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In Vitro Screening and Characterization of Kefir Yeast for Probiotic Attributes

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Abstract

The yeasts constitute a large and heterogeneous group of microorganisms, currently attracting increased attention from scientists and industry as probiotics. Till date only *Saccharomyces boulardii* has been extensively studied for its probiotic effects. Therefore, the current study aims to characterize the probiotic potential of yeast isolated from kefir, a fermented beverage. Out of 22 yeast isolates screened, 13 isolates could survive (>75%) in simulated conditions similar to the gut (pH 2.0 and 1.0% bile salt). The isolates showed high auto-aggregation (>85%) ability and cell surface hydrophobicity (>75%) have also expressed high in-vitro adherence (>90%) to HT-29 cells. A simulation of transit tolerance in the upper human gastrointestinal tract together with auto-aggregation, hydrophobicity, and adherence to HT-29 cells have been vital in reducing the number of yeast strains to 7 promising probiotics. The probiotic yeast strains showed resistance to commonly used antibiotics and exhibited a broad spectrum of antagonistic activity against pathogenic microorganisms (*E. coli, S. typhimurium, S. paratyphi-A, S. aureus, S. sonnei, B. cereus* and *Y. enterocolitica*). The results obtained were compared with the reference culture, *Saccharomyces cerevisiae* ATCC 7745. Based on 5.8S rRNA gene sequencing the isolates were identified as *Pichia kudriavzevii, Candida xylopsoci, Saccharomyces cerevisiae* and *Issatchenkia orientalis*. Overall these results demonstrated the possible use of these isolates in the development of novel functional foods with potential probiotic properties.

Keywords: Probiotics, Yeast, Kefir Grains, Beverage, Fermented Foods, Screening

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Introduction

Yeasts are microorganisms of great economical interest for their numerous applications in traditional and modern biotechnology (Raton, 2004). The products of yeast form the backbone of many commercially important sectors, including various fermented foods (Yarrow, et al. 1998), beverages, pharmaceuticals and industrial enzymes which are attracting increased attention from scientists and industry. Kefir, a natural fermented probiotic beverage is gaining in popularity because of its number of health promoting properties and its distinct flavour, typical of yeast (Farnworth el at. 2005; Lopitz- Otsoa et al. 2006; Miguel et al. 2010; Rattray and O'Connel et al. 2011; Magalhaes et al. 2011a; Ahmed et al. 2013).

The microbes responsible for the fermentation of milk to produce kefir consist of a complex association of lactic acid bacteria (*Lactobacillus, Lactococcus, Leuconostoc,* and *Streptococcus spp.*) and yeasts (*Kluyveromyces, Saccharomyces* and *Torula*) that are immobilized on a polysaccharide and protein matrix of the kefir grains to serve as a starter culture (Garrote et al. 2010; Miguel et al. 2010; Magalhaes et al. 2011a). Kefir has immuno-modulatory properties as well as antimicrobial, antihypertensive, anti-inflammatory, anticarcinogenic, antiallergic, antioxidant activity, with ability to reduce cholesterol levels, and alleviate lactose intolerance (Farnworth et al. 2005; Ahmed et al. 2013). Regardless of their non-human origin, such non-pathogenic yeasts fulfill the major criteria for probiotic definition. The beneficial features thus reported therefore, indicate kefir being as a promising source of new microbial strains including yeast for the development of functional foods.

Probiotics are living microorganisms that exert a health benefit to the host when administered in sufficient amounts (FAO/WHO 2002). The most extensively studied probiotics belong to the genera *Lactobacillus* and *Bifidiobacterium* (Borriello et al. 2003; Tuohy et al. 2003). Despite the occurrence of yeasts in many dairy related products (Fleet et al. 1990; Jakobsen and Narvhus et al. 1996) and in the human gastrointestinal tract (GI) (Knoke et al. 1999; Czerucka et al. 2007), their potential as probiotics has been overlooked. The microbiological and chemical composition of kefir indicates that it is a much more complex probiotic, as the large number of different bacteria and yeast found in it distinguishes it from other probiotic products. The studies have revealed that the yeast component of kefir consists of *Kluyveromyces, Saccharomyces, Candida* and *Torulaspora* (Angulo et al. 1993; Wyder et al. 1997; Lin et al. 1999; Simova et al. 2002; Loretan et al. 2003). Other yeast that have been less frequently associated with kefir include *Pichia/Issatachenkia* (Latorre-Garcia et al. 2007), *Brettanomyces/Dekkera* (Pintado

et al. 1996; Wyder et al. 1997), *Yarrowia* (Loretan et al. 2003), *Zygosaccharomyces* (Witthuhn et al. 2005), and recently reported *Kazachstaniaaerobia* and *Lachanceameyersii* (Zhou et al. 2009; Magalhaes et al. 2011; Gao et al. 2012). The yeasts and bacteria present in kefir grains have undergone a long association; the resultant microbial population exhibits many similar characteristics, making isolation differentiation of the isolated strains more challenging.

The yeast strains recovered from kefir namely, *Saccharomyces cerevisiae, Saccharomyces unisporus, Issatchenkia occidentalis,* and *Kluyveromyces marxianus* have showed acid and bile resistance phenotypes and are thus potentially suitable for probiotic purposes (Diosma et al. 2013). But still the only yeast known for its probiotic effects in humans and often marketed as a dietary supplement (McFarland et al. 2010) and also employed as a therapeutic agent for the treatment of a variety of gut disorders to normalize intestinal flora is, *Saccharomyces cerevisiae* var. *boulardii (Saccharomyces boulardii)* (Szajewska et al. 2007; Zanello et al., 2009; Saad et al. 2013). Further research into novel probiotic yeast isolates is important to satisfy the increasing market demand and to obtain highly active probiotic cultures for improved food products with characteristics that are superior to those present on the market. The search for more yeast with probiotic potential from kefir preparation and with possible application in food industry appears to be a promising area of investigation.

Materials And Methods

Screening of yeast from kefir and growth media

The organic milk kefir grains were inoculated (5%; w/v) to the cooled pasteurized milk (3% fat Nandini milk; Mysore, Karnataka, India) and incubated at 37 °C for 24-48 h. The fermented kefir beverage was filtered to remove the kefir grains. The obtained beverage was serially diluted up to 10^{-6} dilution and plated on MRS (de Man, Rogosa and Sharpe) agar (Himedia, India) plate by spread plate method and incubated at 37 °C for 24-48 h. The morphology and size of different colonies of yeast on MRS agar plates were characterized by microscopic and macroscopic method (Barnett et al. 2000). A total of 22 visually different colonies were isolated.

The colonies grown on MRS agar plates were further cultured on yeast extract peptone glucose agar (YPGA) and potato dextrose agar (PDA) to screen suitable media for the growth of kefir yeast. All the 22 isolates along with reference culture *Saccharomyces cerevisiae* ATCC 7745 were point inoculated on YPGA, PDA and MRSA plates and incubated at 37 °C for 24-48 h. In order to determine morphology of yeasts cells and reproduction type, the yeast isolates were examined microscopically by staining method.

Genotypic characterization

Total genomic DNA of 22 yeast isolates was extracted using the DNA extraction kit (HiPureATM Bacterial and yeast Genomic DNA Miniprep purification spin kit, Himedia, India) following the instructions of the manufacturer. The extracted DNA was quantified using the Nanodrop (Thermo Scientific Nanodrop 2000 Spectrophotometer, Genesis Biosolutions, and India) and diluted to a concentration of 50ng/ml and used for the PCR analysis (Greppi et al. 2013a and 2013b). The amplification of internal transcribed spacer ITS1 and ITS4 with forward and reverse universal primers (ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 5'-TCCTCCGCTTATTGATATGC- 3') targeted to the 5.8S rRNA gene was carried out (Guillamon et al. 1998) in 20 µl of reaction mixture containing 10x PCR buffer (2.0 µl), 25 mM $MgCl_2$ (1.6 µl), 2 mM dNTP (1.0 µl), 0.6 µl each of forward and reverse primers, Taq DNA polymerase (0.1 µl), autoclaved deionized water (13.1 µl) and genomic DNA (1.0 µl). PCR amplification was carried out in a Verti 96 well Thermal cycler (Applied Biosystems, India) with an initial denaturation at 94 °C for 4 min, followed by 35 cycles at 94 °C for 1 min, annealing at 58 °C for 1 min and elongation at 72 °C for 1 min followed by final extension at 72 °C for 10 min. The amplified products were stored at -20 °C until analyzed. Aliquots of the amplification products along with DNA ladder marker were analyzed by electrophoresis in horizontal 1.0% (w/v) agarose gel in 0.5X TBE buffer, stained with ethidium bromide (0.5 µl/ml) at a constant current of 90V and visualized under ultraviolet light. The PCR products obtained through amplification were purified and sent for sequencing to a commercial sequencing facility. Sequences were aligned to 5.8S rRNA gene sequences in the Gen bank database using the BLAST algorithm (Altschul et al. 1997).

Probiotic characterization of kefir yeast

According to recent Food and Agriculture Organization (FAO) and World Health Organization (WHO) guidelines (FAO/WHO, 2002), probiotic organisms used in food must be able to survive passage through the gut i.e., they must have the ability to resist gastric juices and exposure to bile. Furthermore, they must be able to proliferate and colonize the digestive tract. Therefore, the yeast isolates were studied for their prime probiotic properties.

Acid and bile salt tolerance

To determine the acid and bile salt tolerance, 22 selected yeast isolates were propagated twice in YPG broth. Initially yeast cultures were incubated at 37 °C for 24 h in YPG broth and after 24 h, the yeast isolates were inoculated into sterile YPG broth medium acidified to pH 2.0 with 1N HCl and supplemented with 1% ox bile (Syal and Vohra et al. 2013). Samples were drawn immediately (0 h) and after 4 h of incubation at 37 °C, and serial dilutions in saline 0.85% (w/v) was made. Appropriate dilutions were placed on YPGA in order to determine the number of viable cells. The survival rate was calculated as the percentage of colonies grown on YPGA medium after exposure (4 h) to low pH and high bile salt concentration as compared to the initial cell concentrations using standard formula.

$$\% \text{ Survival} = \frac{\log \text{number of viable cells survived (CFU/ml)}}{\log \text{number of initial viable cells inoculated (CFU/ml)}} \times 100$$

Tolerance to synthetic gastric juice (SGJ)

Survivability of yeast isolates in synthetic gastric juice was determined according to the method of Cotter et al., (2001). The composition of SGJ per liter is 8.3 g of protease peptone, 3.5 g of glucose, 2.05 g of NaCl, 0.6 g of KH2PO4, 0.11 g of CaCl2, 0.37 g of KCl, 0.05 g of bile, 0.1 g of lysozyme and 13.3 mg of pepsin (pH 2.5). The media was filter sterilized (Pedersen et al. 2004) using 0.22µ membrane filter (Millipore, India). Samples were drawn immediately (0 h) and after 4 h of incubation at 37 °C, and appropriate dilutions in 0.85% (w/v) saline was inoculated to YPGA by spread plate method in order to determine the number of viable cells and percent survival was calculated using standard formula.

$$\% \text{ Survival} = \frac{\log \text{number of viable cells survived (CFU/ml)}}{\log \text{number of initial viable cells inoculated (CFU/ml)}} \times 100$$

Microbial adhesion to hydrocarbons (MATH) assay

MATH assay was carried out by the method of Syal and Vohra et al. (2013) with slight modification. For the cell surface hydrophobicity, the selected yeast isolates were grown in YPG broth at 37 °C for 24 h. The cells were harvested by centrifugation at 12000 rpm, at 4 °C for 20 min, washed twice and resuspended yeast pellet in phosphate buffer saline (PBS; pH 7.0). The absorbance (OD) was measured using an infinite M200PRO (TECAN) at 600 nm. Aliquots of yeast suspensions were put in contact with hydrocarbons - xylene and toluene, separately (1:3 v/v). The cells were vortexed for 120 sec and the suspension was kept undisturbed at 37 °C for 30 min to allow phase separation. After 30 min, the aqueous phase was removed carefully and the absorbance (OD) was measured at 600 nm. The decrease in the absorbance is the measure

of the cell surface hydrophobicity. The % hydrophobicity is calculated using the equation given below.

% Hydrophobicity = (OD initial - OD final)/OD initial x 100

Adhesion of yeast isolates to intestinal HT-29 cell lines

The colonocyte-like cell line HT-29 was procured from NCCL, Pune, India and were used to determine the adhesion ability of the yeast isolates. The culture and maintenance of the HT-29 cell lines were carried out following standard procedures (Sanchez et al. 2010) using DMEM (Dulbecco's Modified Eagle's medium) supplemented with FBS (Fetal Bovine Serum, Sigma). Intestinal cells were seeded in 24-well tissue culture plate and cultivated until a confluent differentiated state was reached. For adhesion experiments, 9±1 day-old cellular monolayers were used. Yeasts were cultured for 24 h and after washing twice with phosphate buffer solution (PBS), they were re-suspended in the corresponding cell-line media without FBS. Cellular monolayers were also carefully washed with PBS and yeast suspensions (10^8 CFU/ml) were added at a ratio of about 10:1 (yeast: eukaryotic cell). Adhesion experiments were carried out for 1 h at 37 °C , 5% CO2. After incubation, wells were gently washed to release unattached yeast before proceeding with the lysis of cellular monolayer using 0.25% Trypsin–EDTA solution (Sigma). Dilutions of samples, before and after adhesion were made in 0.85% NaCl solution and yeast counts were performed in YPGA plates. The percent of yeast adhering to the intestinal epithelial cells was calculated as,% = CFU adhered yeasts /CFU added yeasts

Auto-aggregation assay

Auto-aggregation assay was performed as described by Collado et al. (2008) with minor modifications. Yeast isolates were grown for 24–48 h at 37 °C in YPG broth. The cells were harvested by centrifugation at 12000 rpm for 10 min, washed twice and resuspended in PBS (pH 7.0). Cell suspensions (5 ml) were mixed by vortexing for 10 sec and auto-aggregation was determined after 3 h and 20 h of incubation at 37 °C. An aliquot (100µl) of the upper suspension of PBS after incubation was transferred to another tube with 3.9 ml of PBS and the absorbance (A) was measured at 600 nm. The auto-aggregation percentage is expressed as: $1-(A_t/A_o)X\ 100$. Where, A_t represents the absorbance at time t = 3 h or 20 h. Ao the absorbance at t = 0 h.All the experiments were performed in triplicates.

Antibiotic susceptibility The antibiotic resistance of yeast isolates was analyzed using various antibiotic discs (HiMedia, India) on YPGA plate seeded with 24 h active cultures of the yeast isolates (Christobell et al. 2012). The antibiotic resistance was assessed against Trimethoprin (TR), Cephotaxim (CTX), Cefixime (CFM), Ofloxacin (OF), Nalidixic acid (NA), Cloramphenicol (C), Amoxyclav (AMC), Oxytetracycline (O), Ceftriaxone (CTR), Tetracycline (TE), Gentamycin (GEN), Erythromycin (E), Streptomycin (S), Ampicillin (AMP), Vancomycin (VA), Polymyxin–B (PB), Pencillin-G (P), Co-trimoxazole (COT), Azithromycin (AZM), Doxycycline Hydrochloride (DO) and Rifampicin (RIF). The antibiotic discs were placed on the surface of agar and the plates were incubated at 37 °C for 24 h. The zone size (mm) interpretative chart for antibiotics was measured according to performance standards.

Antimicrobial activity

The antimicrobial activity of yeast isolates against enteric pathogens (*Escherichia coli* ATCC 10536, *Salmonella typhimurium* MTCC 1251, *Salmonella paratyphi*-A ATCC 9150, *Staphylococcus aureus* ATCC 700699, *Shigella sonnei* ATCC 25931, *Bacillus cereus* ATCC 14579, and *Yersinia enterocolitica* ATCC 23715) was performed by using the agar well diffusion method, as described by Tatsadjieu et al. (2009). The cultures filtrate of the 24-48 h old yeast isolates was inoculated to the wells of YPGA plate containing pathogens. The plates were incubated at 37 °C for 24 h and the diameter of zone of inhibition was measured in millimeter (mm). The zone of inhibition around the wells indicates positive (+) for antimicrobial activity while the absence of zone indicates a negative (-) result.

Results And Discusion

Screening of yeast from kefir and growth media

Out of 26 isolates from kefir, 22 strains were confirmed as yeast based on their colony morphology on MRS agar plates. Colonies were off white in colour, circular in shape with slightly irregular margins, convex elevated with opaque opacity and smooth texture. It was further confirmed by lacto-phenol cotton blue staining and observed under microscope. The screened isolates were preserved on MRS agar plate for further study.

The growth profile of yeast strains on YPGA, MRSA and PDA media is shown in Fig. 1 and it revealed that the culture media had great influence on growth. It was observed that irrespective of the culture medium employed and the conditions maintained, the growth of each strain is found to be diverse (Table I). Based on the result obtained, YPGA is considered suitable media for the rapid growth of yeast strains when compared with that of MRSA and PDA media. Therefore, YPGA medium is selected for further study.

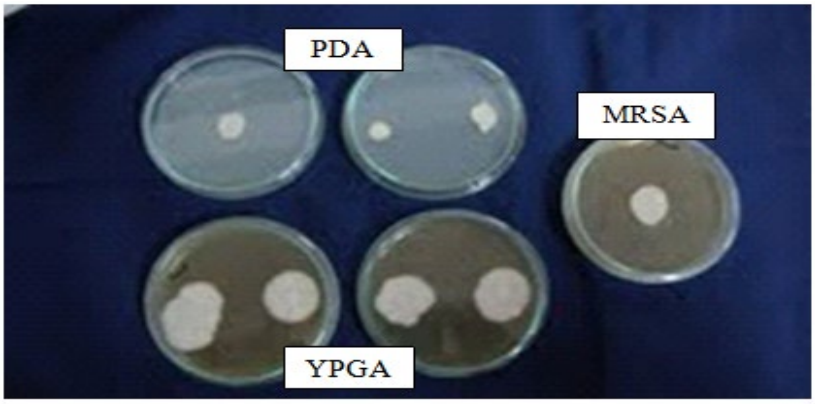

Figure 1 : Yeast strains on PDA, MRSA, and YPGA plate

Yeast strains	GenBank Accession numbers	Diameter of the yeast cultures (cm)		
		YPGA	MRSA	PDA
1y - *Pichia kudriavzevii*	MF685411	5.3	3.1	0.9
2y - *Pichia kudriavzevii*	MF685412	4.5	2.7	0.7
3y - *Pichia kudriavzevii*	MF685413	3.9	2.0	1.1
4y - *Saccharomyces cerevisiae*	MF685414	4.6	3.0	1.2
5y- *Saccharomyces cerevisiae*	MF685415	4.2	2.8	0.8
6y - *Issatchenkia orientalis*	MF685416	3.5	2.3	1.4
7y- *Issatchenkia orientalis*	MF685417	3.2.	2.2	0.7
8y - *Pichia kudriavzevii*	MF685418	3.0	2.2	1.1
9y - *Pichia kudriavzevii*	MF685419	3.7	1.7	1.2
10y - *Issatchenkia orientalis*	MF685420	3.9	2.4	0.8
11y - *Issatchenkia orientalis*	MF685421	3.8	1.5	0.7
12y - *Candida xylopsoci*	MF685422	4.0	2.0	0.9
13y - *Pichia kudriavzevii*	MF685423	3.5	2.4	1.3
14y - *Candida xylopsoci*	MF685424	4.3	1.9	1.1
15y - *Candida xylopsoci*	MF685425	5.0	2.4	0.7
16y - *Candida xylopsoci*	MF685426	3.4	2.5	0.8
17y - *Saccharomyces cerevisiae*	MF685427	3.2	2.7	0.7
18y - *Pichia kudravzevii*	MF685428	5.0	2.5	0.8
19y - *Issatchenkia orientalis*	MF685429	4.5	2.4	0.6
20y - *Pichia kudriavzevii*	MF685430	4.1	2.8	0.9
21y - *Pichia kudriavzevii*	MF685431	3.6	1.7	1.2
22y - *Issatchenkia orientalis*	MF685432	5.0	2.1	1.0

Table 1: Growth profile of yeast strains on YPGA, MRSA, and PDA plate

Genotypic characterization

The 22 yeast isolates screened were identified as *Pichia kudriavzevii* (9), *Issatchenkia orientalis* (6), *Candida xylopsoci* (4), and *Saccharomyces cerevisiae* (3) based on the ITS region of 5.8S ribosomal RNA gene. The sequence of rRNA gene from all the yeast strains was homologous to an extent of 99% with that of other strains. In the current study, *P. kudriavzevii*, being the most prevalent species during fermentation of kefir. *S. cerevisiae* comprised less than 15% of the total isolated microflora in kefir. Previously *S. cerevisiae* has been reported to be one of the predominant yeast species in fermented products (Aditi Sourabh et al. 2012). The fermentation of kefir is often initiated by *I. orientalis*, *C. xylopsoci*. The presence of *P. kudriavzevii*, *I. orientalis* and *C. xylopsoci* has not been previously reported in kefir as probiotics. Overall, these results confirm the importance of these genera for kefir production. Irrespective of the possible failures in the isolation and identification of the yeast in the grains, the results obtained in the present study confirm the high microbial heterogeneity in kefir grains. Further, these yeast strains were characterized for their probiotic properties. Because the complexity of intestinal flora where popular yeast is supposed to serve as a probiotic requires a clear definition of the selection criteria based on which, it could be classified as a new target-specific or site-specific probiotic strain (Collins et al. 1998; Klaenhammer and Kullen et al. 1999; Gueimonde and Salminen et al. 2006).

Resistance at low pH

Ability of the yeast strains to survive at low pH (2.0) for 4 h at 37 °C is shown in Fig. 2. The acidic environments encountered in food and in the gastrointestinal tract provide a significant survival challenge for intestinal flora. Hence, acid tolerance is accepted as one of the indispensable properties used to select potentially probiotic strains. The result obtained indicates that, all the strains were tolerant to pH 2.0 for 4 h despite variation in their degree of viability. Of the 22 strains tested, 15 yeast strains showed significant viable count [10 isolates (*S. cerevisiae* - 4y; *I. orientalis* – 6y, 7y, 11y, 19y; *P. kudriavzevii* - 9y, 13y; *C. xylopsoci* - 12y, 14y, 15y) showed more than 80% and 5 isolates (*C. xylopsoci* - 16y; *S. cerevisiae* - 17y; *P. kudriavzevii* - 18y, 20y; *I. orientalis* - 22y) showed 75% survivability]. The other yeast isolates showed less than 75% tolerance after 4 h of incubation. The yeast isolates showed more than 80% survivability was comparable with the reference culture *S. cerevisiae* ATCC 7745 which showed 85% survivability. The reasons of the factors responsible for this could be cell size, composition of cell wall, extrude protons etc (Czerucka et al. 2007). Exposure of the yeast cells to environmental stresses like low pH and high bile concentration (Arino et al. 2010) can possibly triggers biochemical and gene expression changes (Gasch et al. 2000) and may also causes immediate changes in the cytosolic calcium, an important second messenger in eukaryotic cells. The survivability of kefir yeast at low pH reported by Katarzyna Rajkowska and Styczynska, et al. (2010) and Diosma et al. (2013) was found to be 50% and 90% after 4 hours and 3 hours of incubation respectively, which is significantly less when compared to the survival rate (>80%) of *P. kudriavzevii*, *S. cerevisiae*, *I. orientalis* and *C. xylopsoci* in the current study.

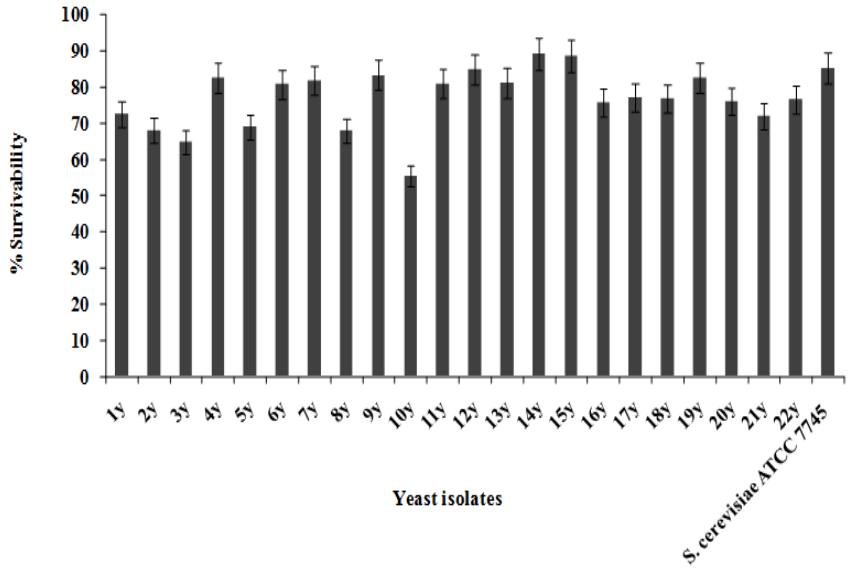

Figure 2: Survivability of yeast isolates at low acid (pH-2.0)

Tolerance to high ox-bile

Once the yeast passes through the acidic stomach condition, it is important for the yeast to survive in the high bile salt environment of the small intestine for growth, colonization and metabolic activity in the host's gut (Liong and Shah et al. 2005). The small intestine and colon of humans and animals contain relatively high concentrations of bile salt, which can inhibit growth or kill many bacteria. Therefore, it is essential that probiotic bacteria, to be effective, should be able to grow in 0.3-1.0% ox-bile (Goldin and Gorbach et al. 1992). The 22 yeast strains were tested for their tolerance to 1% ox-bile concentrations for 4 h at 37 °C. In the current study, a total of 16 yeast strains survived at 1.0% ox-bile concentration with the survival rate of more than 75%. The % survivability (>80%) exhibited by *I. orientalis* - 7y, 19y, 22y; *C. xylopsoci* - 12y, 15y; *S. cerevisiae* - 17y; *P. kudriavzevii* - 9y, 18y, 21y was highly comparable with the survival rate (86%) of the reference culture *S. cerevisiae* ATCC 7745. However, other yeasts (*P. kudriavzevii* - 1y; *S. cerevisiae* - 4y, 5y; *I. orientalis* - 6y, 10y, 11y, *C. xylopsoci* - 16y) showed good survivability (>75%) after 4 hour of incubation (Fig. 3). The difference in the level of bile tolerance of yeast strains in the present study may probably be due to the differences in their ability to grow and colonize the intestinal tract (Usman and Hosono et al. 1999; Kheadr et al. 2006). The kefir yeasts reported by Rajkowska and Kumicka-Styczynska (2010) showed least bile salt tolerance in 1% bile salt concentration. The yeast strains investigated in the current study displayed good resistance to 1% ox-bile as all could replicate and hence survive the exposure to bile salts. There was considerable variability in resistance to bile salts between the different species of yeasts, supporting the importance of assessing the bile tolerance of isolates in selecting potential probiotics. Hence the yeast strains survived >80% were considered as the most bile salt tolerant strains.

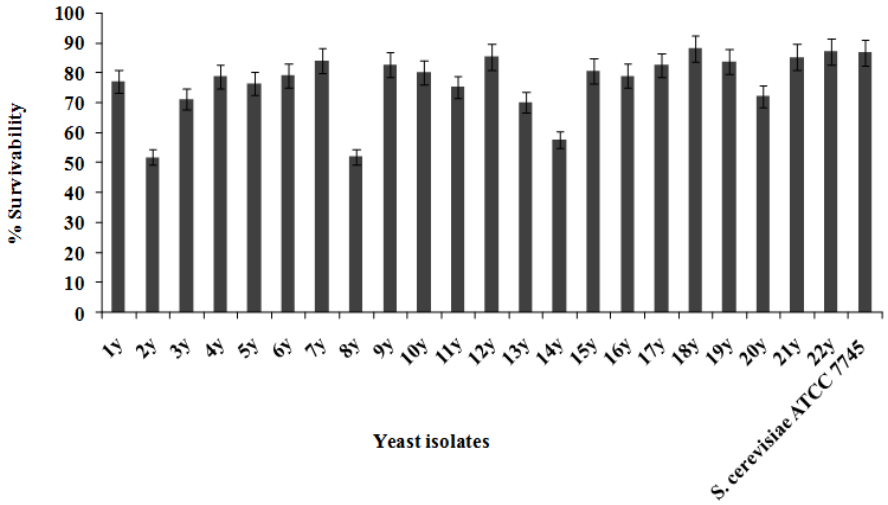

Fig 3: Survivability of yeast isolates at high bile salt concentration (1% ox bile)

Tolerance to simulated gastric condition

The effects of simulated gastric condition on the viability of yeast strains are depicted in Fig. 4. Tolerance to simulated gastric juice is an important trait of probiotic microorganisms because the probiotics entering the gastro intestinal tract must be resistant to local stresses such as the presence of gastro intestinal enzymes besides pH and bile salt. The results showed that the 13 yeast strains (*P. kudriavzevii* - 2y, 3y, 9y, 18y, 21y; *I. orientalis* - 7y, 19y, 22y; *C. xylopsoci* - 12y, 14y, 15y, 16y; *S. cerevisiae* - 17y) survived (>75%) under simulated gastric condition. Yeast strains able to survive conditions mimicking the gastro intestinal environment together with low pH and high bile salt concentration, have been very important in decreasing the number to 9 strains (*I. orientalis* - 7y, 19y, 22y; *P. kudriavzevii* - 9y, 18y; *C. xylopsoci* - 12y, 15y, 16y, *S. cerevisiae* - 17y).

The possible use of yeasts as probiotics is encouraged by the observation of the ability of *S. cerevisiae* members to survive passage through the intestinal tract (Lourens-Hattingh and Viljoen et al. 2001). The kefir yeast strains demonstrated high tolerance to simulated gastric environment and thus they offer a better source of potential probiotics, apart from lactic acid bacteria. This finding suggests that these strains have the potential to survive the passage through the stomach, small and large intestine.

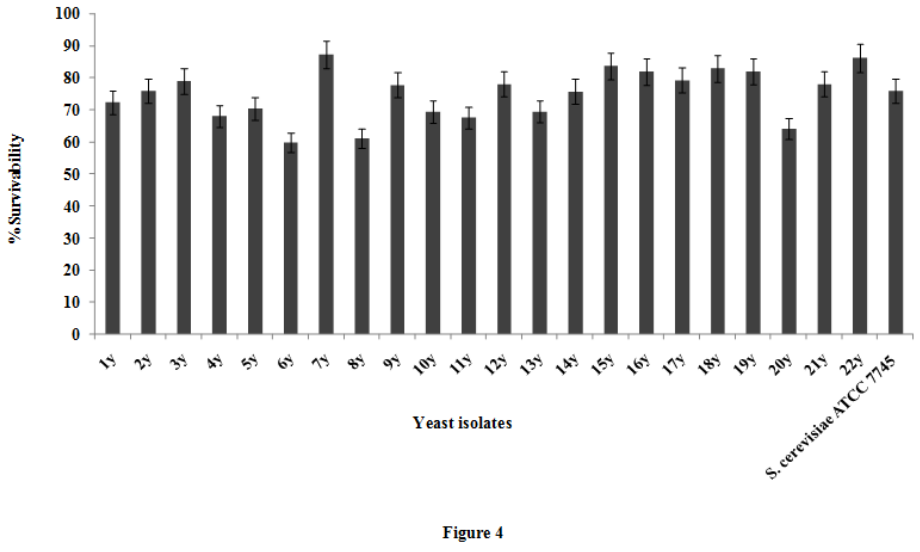

Fig 4: Survivability of yeast isolates in simulated gastric juice

Hydrophobicity, auto-aggregation and adhesion ability to intestinal HT-29 cell line

One of the important properties of probiotic microorganisms is their ability to adhere to the target sites for their colonization in the gut for expressing optimal functionality. The yeast strains (*P. kudriavzevii* - 2y, 3y, 9y, 18y, 21y; *I. orientalis* - 7y, 19y, 22y; *C. xylopsoci* - 12y, 14y, 15y, 16y; *S. cerevisiae* - 17y) showed more than 75% survivability in simulated gastric condition was undertaken with the objective to elucidate the adherence potential of yeasts under in vitro conditions based on their ability to adhere HT-29 cells, hydrocarbons and auto-aggregation (Fig. 5). Auto-aggregation and hydrophobicity value of 67.59 ± 0.27% and 58.21 ± 1.09% reported for *S. cerevisiae* (Sourabh et al. 2011) falls between the auto-aggregation (%) and hydrophobicity values in the present study which is in the range of 51.11% to 91.30% and 40.74% to 86.79% respectively. Strains possessing high hydrophobicity and auto-aggregation ability have been more strongly associated to adhesion property (Del Re et al. 2000; Pan et al. 2006; Rahman et al. 2008) since, adhesion is a prerequisite for colonization (Yongchen Zheng 2013). Aggregation between the cells of same strains (auto-aggregation) is of considerable importance in the human gut where probiotics are to be active and such abilities favour colonization in the gastrointestinal tract (Venkatasatyanarayana Nallala and Jeevaratnam 2015). In the present study, *I. orientalis* - 7y, 19y and 22y; *P. kudriavzevii* – 9y and 18y, *C. xylopsoci* - 16y; *S. cerevisiae* - 17y showed strong (>85%) hydrophobicity and auto-aggregation properties. Microbial adhesion is initially based on non-specific physical interactions between two surfaces, which then enable specific interactions between adhesins (usually proteins) and complementary receptors. Adhesion scores of *I. orientalis* (7y, 19y and 22y), *P. kudriavzevii* (9y and 18y), *C. xylopsoci* (16y) and *S. cerevisiae* (17y) were more than 90% on HT-29 cell lines which is highly comparable with the reference culture (84%) and therefore, these isolates can be regarded as strongly adhesive to HT-29 cell line. These strains demonstrated their ability to adhere to epithelial cell and exhibited strong hydrophobicity under in vitro conditions, and thus could have better prospects to colonize the gut with extended transit. Hence simulation of transit tolerance in the upper human gastrointestinal tract, together with auto-aggregation and hydrophobicity, has been decisive in reducing the number of promising probiotic yeast. Therefore, out of 13 yeast strains, only 7 isolates (7y, 9y, 16y, 17y, 18y, 19y and 22y) could fulfill the preliminary in vitro selection criteria for being designated as probiotic.

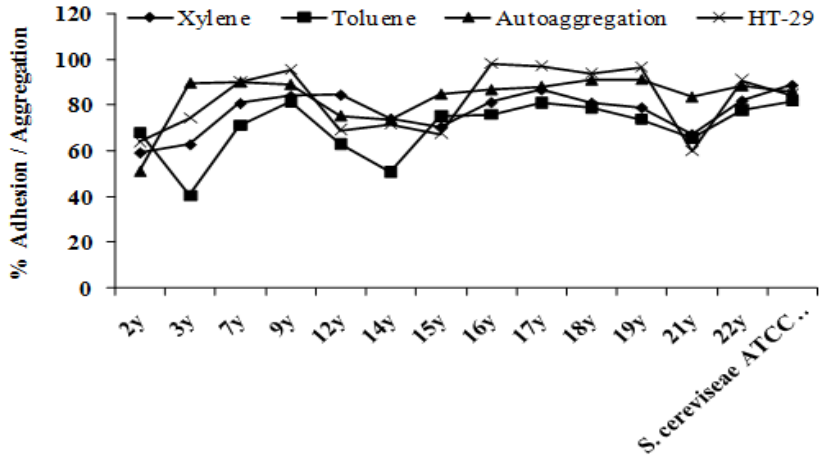

Fig 5: Adhesion and auto-aggregation of yeast isolates

Antibiotic susceptibility

Antibiotics taken during illness not only kill the disease causing microorganisms but also disrupt the normal microbial balance of the gut leading to a number of side effects and encouraging the patients to restore their natural gut microflora with the intake of probiotics (Natt and Garcha 2011). The results showed that all the probiotic yeasts (*P. kudriavzevii*, *C. xylopsoci*, *S. cerevisiae*, and *I. orientalis* - 7y, 9y, 16y, 17y, 18y, 19y and 22y) were resistant to most of the commonly used antibiotics except Polymyxin-B (Fig. 6). This could be due to the surface active bactericidal and fungicidal Polymyxin-B altered the cell envelope by binding to the lipid-A portion of lipopolysaccharide and also to phospholipids component of the membrane and caused cell lysis (Schwartz et al. 1972).

There have been a number of studies carried out to determine the benefit of taking a probiotic supplement to help reduce antibiotic-associated diarrhoea, of which an encouraging number have yielded a positive result. In double-blind placebo-controlled randomized studies, probiotic *S. boulardii* (Surawicz et al. 1989; McFarland et al. 1995; D'Souza et al. 2002) significantly decreased the incidence of diarrhea in healthy subjects and patients treated with antibiotics. Most of the probiotic microorganisms are bacteria and many of them are not able to resist or tolerate antibiotics, whereas, yeasts have a natural resistance against antibiotics. Thus in the present study, the probiotic yeasts showed resistance to antibiotics could be used for patients undergoing antibiotic treatment, indicating their potential to be used in therapeutics.

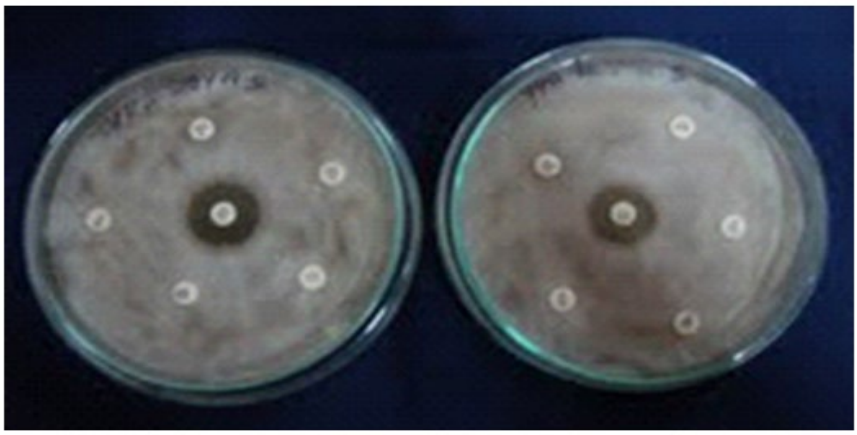

Fig 6: Probiotic *P. Kudriavzevii* and *S. cerevisiae* showing antibiogram against polymyxin-B

Antimicrobial property

One of the most desirable properties of probiotic yeasts is the antimicrobial activity against pathogens that penetrate various mucosa sites (Syal and Vohra et al. 2013). The use of antagonistic bacteria to inhibit pathogenic bacteria has been studied extensively over the years, while little attention has been given to yeasts in a similar role. Therefore, the probiotic yeast strains exhibited the spectrum of antimicrobial activity against few of the food borne pathogens (*E. coli, S. typhimurium, S. paratyphi-A, S. aureus, S. sonnei, B. cereus,* and *Y. enterocolitica*) was determined. The inhibitory action was observed as a clear zone of 10mm-30mm (Table 2) around the colonies of the pathogen against the lawn of the growth of probiotic yeasts (Fig. 7). The probiotic yeasts showing inhibition zones with, at least, more than 10 mm of diameter were considered mycocin-producing strains and the latter the yeast demonstrated a strong antimicrobial activity against tested pathogens. The mechanisms involved in yeasts antibacterial property to act against enteric pathogens are the prevention of bacterial adherence and translocation in the intestinal epithelial cells, production of factors that neutralize bacterial toxins and modulation of the host signaling pathway with proinflammatory response during bacterial infection (Czerucka et al. 2007; Martins et al. 2011; Tiago et al. 2012).

A study of Andreas et al., (2010) showed that the yeast strains isolated from feta cheese (*S. cerevisiae*) and infant's faeces (*S. boulardii* and *I. orientalis*) had no antibacterial or antagonistic activity against the selected food borne pathogens. It has been proposed that the most adhering *Lactobacillus* strains inhibit the *S. typhimurium* attachment and cell entry to human enterocyte-like Caco-2 cells (Gorbach and Newton, 1996). Since the eukaryote microbes have ten times surface area than bacteria, greater protection to intestinal cell walls is expected. The antagonistic activity exerted by probiotic yeasts (*P. kudriavzevii-9y, C. xylopsoci-16y, S. cerevisiae-17y,* and *I. orientalis*-19y), which showed the best adhesion properties (>90%) to HT-29 cells, could be the most important inhibitor of *S. typhimurium* attachment. Likewise the adhesion capacity of the probiotic yeast would have inhibited the attachment of other pathogens studied.

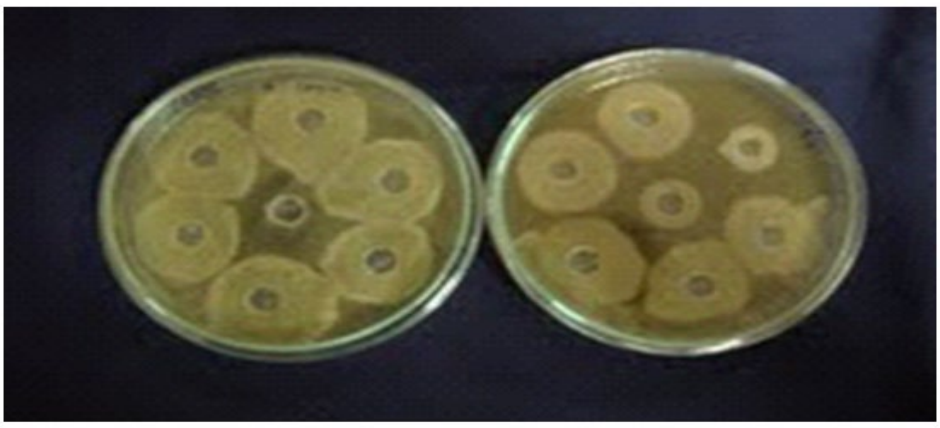

Fig 7: Probiotic yeasts showing zone of inhibition against pathogens

Zone of inhibition (mm)							
Probiotic yeasts	***E. coil*** ATCC 10536	***S. paratyphi-A*** ATCC 9150	***S. aureus*** ATCC 700699	***B. cereus*** ATCC 14579	***S. typhimurium*** MTCC 1251	***S. sonnei*** ATCC 25931	***Yersinia enterocolitica*** ATCC 23715
I. orientalis-7y	28	22	19	28	26	23	12
P. kudriavzevii-9y	29	31	18	18	24	22	14
C. xylopsoci-16y	20	26	17	23	24	24	16
S. cerevisiae-17y	17	19	13	22	30	20	18
P. kudriavzevii-18y	17	15	14	24	28	18	14
I. orientali-19y	21	14	13	26	26	21	15
I. orientalis-22y	18	16	14	15	14	17	13
S. cerevisiae ATCC 7745	17	19	19	16	15	14	10

Table 2: Antimicrobial activity (mm) of probiotic yeast against food borne pathogens

Conclusion

Kefir grains are considered as an excellent source of beneficial probiotics and a logical natural product to investigate for new probiotic strains. Although, lactic acid bacteria and bifidobacteria group are most widely studied for probiotic properties, the use of yeast as a probiotic food supplement is gaining relevance (Fleet and Balia 2006). *Saccharomyces boulardii* is a strain of yeast which has been extensively studied for its probiotic effects. Research into novel probiotic strains is important to satisfy the increasing market demand and to obtain highly active probiotic cultures for improved products (Bertazzoni et al. 2004) with probiotic characteristics that are superior to those presently on the market. Therefore an attempt has been made to identifying and characterizing the probiotic potential of kefir yeast. In summary, *I. orientalis* (7y, 19y and 22y) *P. kudriavzevii* (9y and 18y), *C. xylopsoci* (16y), *S. cerevisiae* (17y) exhibited promising probiotic properties such as excellent pH and bile tolerance, cell surface traits like hydrophobicity, aggregations, and suppression of pathogen growth under in vitro conditions. Moreover, these probiotic yeast strains were sensitive only to Polymyxin-B. It is interesting to note that even after several decades of investigation, the potential of yeast, especially those of kefir origin, has not yet been fully exploited for probiotic properties. Hence, more research is needed to exploit other potential probiotic and functional properties of these strains. Further, in vivo trials are needed to determine whether they function as probiotics in ideal therapeutic conditions.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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