Characterization of yeasts isolated from traditional kefir grains for potential probiotic properties

Characterization of yeasts isolated from traditional kefir grains for potential probiotic properties

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Abstract

Kefir is a mixed fermented product with numerous attributed health benefits due to presence of a complex culture composed of bacteria and yeasts in an exopolysaccharide matrix. This work aimed at isolating and identifying culturable yeast species from two types of traditional kefir grains and establishing some potential probiotic properties including survival in the gastrointestinal tract, auto-aggregation, hydrophobicity and hydrolytic enzymes production. All the isolates showed good survival rates in simulated gastrointestinal tract solution, with <0.5 $\log_{10}$ reduction. *Kluyveromyces lactis* was characterized with a high level of hydrophobicity (88.75%) but moderate auto-aggregation whereas *S. unisporus* showed moderate hydrophobicity and auto-aggregation. Indicator enteric bacteria adhered onto both viable and non-viable yeast isolates and controls. In comparison to *Saccharomyces boulardii* strains used as controls, both kefir yeast strains showed low alpha hemolytic and proteolytic activities, but exhibited no phospholipase activity. *Kluyveromyces lactis* and *Saccharomyces unisporus* isolated, were identified on the basis of 26s rDNA and ITS region sequencing. Overall, the yeast isolates showed some potential probiotic properties.

Key words: *Kluyveromyces lactis;* Probiotics; Kefir; *Saccharomyces unisporus; Yeasts*

1. Introduction

Kefir is an acidic and low alcoholic probiotic product made from kefir grain, which is a consortium of exopolysaccharides and many microorganisms (Plessas et al., 2016; Prado et al., 2015). The term kefir is derived from *kef*, a Turkish word which is translated as 'pleasant taste' (Arslan, 2015). Chemically, kefir grains are generally composed of 890-900 g/kg water, 2 g/kg fat, 30 g/kg protein, 60 g/kg sugars and 7 g/kg ash, and these may vary depending on the grain. Physically, grains appear as cauliflower florets with size ranging from 0.3 to 3.5 cm in diameter (Garrote, Abraham, & De Antoni, 1997). Bacterial components of kefir grains include *Lactobacillus, Lactococcus, Leuconostoc* and *Streptococcus* genera while yeast genera include *Kluyveromyces, Candida, Saccharomyces and Pichia* (Plessas et al., 2016). Spatial distribution of microorganisms in kefir is still controversial, however, it has been generally reported that yeasts are located in the inner and intermediate inner section of the grains while bacteria exist on the surface areas of grains (de Oliveira Leite et al., 2013). Kefir can be produced commercially using two-step fermentation process (Russian method) or traditional one-step fermentation method by inoculating milk, fruit juices or molasses with kefir grains (Plessas et al., 2016).

Kefir is widely consumed in Caucasus Mountains of Russia, Europe, Asia, South and North America for health benefits conferred by probiotic microorganisms (Plessas et al., 2016). Probiotics are defined by the World Health Organization (WHO) and Food and Agriculture Organization (FAO) as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002). Kefir consumption has been associated with benefits in management and treatment of gastrointestinal problems, hypertension, allergies, cancers, and ischemic heart disease. Furthermore, antibacterial properties against pathogenic bacteria such as

Salmonella have been reported (Zavala et al., 2016). These prophylactic and therapeutic properties are associated with probiotic microorganisms' interactions with the hosts. Moreover, probiotics prophylactic and therapeutic properties are also attributed to their bioactive metabolites including organic acids, bacteriocins, carbon dioxide, hydrogen peroxide, ethanol and diacetyl (de Oliveira Leite et al., 2013). Probiotics are expected to meet certain criteria including; the ability to persist and multiply in the gastrointestinal tract (GIT) (resistance to acidic gastric juice, basic pancreatic juice, lysozyme, and bile salts), ability to auto-aggregate and to form normal sustaining flora, and should be non-pathogenic (Gut, Vasiljevic, Yeager, & Donkor, 2018). Proteinases, phospholipases and hemolysins are some of the key hydrolytic enzymes that may contribute to invasiveness, persistent infections, host immune evasion, proliferation and colonization, as well as provision of nutrients to pathogenic yeasts such as *C. albicans* (Anoop, Rotaru, Shwed, Tayabali, & Arvanitakis, 2015; Ramesh et al., 2011). However, some of these hydrolytic enzymes may be beneficial in treatment of proteinous toxins producing bacteria (Gut et al., 2018).

This work thus was focused on isolating, identifying and characterizing the culturable kefir yeasts from two traditional kefir grains. The isolates were examined for GIT survival, auto-aggregation, growth at 37 °C, hydrophobicity, antibacterial properties as well as screened for hydrolytic enzymes including proteolysins, phospholipases and hemolysins.

2. Materials and methods

2.1. Isolation and enumeration of yeast isolates

Kefir grains were obtained from the Werribee starter culture collection (Victoria University, Melbourne, Australia). They had two origins - kefir grain coded TVR was

originally from Russia while the other grain coded HSK originated from Kazakhstan. Yeast species were isolated as previously reported with some modifications (Garofalo et al., 2015). Briefly, samples of kefir grains were initially cultivated in pasteurized milk to initiate their growth proliferation. They were subsequently removed from the fermented milk and washed with sterile water. The grain samples were inoculated into 200 mL ultra-high temperature (UHT) milk (Devondale, Murray Goulburn, Melbourne, Australia), incubated at 30 °C for 24 hours, and then moved to 25 °C incubator (Thermoline, wetherill park, Australia) for further 24 hours. Ten grams of the freshly cultured (TVR and HSK) grains were diluted in 90 mL 0.1% peptone water (Oxoid, Basingstoke, United Kingdom) and each mixture was thoroughly homogenized using a BagMixer (Interscience, Saint Nom, France) for 2 minutes. Ten grams each of remaining kefir (devoid of grains) were also diluted in 90 mL of 0.1% peptone water. Serial dilutions from 10^{-1} to 10^{-5} were performed and 100 µL were inoculated onto Rose-Bengal chloramphenicol agar (RBCA, Oxoid, Basingstoke, United Kingdom)**.** Chloramphenicol was prepared by following manufacturer's protocols and 3 mL of sterile deionized water was added to vial and mixed thoroughly. The vial content was then added to 500 mL RBCA agar base. Chloramphenicol was added to RBCA in order to inhibit growth of bacteria component of kefir. Plates were incubated aerobically at 25 °C for 5 days. Yeast growth on agar plates were counted and morphologically grouped as reported in a previous study (Garrote et al., 1997). Briefly, colonies were grouped based on color, size, form, elevation and margin, and also on the basis of cell morphology using optical microscopy (Olympus Optical, Tokyo, Japan). Colonies from HSK and TVR were coded as HSK18099-11 and TVR18099-12 respectively.

2.2. Probiotics potential evaluations

2.2.1 Growth at human body temperature

Well isolated colonies of the yeast isolates were streaked onto yeast YEPD agar and incubated at 37 °C aerobically for 10 days.

2.2.2. Survival in simulated gastrointestinal tract conditions

Survival of kefir yeast isolates in simulated GIT was performed as reported (Minekus et al., 2014) with some modifications. Briefly, 10 mL of kefir yeast isolates and controls *(S. boulardii* SB48 MYA-796, and *S. boulardii* SB49 MYA-79) in YEPD broth initially incubated at 25°C for 24 hours in a shaking incubator at 100 horizontal strokes/min (Innova 4230 New Brunswick Scientific, Edison, NJ, USA) was added to 10 mL sterile 0.1 % peptone water. The yeast mixture was serially diluted up to 10^{-7} using 0.1 % peptone water, 100 µL of each dilution was plated onto YEPD agar, and incubated at 25 °C aerobically for 5 days. This was used as a control.

Another 10 mL aliquot of the yeast cultures mentioned above were mixed with 7.5 mL of simulated gastric fluid (SGF) containing 6.9 mL KCl (0.5 *M*), 0.9 mL KH_2PO_4 (0.5 *M*), 12.5 mL $NaHCO_3$ (1 *M*), 11.8 mL NaCl (2 *M*), 0.4 mL $MgCl_2{\cdot}6H_2O$ (0.15 *M*) and 0.5 mL $(NH_4)_2CO_3$ (0.5 *M*). Two milliliters of 20,000 U/mL (3.7 g 543 unit/g in 100 mL sterile milli-Q water) porcine pepsin solution was added. Furthermore, 5 µL 0.*3* M $CaCl_2$ solution and 0.295 mL of water were added. Final pH was adjusted to 3.0. Twenty milliliters of the mixtures containing yeasts were incubated at 37 °C aerobically for 2 hours in a shaking incubator as above. The simulated gastric chymes containing yeast were further mixed with 11 mL of simulated intestinal fluid (SIF). The SIF solution contained 6.8 mL KCl (0.5 *M*), 0.8 mL KH_2PO_4 (0.5 *M*), 42.5 mL $NaHCO_3$ (1 *M*), 9.6 mL NaCl (2 *M*), 1.1 mL $MgCl_2{\cdot}6H_2O$ (0.15 *M*), 5 mL pancreatin stock solution (800 U/mL in simulated intestinal fluid electrolyte), 2.5 mL bile salt (160 m*M*), 40 µL $CaCl_2$ (0.3 *M*) and 1.31 mL sterile water. Final pH was adjusted to 7.0, and the

solutions were incubated at 37 °C aerobically for 2 hours in a shaking incubator at 100 horizontal strokes/min. Serial dilutions from the simulated GIT mixture were performed as above and YEPD agar was inoculated with 100 µL of each dilution and incubated at 25 °C aerobically for 5 days.

2.2.3. Hydrophobicity

Hydrophobicity experiment was carried out as previously described (Fadda, Mossa, Deplano, Pisano, & Cosentino, 2017a) with some modifications. Yeast cultures in YEPD broth incubated at 25 °C for 24 hours were centrifuged (Eppendorf AG, Hamburg, Germany) at 4,000 g for 15 minutes at 4 °C and washed twice with 1X PBS. The yeast pellets were re-suspended in 1X PBS and optical density at 560 nm (OD_{560nm}) was measured using spectrophotometer (Shimadzu, Kyoto, Japan). The yeast cultures in 1X PBS optical densities were adjusted to range between 0.4 to 1 using the same buffer. Three milliliters of the yeasts suspension and 0.6 mL n-hexadecane (Sigma, St. Louis, USA) were mixed and vortexed for 2 minutes before incubation at 37 °C aerobically for 1 hour to separate n-hexadecane and aqueous phases. The aqueous phase optical density of the solution was measured using spectrophotometer (Shimadzu, Kyoto, Japan) at 560 nm. Percentage hydrophobicity was calculated as a reduction in OD_{560nm} using the formula:

$$\% \text{ hydrophobicity} = \frac{OD0 - OD}{OD} \times 100$$

Where OD0 and OD are OD_{560nm} before and after extraction with n-hexadecane respectively.

2.2.4. Auto-aggregation

Auto-aggregation experiment was performed as reported in the literature (Fadda et al., 2017a) with some modifications. Yeast cultures incubated in YEPD broth at 25 °C

aerobically for 24 hours were centrifuged at 4000 g for 15 minutes at 4 °C and washed twice in 3 mL 1X PBS per wash after each centrifugation. The yeast cultures in 1 X PBS optical densities were adjusted to range between 0.4 to 1 using the same buffer. OD_{560nm} of the suspensions were measured using spectrophotometer (Shimadzu, Kyoto, Japan) before incubation at 37 °C for 2 hours. The top phase of the solution was carefully removed after incubation and OD measured again at 560_{nm}. Percentage auto-aggregation was calculated as follows:

$$\% \text{ auto-aggregation} = [1 - \left(\frac{ODt}{D0}\right)] \times 100$$

Where OD is OD_{560nm} before incubation and OD_t is OD_{560nm} after incubation.

2.3. Safety screening

2.3.1. Phospholipase production

Phospholipase production screening was performed as described in literature (Deorukhkar, Saini, & Mathew, 2014) with some modifications. Ten microliters of yeast suspension from YEPD broth was spotted onto Sabouraud dextrose agar (SDA) plus egg yolk (16.25 g SDA, Oxoid, Basingstoke, United Kingdom; 20 mL egg yolk emulsion, Sigma-Aldrich, Castle Hill, Australia, 230 mL sterile distilled water), with a final pH 7. Plates were incubated at 30 °C and 37 °C aerobically for up to 5 days. Phospholipase activity was expressed as ratio of diameter of colony to that of colony plus translucent zone around the colony. Diameter was measured using digital caliper (Instrument Choice, Dry Creek, Australia). *C. albicans* (ATCC 10231) was used as a positive control.

2.3.2. Hemolytic activity

Hemolysin production was screened as described in literature (Deorukhkar et al., 2014; Luo, Samaranayake, & Yau, 2001) with some modifications. Briefly, spot inoculation with 10 µL yeasts suspension in YEPD onto SDA enriched with 7% (of final volume) sheep defibrinated blood (Oxoid, Basingstoke, United Kingdom) was performed. Final medium pH was adjusted to 5.6. Plates were incubated at 30 °C and 37 °C for up to 5 days. Hemolytic activity was expressed as ratio of diameter of colony to that of colony plus translucent or clear zone around the colony. Diameter was measured using digital caliper (Instrument Choice, Dry Creek, Australia). *C. albicans* was used as a positive control.

2.3.3. Proteolytic activity

Screening for proteolytic enzymes production was performed as previously described (AlGburi et al., 2016; Deorukhkar et al., 2014) with some modifications by spot inoculating 10 µL of yeast suspensions in YEPD broth onto SD plus milk agar (100 mL UHT skim milk, 16.25 g SDA, and 100 mL deionized sterile water), with a final medium pH of 7.3. Plates were incubated at 30 °C and 37 °C for up to 5 days. Proteolytic activity was expressed as ratio of diameter of colony to that of colony plus clear zone around the colony. Diameter was measured using digital caliper (Instrument Choice, Dry Creek, Australia). *C. albicans* was used as a positive control.

2.4. Identification of yeast isolates

Different colony types from RBCA were picked and streaked onto yeast extract peptone dextrose (YEPD) agar (Oxoid, Basingstoke, United Kingdom) and incubated at 30 °C for 3 days. These isolates on YEPD agar were sent to Microgenetix, a National Association of Testing Authorities (NATA) accredited laboratory for identification using 26s fungal ribosomal DNA. MicroSEQ® D2 LSU rDNA Fungal Identification Kit was

used as per manufacturer's protocols (Scientific, 2015). Further identification work was performed on TVR18099-12 colonies using internal transcribed spacer (ITS) region sequencing and Accugenix ITS database (AccuBLAST) by the same laboratory as per literature (Schoch et al., 2012). 26s rDNA was previously used in identification of kefir grains yeast isolates including Saccharomyces cerevisiae, Saccharomyces unisporus, Issatchenkia occidentalis and Kluyveromyces marxianus. (Diosma, Romanin, Rey-Burusco, Londero, & Garrote, 2014).

2.5. Antibacterial properties

2.5.1. Growth inhibition

Bacteriostatic and bactericidal analysis was performed as described in the literature (Katarzyna Rajkowska & Kunicka-Styczyńska, 2012) with some modification. Slabs of yeasts isolates previously grown on YEPD agar at 30 °C for 24 hours were placed on Muller-Hinton agar (Oxoid, Basingstoke, United Kingdom) previously inoculated with 10^4 cfu/mL *Escherichia coli* ATCC 43895 (*E.coli*) and *Enterobacter aerogenes* VUN 00025 (*E. aerogenes*) as spread plate and incubated at 37 °C for 24 hours.

Production of antibacterial molecules or pH effects analysis was carried out as reported (Bajaj, Raina, & Singh, 2013) with modification. Fermentation was performed by growing yeast isolates and controls in killer toxin medium (KTM) consisting of YEPD plus glycerol (50 g/L, Sigma, St. Louis, USA), buffered at pH 5 using 50 mM citrate-phosphate buffer, and fermented at 30 °C under shaking (180 rpm) for 24–72 hrs. Fermented broth was centrifuged (Eppendorf AG, Hamburg, Germany) at 4,000 g for 30 minutes at 4 °C. Supernatant from KTM was used for well diffusion assay as described previously (Bajaj, Raina, & Singh, 2013).

2.5.2. Sedimentation and adhesion assay.

Adhesion of bacteria onto yeast cells was performed as previously described (Tiago et al., 2012) with some modification. Briefly, 1 mL (approximately 10^8 cfu/mL) yeast in YEPD broth initially incubated at 25 °C for 24 hours in a shaking incubator at 100 horizontal strokes/min (Innova 4230 New Brunswick Scientific, Edison, NJ, USA) was added to a 15 mL centrifuge tube with 0.5 mL of *E. coli* and *E. aerogenes* (approximately10^9 cfu /mL). The bacteria-yeast mixture was vortexed for 1 minutes and incubated at 37 °C for 4 hours. Slide agglutination was also performed and macroscopically observed. One hundred microliter aliquot of supernatant was serially diluted and plated onto Nutrient agar (Oxoid, Basingstoke, United Kingdom) supplemented with 0.1% cycloheximide (Sigma, St. Louis, USA) to suppress yeasts growth. Plates were incubated at 37°C for 24 hours. Indicator bacterial colonies were counted and expressed as $\log_{10}$ cfu/mL. For the controls, 1 mL of sterile YEPD broth was added to 0.5 mL of indicator bacteria and treated as above. The procedure was repeated for non-viable yeasts sedimentation and adhesion assay after the yeasts initially grown in YEPD broth were inactivated by autoclaving at 121°C for 15 minutes.

To visualize the adherence of bacteria onto yeast cells, 10 µL of the sediments were smeared onto microscopic slides. Gram stain was performed as described (Claus, 1992) and analyzed under optical Motic microscope (Motic, Melbourne Australia).

2.6. Statistical analysis

Experiments were replicated at least twice with subsequent three subsampling. The data was analyzed with a randomized split plot block design, using replications as the block. The isolates at two levels were the main plot. All data were expressed as mean and with standard error of the mean. Statistical analysis was performed using SPSS Statistical software (IBM, New York, USA).

3.0. Results and discussions

3.1. Isolation and enumeration of yeast isolates

Table 1 summarizes yeast species isolated from traditional kefir product. Two distinct colony morphologies were isolated on RBCA shown in figure 1. HSK18099-11 colonies morphology from both grain and kefir appeared similar, likewise, the morphology appearance of colonies for TVR. Both colony and cellular morphologies of these kefir isolates were consistent with previous study in terms of colonies and cell appearance (Garrote et al., 1997). Figure 2 shows traditional kefir grains used in this study after separation from kefir, and washed with sterile water. The appearances of the grains were consistent with the description of kefir grains in literature (Garrote et al., 1997). It appeared TVR18099-11 was readily released into kefir, hence the high cell count in kefir compared to the grain. On the other hand, HSK18099-12 was retained in the grain resulting in low cell count (Table 1). The numbers of yeasts in these traditional kefirs were similar to a previous study (Silva, Santos, Santana, Silva, & Coaceicao, 2018) which recorded 5.6 cfu/g (log_{10}) yeasts cells in kefir drinks.

3.2. Survival in simulated GIT

Kefir yeast isolates and 2 strains of *S. boulardii* used as controls decreased by <0.5 log_{10} under GIT simulated conditions for 4 hours (Table 2). All the kefir yeasts isolates showed high survival rates in simulated GIT conditions comparable to controls strains (SB48/MYA 796^{TM} and SB49/MYA 797^{TM}) currently used as prophylactic and therapeutic strains in some human ailments (Czerucka, Piche, & Rampal, 2007; Hudson et al., 2016; Palma et al., 2015). MYA 796 was the least affected yeast strain by GIT conditions, and the variation of log_{10} reduction may be due to strain differences. The ability of these kefir yeast isolates to survive in the GIT may be due to the fact that

kefir is an acidic and low alcohol beverage (Prado et al., 2015) and therefore have likely developed resistance to harsh conditions. HSK and TVR kefir pH were 4.7 and 4.5 respectively after 48 hours of incubation as described above. Resistance of *S. boulardii* MYA 797 to GIT conditions has been postulated to be due to thicker cell walls compared to other *Saccharomyces cerevisiae* strains including W303 and BY4741 (Hudson et al., 2016). However, exposure of *S. boulardii* MYA 797 to Caspofungin, an antifungal drug that interferes with synthesis of cell wall, was found to significantly reduce resistance of these strains to GIT simulated environment (Hudson et al., 2016). Role of cell wall thickness of kefir yeast isolates in GIT survival in this study needs further investigation. Survival in the GI tract is an important criteria for microorganisms to be classified as probiotic, and involves being able to resist acidic gastric juice such as pepsin, basic pancreatic enzymes lysozyme, and bile salts at physiological temperature (Gut et al., 2018). Survival of these yeast strains in simulated GIT showed their resistance to digestive enzymes including pepsin, pancreatins as well as bile salts and low pH. *S. cerevisiae* CIDCA8112 and *Kluyveromyces marxianus* were reported to exhibit immunomodulatory properties which depended on viability of the yeast species (Romanin et al., 2010). Furthermore, viability of yeast probiotics is associated with several antagonistic properties towards enteropathogenic bacteria including competition for nutrients, binding sites and production of antibacterial molecules (Revolledo, Ferreira, & Ferreira, 2009).

3.3. Growth at human body temperature

Growth for both isolates and controls is shown in table 2. Both the controls and TVR18099-12 were able to grow at 37 °C whereas HSK18099-11 did not grow at 37° C (Table 2). This was in agreement with previous studies where some yeast species grew at 30 °C but no at 37 °C (Lodder & Kreger-Van, 1952). Failure of HSK18099-11

to grow at this human body temperature may not disqualify it as a potential probiotic since prophylactic and therapeutic potentials of yeasts are not limited to viable and proliferating cells only. Prophylactic and therapeutic efficacy of non-viable yeast cells have been reported (Gut et al., 2018).

3.4. Auto-aggregation

Auto-aggregation of kefir isolates and controls are shown in table 2. It is defined as aggregation among yeast cells to form flocs or flor which provides competitive advantage over other microorganisms including enteric bacterial pathogens in a harsh environment such as human GIT (Brückner & Mösch, 2012). Auto-aggregations of kefir isolates were slightly comparable to that of *S. boulardii* strains used as controls. The percentage auto-aggregations of isolates and controls were consistent with previous studies (Fadda et al., 2017a; Gil-Rodríguez , Carrascosa, & Requena, 2015). Formation of cell aggregates provides shielding to cells in the center against harmful environmental conditions (Suvarna, Dsouza, Ragavan, & Das, 2018). *In vitro* auto-aggregation can be influenced by duration of incubation used during analysis to separate aqueous and n-hexadecane phase (Gil-Rodríguez, Carrascosa, & Requena, 2015). On the other hand, yeasts auto-aggregation has been reported to be strain-specific (Gil-Rodríguez et al., 2015; Suvarna et al., 2018). Therefore, variations of auto-aggregation in the current study was likely due to these factors which have similarly been reported by Fadda et al. (2017) using similar strains.

3.5. Hydrophobicity

Kefir yeast isolates were analyzed for hydrophobicity (Table 2). Hydrophobicity is defined as a non-specific interaction between microbial and host cells. This interaction is mediated by cell-surface proteins and lipoteichoic acids (Todorov et al., 2008). In

this study, TVR18099-12 showed significantly higher hydrophobicity and therefore was capable of interacting with other cell bodies compared to HSK18099-11 and controls. Similar findings showed significantly lower hydrophobicity for control strains compared to other strains (Fadda et al., 2017a). Hydrophobicity is crucial in adhesion of probiotic microorganisms onto GIT epithelial cells where they may provide prophylactic and therapeutic benefits (Fadda et al., 2017a). Hydrophobicity is species and strain specific as demonstrated in this study (Fadda, Mossa, Deplano, Pisano, & Cosentino, 2017b; Suvarna et al., 2018). Furthermore, similar to a previous study, there was no correlation between auto-aggregation and hydrophobicity (Fadda et al., 2017a).

3.6. Hydrolytic enzymes screening

3.6.1. Phospholipase activity

The kefir yeast isolates and probiotic controls did not produce these enzymes (Figure 3). Only positive control (C*. albicans*) produced phospholipase hence the zone of precipitation around the colony shown in Figure 3 (Mayer, Wilson, & Hube, 2013; Park, Do, & Jung, 2013). This activity was in agreement with previous studies in which C*. albicans* produced this enzyme (Deorukhkar et al., 2014; Ramesh et al., 2011; Yang, 2003). Lack of phospholipase production by kefir grain yeasts isolates make them safe in respect to this enzyme as it is associated with yeast virulence (Ramesh et al., 2011).

3.6.2. Hemolytic activity

All the kefir yeast isolates and *S. boulardii* strains produced low levels of alpha hemolysins comparable to *C. albicans* as shown in Figure 4. However, only *C. albicans* produced weak beta-hemolysis on further incubation up to 72 hours, as illustrated by greenish-black halo around the colony (Figure 5E). Similar findings have been

reported previously in which *C. albicans* produced beta hemolysis after 48 hours incubation (Luo et al., 2001). Mammalian systems lack free iron which is essential for microbial proliferation and pathogenesis. Some pathogenic microorganisms possess hemolysins which assist with breakdown of hemoglobin in order to access hemoglobin-bound iron (Luo et al., 2001). Hemolysins are either proteinaceous enzymes or non-proteinaceous toxins which cause cell lysing. The mechanism involve creating a pore in the cell membrane. Fungal hemolysins are reported to act slowly on cells resulting in cell death (Vesper & Jo Vesper, 2004). Alpha hemolysin in the blood may cause partial breakdown of red blood cells whereas beta hemolysin breakdown cells completely (Vesper & Jo Vesper, 2004). Histamine release induced by *E.coli* alpha hemolysin through immunomodulation was reported in rat model (Gross-Weege, König, Scheffer, & Nimmich, 1988; Scheffer, König, Braun, & Goebel, 1988). The production of alpha hemolysin by the isolates, is an important property which may be employed to fight infection by pathogenic bacteria.

3.6.3. Proteolytic activity

All kefir yeast isolates and controls showed very weak proteolytic activity shown in Figure 5. Proteolytic enzymes are associated with active entry of pathogens into the host tissue (Mayer et al., 2013; Sacristan et al., 2011). However, production of proteolytic enzymes by yeasts may provide prophylactic and therapeutic benefits to the host as *S. boulardii* serine protease has been reported to break down *Clostridium difficile* and *Clostridiums perfringens* toxins (Czerucka et al., 2007; Hudson et al., 2016; Palma et al., 2015). Therefore, production of proteolytic enzymes may protect host against infections by toxins produced by enteropathogenic bacteria such as *Salmonella, Vibrio, Clostridiums, E.coli* and *Bacillus* species (Gut et al., 2018).

3.7. Identification of yeast isolates

The yeast isolates from two traditional kefir grains showed potential probiotic properties and after identification and characterization, two phylogenetic trees were obtained as shown in Figure 6. A phylogenetic tree by definition shows evolutionary relationships among species (Mooers & Heard, 1997). Isolates HSK18099-11 and TVR18099-12 were identified as *Saccharomyces unisporus* ATCC 10612 (*S. unisporus*) and *Kluyveromyces lactis* var. *lactis* ATCC 56498 *(K. lactis) /Kluyveromyces marxinus ATCC16045* (*K. marxinus)* respectively. The first part of the figure shows a good separation of *S. unisporus* from its evolutionarily related species including *Kazachstania africana*, *Saccharomyces cerevisiae* and *Saccharomyces bayanus*. Therefore, further differentiation identification method was not required. However in a previous study *S. unisporus* isolated from Tibetan kefir grains could not be differentiated from Kazachstania unisporus and Kazachstania exigua using 26s rDNA (Zhou, Liu, Jiang, & Dong, 2009). On the other hand, the second part of the figure showed close similarity between *K. lactis* and *K. marxinus,* which were not effectively differentiated using 26s ribosomal DNA. This was in agreement with a previous study in which two *Kluyveromyces* species could not be differentiated on the basis of their amino acid sequence (Lertwattanasakul et al., 2015). However, in another study, it was reported that *K. lactis* and *K. marxinus* were clearly separated and identified from kefir grain using 26s rDNA (Zhou et al., 2009). The TVR18099-12 colonies labelled as *K. lactis*/*K. marxinus* by 26s rDNA (Figure 6) were further analyzed using ITS sequencing, and identified as *K. lactis* ATCC 56498*.* ITS sequencing was used successfully to identify *Kluyveromyces* species and strain levels in previous study (Belloch, Barrio, García, & Querol, 1998).

406 The isolation and identification of culturable yeasts species in the two traditional grains
407 were consistent with a FAO report which states that kefir grain contains
408 *Saccharomyces unisporus* and *Kluyveromyces* species*, Saccharomyces cerevisiae,*
409 *Issatchenkia occidentalis* (Diosma, Romanin, Rey-Burusco, Londero, & Garrote,
410 2014; Magalhães, Pereira, Campos, Dragone, & Schwan, 2011).

411 **3.8. Antibacterial properties**

412 **3.8.1. Bacterial growth inhibition**

413 *K. lactis* and the controls slabs on the lawn and well diffusion assay showed no growth
414 inhibition of indicator enteric bacteria, however *S. unisporus* showed weak growth
415 inhibition of *E.coli* and *E. aerogenes* just under the slab (figure not shown). The
416 controls results are consistent with previous study in which *S. boulardii* did not inhibit
417 some gram negative enteropathogenic bacteria growth when tested using this method
418 (K. Rajkowska, Kunicka-Styczyńska, & Rygala, 2012). Both control and kefir yeast
419 isolates supernatants showed no growth inhibition effects (picture not shown). There
420 are controversies on bacteriostatic and bactericidal effects of Saccharomyces species
421 including the controls and *S. unisporus* (K. Rajkowska et al., 2012), and the fact that
422 insufficient data on antibacterial properties of *K. lactis* is available, further in-depth
423 research is needed*.*

424 **3.8.2. Adherence of bacteria onto yeasts cells**

425 Adherent of enteric bacteria indicators was analyzed both qualitatively and
426 quantitatively. Table 4 shows both viable and non-viable kefir isolates and controls
427 with statistical significant differences. The data above was also supported by slide
428 agglutinations (figures not shown) and optical microscopic examination as shown in
429 figure 7. Two mechanisms of adherence of bacteria onto yeast cells are proposed.

Specific binding using type 1 fimbriae on bacteria such as *E.coli*, *E. aerogenes* and *Salmonella* cell with mannan oligosaccharides on yeast cells, and non-specific binding such as electrostatic and hydrophobic (Adegbola & Old, 1985; Pérez-Sotelo et al., 2005; Tiago et al., 2012). These results are consistent with previous results where *E.coli* was reported to bind both viable and non-viable *S. boulardii* and *Saccharomyces cerevisiae* UFMG 905. Binding of enteric bacteria onto yeast cells is reported to be irreversible leading to transient passage of bacteria through GIT. *S. boulardii* does not bind to GIT (Gut et al., 2018). The adherence of enteric bacterial pathogen is postulated to be responsible for probiotic effects such as inhibition of signalling transduction pathway activation and subsequent translocation (Tiago et al., 2012) and hence their prophylactic and therapeutic application in human (Gut et al., 2018), and animal husbandry to promote health possibly through reduction of infection (Perez-Sotelo et al., 2005). Survival in GIT of these yeast isolates as shown in this study may lead to increased numbers, and hence increase capacities to scavenge (adhered bacterial cells) potential pathogenic gram negative enteric bacteria from the gut and subsequent flushing out in the feces. Moreover, since these yeasts survive in GIT and are not affected by antibiotics (for example, not affected by Chloramphenicol in this study, data not shown) aimed at bacteria, their use as complementary therapy with antibiotics during enteric bacterial infection may also improve treatment through increased numbers and subsequent mopping out enteric bacteria from the GIT. Furthermore, the binding of opportunistic enteric bacteria onto non-viable yeasts is of great significance since consumption of viable yeast probiotics is associated with fungemia especially in immunocompromised individual or those with GIT issues (Gut et al., 2018).

4. Conclusion and future perspective

The two traditional kefir grains contained yeasts with potential probiotic properties. GIT survival, hydrophobicity, auto-aggregation and hydrolytic enzymes production of kefir yeast isolates was comparable to *S. boulardii* strains. Both kefir yeast isolates and *S. boulardii* produced weak alpha hemolytic and proteolytic activities, but none produce phospholipases at 30 °C. None of the yeasts produced hydrolytic enzymes at 37 °C. The 2 isolates showed adherence to enteric bacteria comparable to the controls. However, further in-depth studies are needed to establish their prophylactic and therapeutic properties. The isolates were identified as *S. unisporus* and *K. lactis.*

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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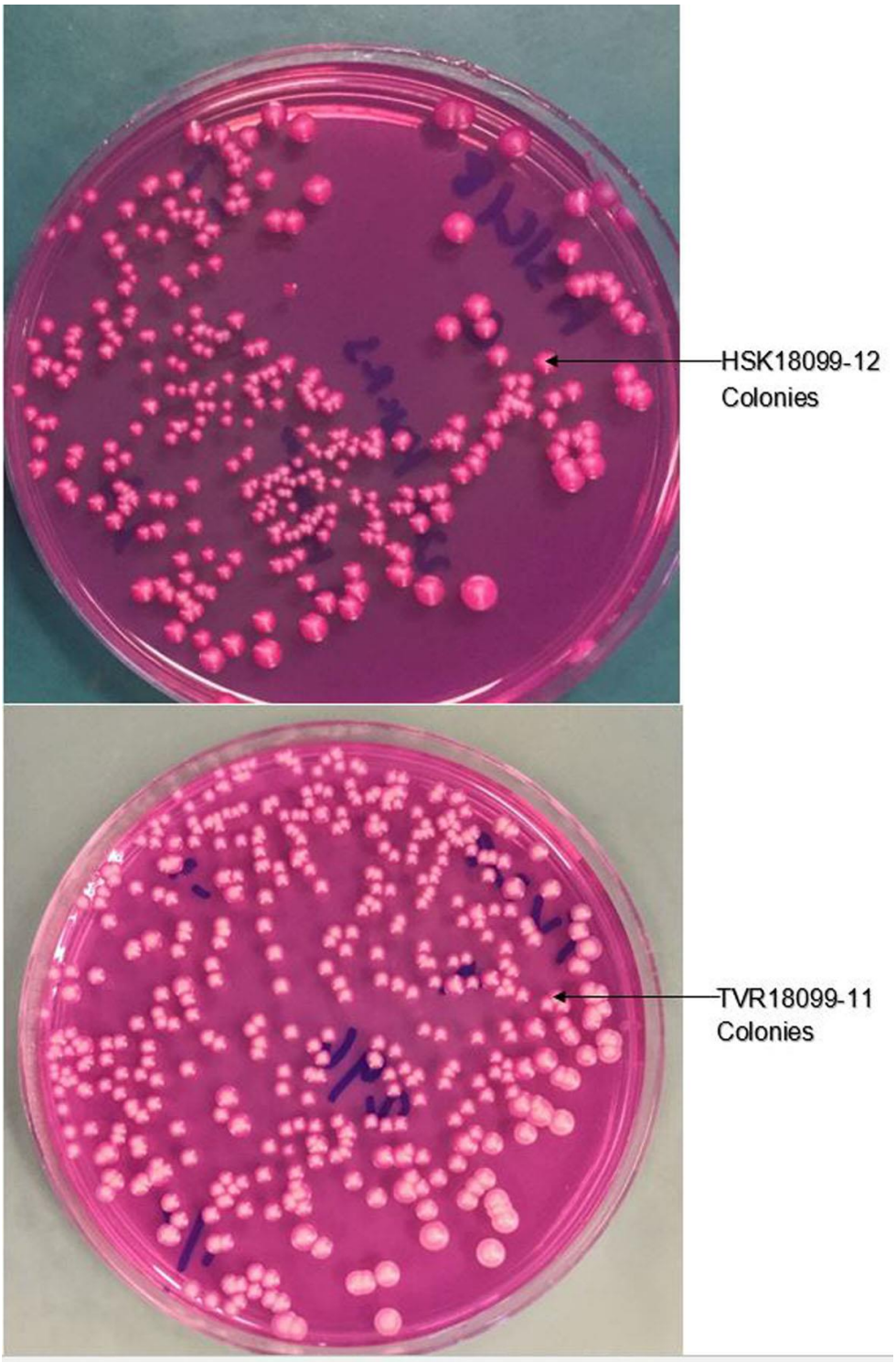
List of figures

D = *E. aerogenes* plus MYA 797™, E = *E.coli* plus *K. lactis,* F = *E.coli* plus *S. unisporus,* G = *E. coli* plus MYA 796™ and H = *E. coli* plus MYA 797™.

List of Tables

711 **Figure**



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713 Figure 1

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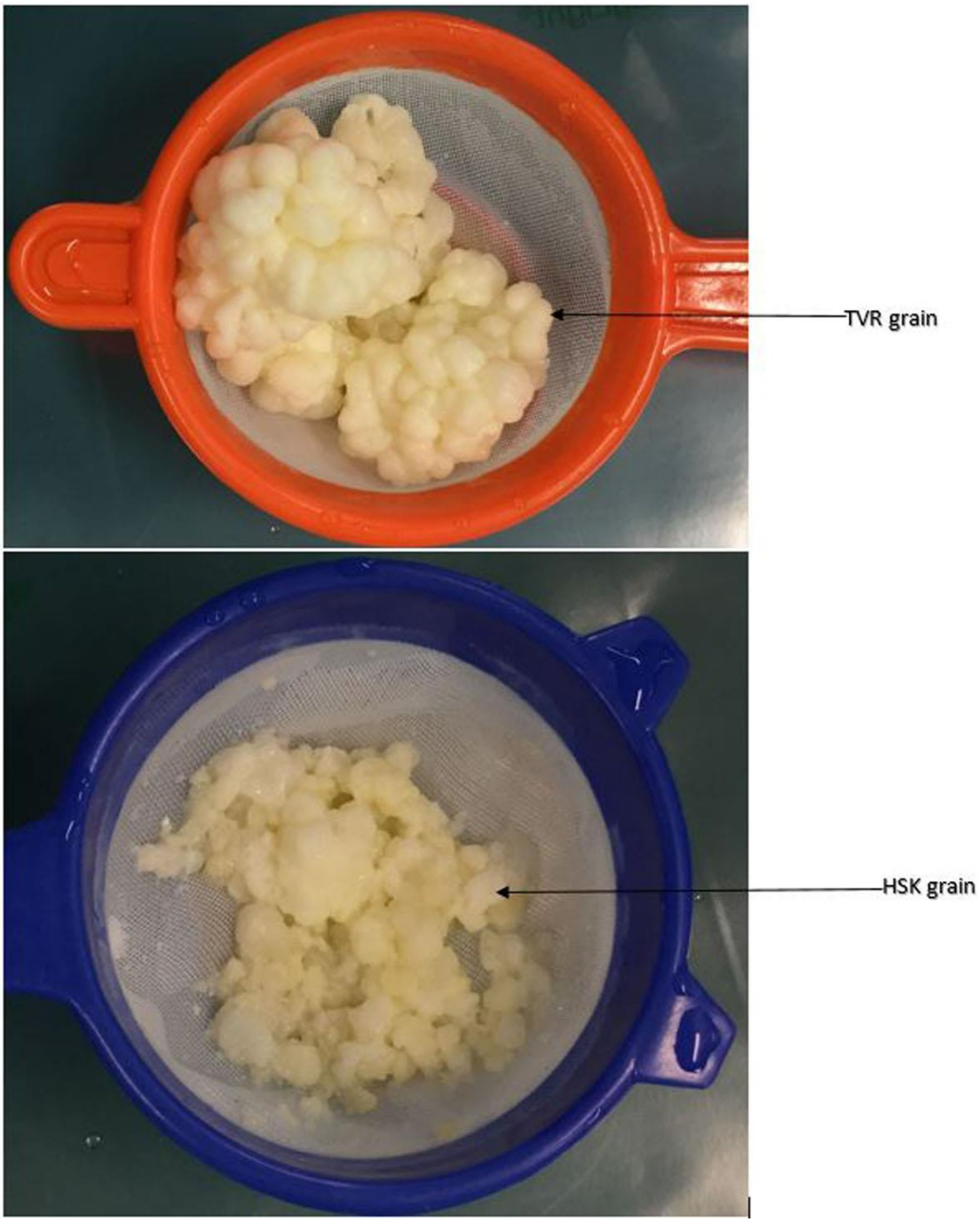
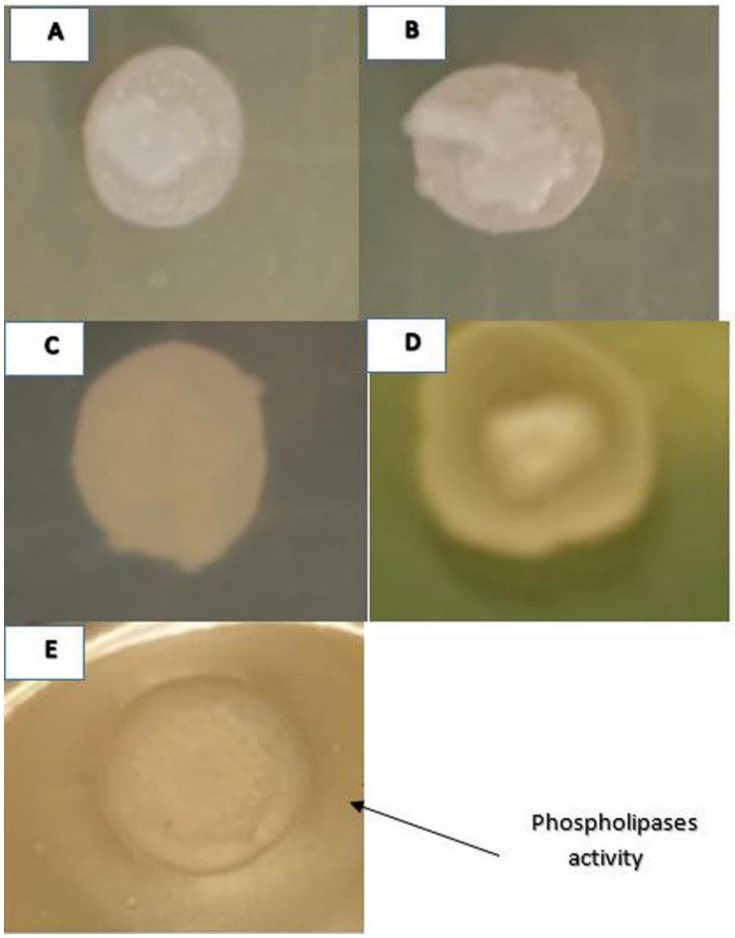


Figure 2



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721 Figure 3

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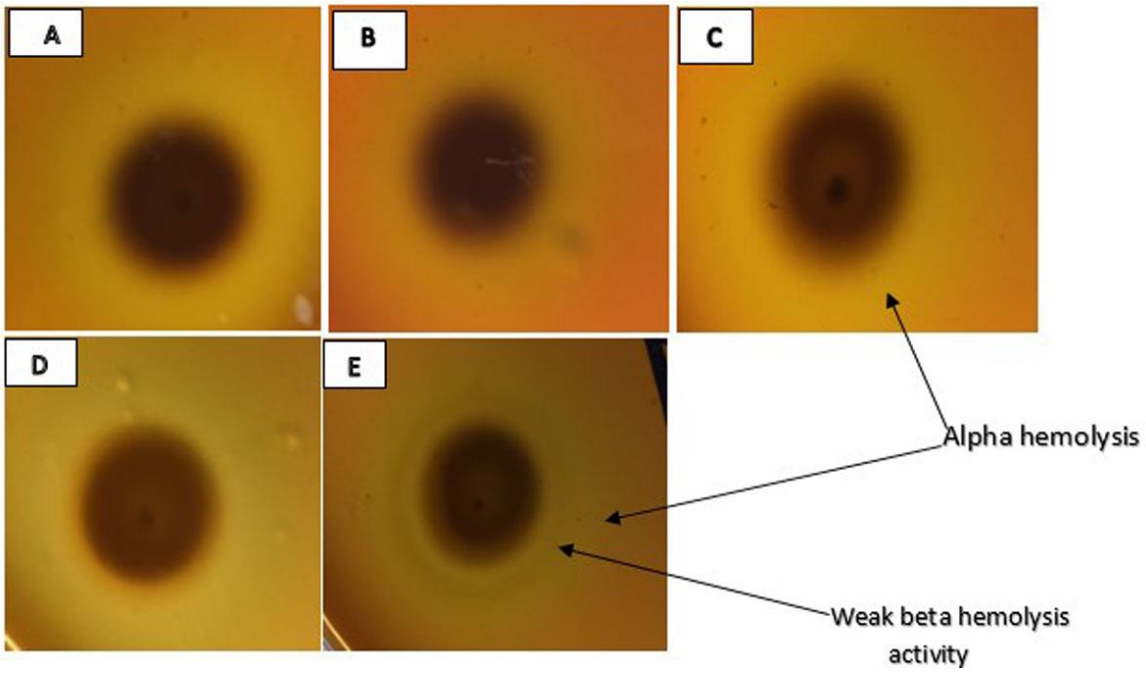

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725 Figure 4

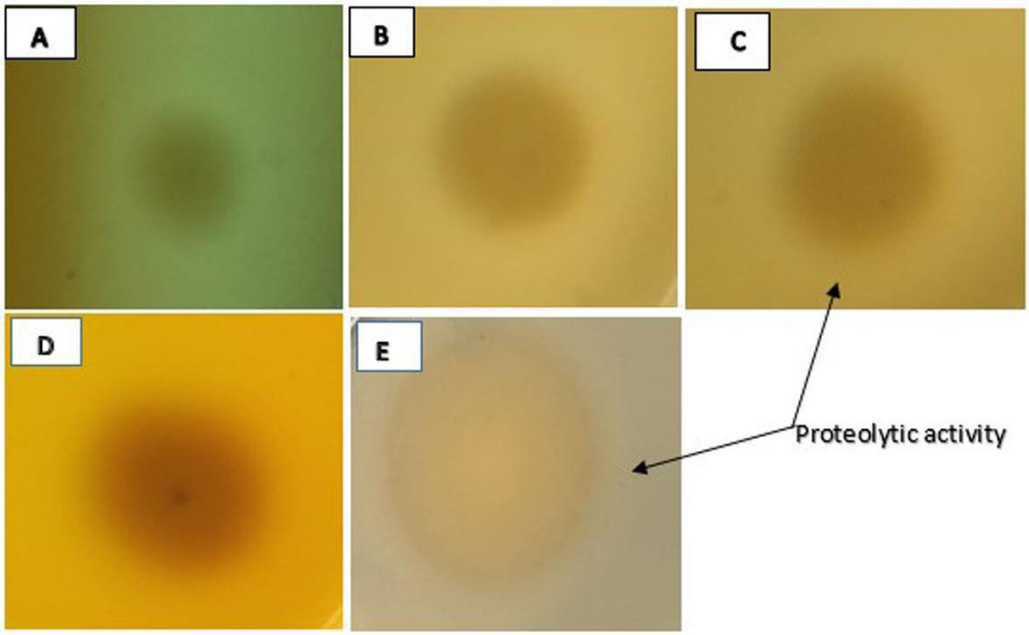

Figure 5

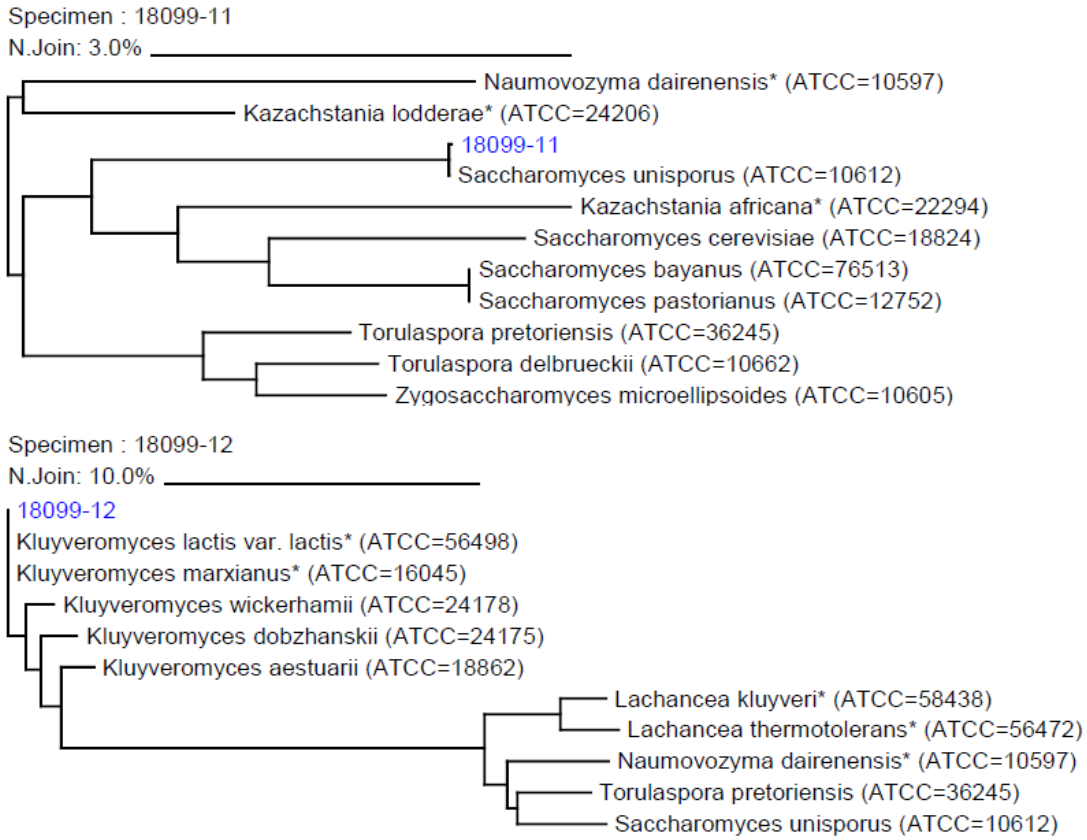

Figure 6

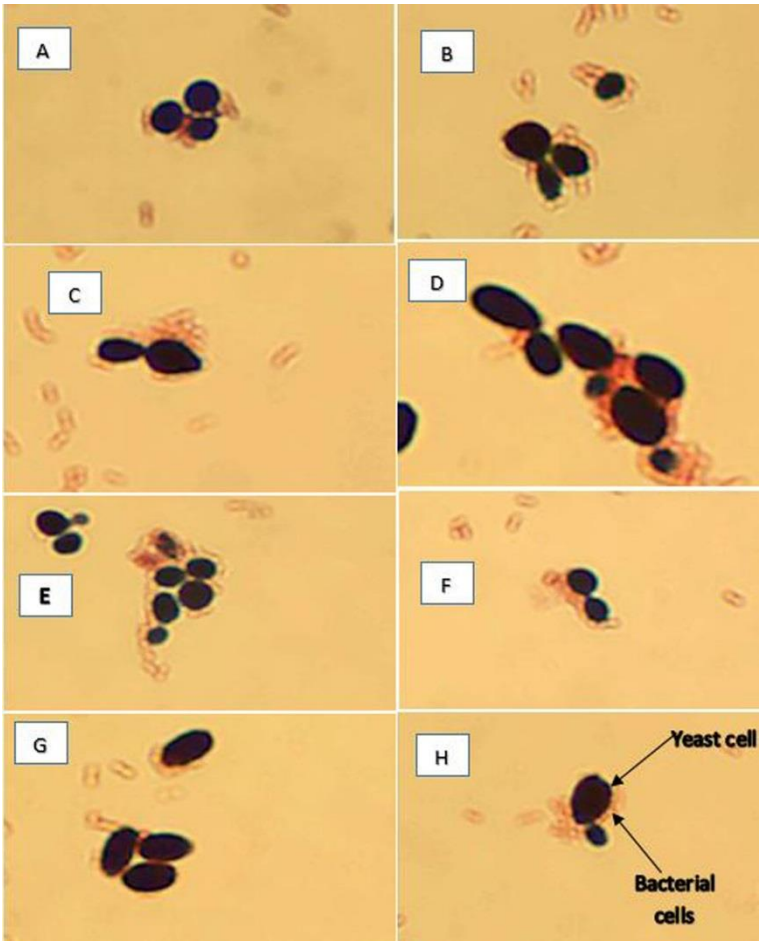

Figure 7

746 **Tables**

747

748 Table 1. Traditional kefir grains morphological and numerical characterization

Yeast strains	TVR18099-12	HSK18099-11
Colony morphology	White centre with pink edge, convex and round	Round smooth, shiny pink and convex colonies
Cellular morphology	Globose to ellipsoidal unicellular and some budding	Globose to ellipsoidal, unicellular and budding
Count in grain Log_{10} cfu /g	5.4	6.28
Count in Kefir Log_{10} cfu /g	6.05	5.81

749 Mean SD (SE) = 0.05

750

751 Table 2. Yeasts isolates probiotics properties

Yeast strains	Initial mean count (Log_{10} cfu /mL)- T0 [a]	GIT survival (Log_{10} cfu /mL) - T1 [a]	Decrease in viability (Log_{10} cfu /mL) - T2[a]	Growth at 37 °C	Hydrophobicity (% index)[b]	Auto-aggregation (% index)[c]
TVR18099-12	7.06	6.82	0.24	+++	88.75	35.48
HSK18099-11	6.80	6.54	0.26	-	30.00	43.33
MYA 796™	7.04	6.95	0.09	+++	15.58	42.86
MYA 797™	7.07	6.66	0.41	+++	21.18	30.59

752 - = no growth; +++ = very good growth; [a] is standard error mean for GIT = 0.06; [b] is
753 standard error mean for hydrophobicity = 0.03; [c] is standard error mean for auto-

aggregation= 0.03. MYA 796^{TM} and MYA 797^{TM} are two strains of *S. boulardii* corresponding to SB48 and SB49 respectively. The difference between GIT treated and untreated counts for both isolates and controls are statistically significant.

Table 3. Hydrolytic enzymes activity of yeasts

Yeast strains	Hemolytic activity[1]		Proteolytic activity[2]	Phospholipase production[3]
	α-hemolysis	β-hemolysis		
TVR18099-12	0.90	1	0.92	1
TVR18099-11	0.76	1	0.67	1
MYA 796^{TM}	0.77	1	0.78	1
MYA 797^{T}	0.84	1	0.82	1
Calb	0.78	0.94	0.76	0.72

Pz = diameter of colony to the colony plus clearing/precipitation diameter ratio; high, Pz < 0.40; medium, Pz = 0.41–0.60; low, Pz = 0.61–0.99; none, Pz = 1. Calb = *Candida albicans*; [1] is Hemolytic activity Mean SD (SE) = 0.01; [2] is proteolytic activity Mean SD (SE) = 0.02; [3] is phospholipases activity SEM = 0.02. MYA 796^{TM} and MYA 797^{TM} are two strains of *S. boulardii* corresponding to SB48 and SB49 respectively. Calb = *Candida albicans*.

772 Table 4. Adhesion and sedimentation assay

	Viable yeasts					Non-viable				
Indicator bacteria	Control	*MYA 796™*	*MYA 797™*	KL	SU	Control	*MYA 796™*	*MYA 797™*	KL	SU
E. aerogenes	9.05	8.08	8.29	8.47	8.46	8.85	8.18	8.23	8.31	8.34
E. coli	8.89	8.29	8.52	8.50	8.69	8.73	8.38	8.42	8.39	8.48

773 The indicator bacterial count in supernatants of yeasts-bacteria mixture are statistically
774 lower compare to the control (Paired T sample t-test, $p < 0.05$). Mean SD (SE), 0.03

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