samples from Mwanza (TFMMw) showed the highest yeasts count of 10^5 CFU/ml unlike that from Dar es Salaam (DSMK, DSMI, and DSMT), Kilimanjaro (KLM) and Morogoro (Mo) which exhibited yeast counts below 10^4 CFU/ml (Fig.2). This may be contributed by the fact that raw milk is used for preparation of *mtindi* in Mwanza. The mean yeast counts in Tanzania's *mtindi* were lower (p = 0.0001) than that reported by [27] in Traditional Tunisian Leben and [28] in Sudanese fermented milk.

Yeast counts in yoghurts were lower (p = 0.0052) than that reported by [8] and [9] but were similar to the counts observed in yoghurt samples of Tunisian industrial Leben [27] , which were reported to be in excess of 10^4 CFU/g, while, values between 10^3 to 10^5 CFU/ml were reported in dairy products from Egypt [29]

The presence of *C. tropicalis* in *mtindi* from Mwanza was an interesting observation. The source of this pathogenic yeast may have been from the handler's hands at the time of preparations or during milking. *C. tropicalis* has previously been found in Zimbabwe traditionally fermented milk [30] also in Turkish-style homemade dairy products [13] in Tunisia traditional fermented milk, *Leben* [27] and in raw milk and cheese [33, 36].

Different degrees of yeast diversity were observed between yoghurt and *mtindi* samples. *Mtindi* from Mwanza contained diverse yeast flora consisting of five species whereas yoghurt brand C and traditionally inoculated fermented milk from Kilimanjaro and Morogoro contained a single but different yeast species. *P. guilliermondii*, *G. geotrichum*, *Y. lipolytica*, *D. Hansenii* were commonly identified across different products. While *P. guilliermondii* was only detected in *mtindi* samples, *Y. lipolytica* and *D. Hansenii* were detected in yoghurts only. Interestingly, *G. geotrichum* was the only species that was detected in both *mtindi* and yoghurt. However, *C. tropicalis*, *C. pararugosa*, *Cl. lusitaniae* (*C. lusitaniae*) and *I. Orientalis* were only detected in *mtindi* from Mwanza. Fermented milk from Mwanza is usually made by spontaneous fermentation of raw milk for about two days with the removal of cream and it has shown in this study to contain the most diverse yeast flora than the rest. The technic or utensils used in removing the cream may be associated with the introduction of more yeast in to mtindi in Mwanza. *Mtindi* from Kilimanjaro had single yeast species *G. geotrichum* while both *G. geotrichum* and *P. guilliermondii* were isolated from *mtindi* from Dar es Salaam

In this study, the most frequently yeast isolated from both yoghurt and *mtindi* was *G. geotrichum* (Anamoph: *Geotrichum candidum*). The proteases of *G. geotrichum* has been associated with development of distinctive

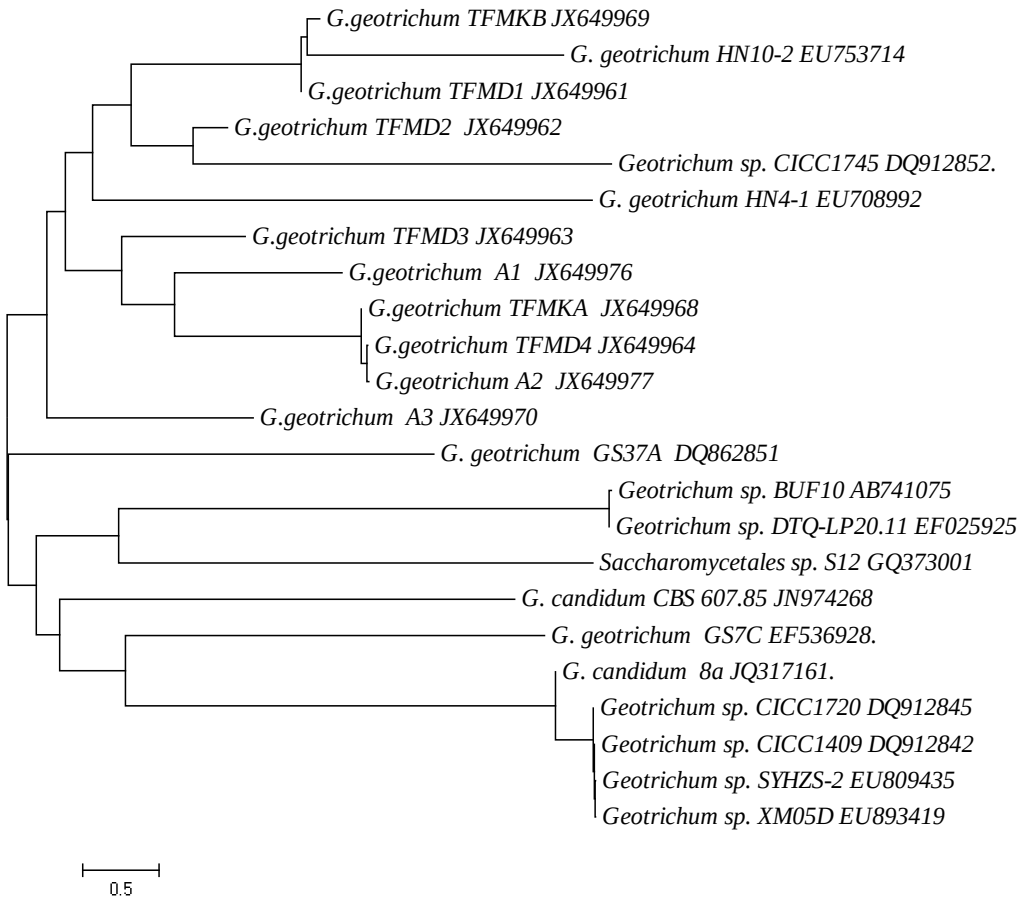


Figure 4: Neighbor-Joining phylogenetic tree of the polymorphic sequences of the D1/D2 domains of the 26S rDNA of *Galactomyces geotrichum*.

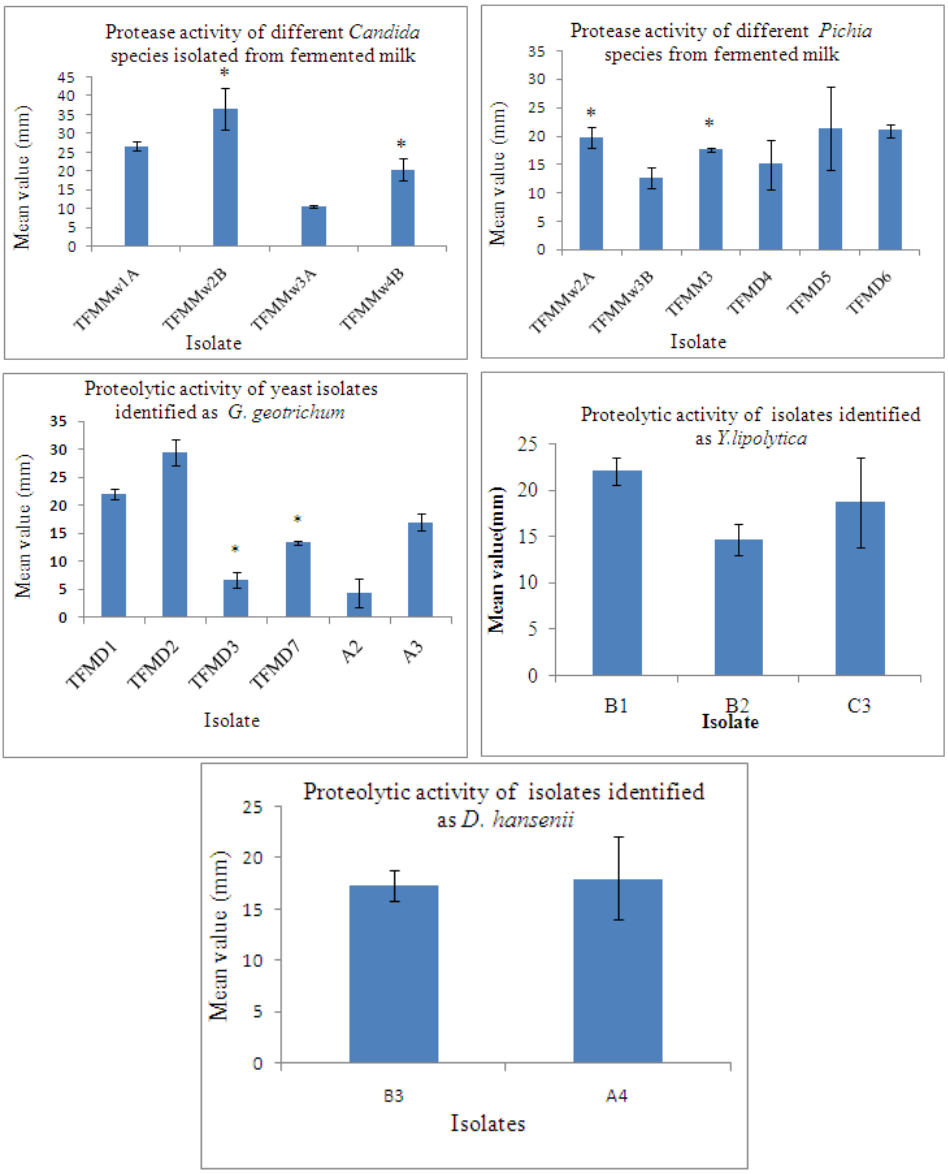


Figure 5: Proteolyitic activity among various yeast species isolated from yoghurt and mtindi. The results are mean values of three independent experiments. *Represent a significant difference ($p < 0.05$) according to Bonferroni Multiple Comparison Test

flavours and other qualities of traditionally made cheese and also reduction of bitterness in industrially made cheese [32]. Another enzyme, lipases from *G. geotrichum* were also reported to contribute to its good qualities. Of all *G. geotrichum* tested, isolate TFMD2 produced the strongest protease activity (29+2.333; p=0.0119). The observed variation in proteolytic activity among *G. geotrichum* in this study might be explained by two circumstances first, the possibility of slight genetic variation in isolate TFMD2 from the rest of *G. geotrichum* of this study, Maximum likelihood tree based on 26S rDNA sequences (Fig.4) displayed isolate TFMD2 and *Geotrichum sp.* CICC1745 (Accession number, DQ912852) from China which is a novel *Geotrichum* species. Secondly, could be due to the three basic morphological variations among *G. geotrichum* strains. Yeast like colonies with abundant arthrospores are associated with low proteolytic activity while those with white colonies and filamentous fungi look-alike and hyphae have high proteolytic activity, and others fall in between [32, 34]. Another fascinating observation was the detection of *G. geotrichum* in yoghurt and *mtindi* made by addition of a portion of inoculums of previously fermentation in to pre-boiled milk only and not in naturally fermented mtindi (TFMMw1, TFMMw2, TFMMw3, TFMMw4 and TFMM3). Marian and others [33] reported that *G. geotrichum* appeared to be one of the most dominating microbial ecology of biofilms. Their presence in yoghurt may be due to their ability to form biofilm on the surface on the factory while in *mtindi* it may be due to the use of the same holding containers with biofilm dominated by *G.geotricum*. We may also speculate the possible association between protease activity and biofilm formation.

Conclusions

Even though the microbiology of dairy products is generally subjected to debates of Lactic acid bacteria, an extensive literature is now describing the importance of the role of yeasts in flavour and texture development of a

number of dairy products. Since fermented dairy products have got favourable growth conditions for yeasts, it is not suprising to find yeasts prolifying in these products. Indeed, the presence of yeasts and their proteases among other metabolites in dairy products is of major importance as they may either have beneficial or harmful outcome with contribution to flavour development and rippening.

This study demonstrates that diverse yeast species existed in the examined Tanzanian traditional (*mtindi*) and industrially fermented milk (yoghurt) in which *mtindi* from Mwanza contains the most diverse yeast flora. To our knowledge this reports for the first time genetically characterized yeast isolated from Tanzanian fermented milk and their proteolytic characteristsics with either potential biotechnological benefits or harmful effects. In the present study, we amplified and sequenced the domains (D1 and D2) regions of the 26S rRNA gene for the identification of yeasts from industrially fermented milk, yoghurt and traditional Tanzanian fermented milk, *mtindi* obtained from different firms and localities of Tanzania. Findings showed a diverse degree of yeast population and protease activity in examined products.

Further research concerning characterization of the protease from yeasts is necesssary to explore the mechanism of their actions in relation to the biochemical nature of the fermentation processes. This may further explore the identity of extracellular protease for novel agents in bio-control of food spoilage and the identity of yeast with the ability to produce them.

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