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1 **Characterization of yeasts isolated from traditional kefir grains for potential**
2 **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*

44 ## 1. Introduction

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

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

69 *Salmonella* have been reported (Zavala et al., 2016). These prophylactic and
70 therapeutic properties are associated with probiotic microorganisms' interactions with
71 the hosts. Moreover, probiotics prophylactic and therapeutic properties are also
72 attributed to their bioactive metabolites including organic acids, bacteriocins, carbon
73 dioxide, hydrogen peroxide, ethanol and diacetyl (de Oliveira Leite et al., 2013).
74 Probiotics are expected to meet certain criteria including; the ability to persist and
75 multiply in the gastrointestinal tract (GIT) (resistance to acidic gastric juice, basic
76 pancreatic juice, lysozyme, and bile salts), ability to auto-aggregate and to form normal
77 sustaining flora, and should be non-pathogenic (Gut, Vasiljevic, Yeager, & Donkor,
78 2018). Proteinases, phospholipases and hemolysins are some of the key hydrolytic
79 enzymes that may contribute to invasiveness, persistent infections, host immune
80 evasion, proliferation and colonization, as well as provision of nutrients to pathogenic
81 yeasts such as *C. albicans* (Anoop, Rotaru, Shwed, Tayabali, & Arvanitakis, 2015;
82 Ramesh et al., 2011). However, some of these hydrolytic enzymes may be beneficial
83 in treatment of proteinous toxins producing bacteria (Gut et al., 2018).

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

89 2. Materials and methods

90 2.1. Isolation and enumeration of yeast isolates

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

93 originally from Russia while the other grain coded HSK originated from Kazakhstan.
94 Yeast species were isolated as previously reported with some modifications (Garofalo
95 et al., 2015). Briefly, samples of kefir grains were initially cultivated in pasteurized milk
96 to initiate their growth proliferation. They were subsequently removed from the
97 fermented milk and washed with sterile water. The grain samples were inoculated into
98 200 mL ultra-high temperature (UHT) milk (Devondale, Murray Goulburn, Melbourne,
99 Australia), incubated at 30 °C for 24 hours, and then moved to 25 °C incubator
100 (Thermoline, wetherill park, Australia) for further 24 hours. Ten grams of the freshly
101 cultured (TVR and HSK) grains were diluted in 90 mL 0.1% peptone water (Oxoid,
102 Basingstoke, United Kingdom) and each mixture was thoroughly homogenized using
103 a BagMixer (Interscience, Saint Nom, France) for 2 minutes. Ten grams each of
104 remaining kefir (devoid of grains) were also diluted in 90 mL of 0.1% peptone water.
105 Serial dilutions from 10^{-1} to 10^{-5} were performed and 100 µL were inoculated onto
106 Rose-Bengal chloramphenicol agar (RBCA, Oxoid, Basingstoke, United Kingdom).
107 Chloramphenicol was prepared by following manufacturer's protocols and 3 mL of
108 sterile deionized water was added to vial and mixed thoroughly. The vial content was
109 then added to 500 mL RBCA agar base. Chloramphenicol was added to RBCA in order
110 to inhibit growth of bacteria component of kefir. Plates were incubated aerobically at
111 25 °C for 5 days. Yeast growth on agar plates were counted and morphologically
112 grouped as reported in a previous study (Garrote et al., 1997). Briefly, colonies were
113 grouped based on color, size, form, elevation and margin, and also on the basis of cell
114 morphology using optical microscopy (Olympus Optical, Tokyo, Japan). Colonies from
115 HSK and TVR were coded as HSK18099-11 and TVR18099-12 respectively.

116 **2.2. Probiotics potential evaluations**

117 **2.2.1 Growth at human body temperature**

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

120 2.2.2. Survival in simulated gastrointestinal tract conditions

121 Survival of kefir yeast isolates in simulated GIT was performed as reported (Minekus
122 et al., 2014) with some modifications. Briefly, 10 mL of kefir yeast isolates and controls
123 (*S. boulardii* SB48 MYA-796, and *S. boulardii* SB49 MYA-79) in YEPD broth initially
124 incubated at 25°C for 24 hours in a shaking incubator at 100 horizontal strokes/min
125 (Innova 4230 New Brunswick Scientific, Edison, NJ, USA) was added to 10 mL sterile
126 0.1 % peptone water. The yeast mixture was serially diluted up to 10^{-7} using 0.1 %
127 peptone water, 100 µL of each dilution was plated onto YEPD agar, and incubated at
128 25 °C aerobically for 5 days. This was used as a control.

129 Another 10 mL aliquot of the yeast cultures mentioned above were mixed with 7.5 mL
130 of simulated gastric fluid (SGF) containing 6.9 mL KCl (0.5 *M*), 0.9 mL KH_2PO_4 (0.5 *M*),
131 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
132 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
133 milli-Q water) porcine pepsin solution was added. Furthermore, 5 µL 0.3 M
134 $CaCl_2$ solution and 0.295 mL of water were added. Final pH was adjusted to 3.0.
135 Twenty milliliters of the mixtures containing yeasts were incubated at 37 °C aerobically
136 for 2 hours in a shaking incubator as above. The simulated gastric chymes containing
137 yeast were further mixed with 11 mL of simulated intestinal fluid (SIF). The SIF solution
138 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
139 mL NaCl (2 *M*), 1.1 mL $MgCl_2{\cdot}6H_2O$ (0.15 *M*), 5 mL pancreatin stock solution (800
140 U/mL in simulated intestinal fluid electrolyte), 2.5 mL bile salt (160 m*M*), 40 µL
141 $CaCl_2$ (0.3 *M*) and 1.31 mL sterile water. Final pH was adjusted to 7.0, and the

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

146 **2.2.3. Hydrophobicity**

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

160 % hydrophobicity = $\frac{OD0-OD}{OD} \times 100$

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

163 **2.2.4. Auto-aggregation**

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

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

173 % auto-aggregation = $[1 - \left(\frac{ODt}{D0}\right)] \times 100$

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

175 **2.3. Safety screening**

176 **2.3.1. Phospholipase production**

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

187 **2.3.2. Hemolytic activity**

188 Hemolysin production was screened as described in literature (Deorukhkar et al., 189 2014; Luo, Samaranayake, & Yau, 2001) with some modifications. Briefly, spot 190 inoculation with 10 µL yeasts suspension in YEPD onto SDA enriched with 7% (of final 191 volume) sheep defibrinated blood (Oxoid, Basingstoke, United Kingdom) was 192 performed. Final medium pH was adjusted to 5.6. Plates were incubated at 30 °C and 193 37 °C for up to 5 days. Hemolytic activity was expressed as ratio of diameter of colony 194 to that of colony plus translucent or clear zone around the colony. Diameter was 195 measured using digital caliper (Instrument Choice, Dry Creek, Australia). *C. albicans* 196 was used as a positive control.

197 **2.3.3. Proteolytic activity**

198 Screening for proteolytic enzymes production was performed as previously described 199 (AlGburi et al., 2016; Deorukhkar et al., 2014) with some modifications by spot 200 inoculating 10 µL of yeast suspensions in YEPD broth onto SD plus milk agar (100 mL 201 UHT skim milk, 16.25 g SDA, and 100 mL deionized sterile water), with a final 202 medium pH of 7.3. Plates were incubated at 30 °C and 37 °C for up to 5 days. 203 Proteolytic activity was expressed as ratio of diameter of colony to that of colony plus 204 clear zone around the colony. Diameter was measured using digital caliper (Instrument 205 Choice, Dry Creek, Australia). *C. albicans* was used as a positive control.

206 **2.4. Identification of yeast isolates**

207 Different colony types from RBCA were picked and streaked onto yeast extract 208 peptone dextrose (YEPD) agar (Oxoid, Basingstoke, United Kingdom) and incubated 209 at 30 °C for 3 days. These isolates on YEPD agar were sent to Microgenetix, a National 210 Association of Testing Authorities (NATA) accredited laboratory for identification using 211 26s fungal ribosomal DNA. MicroSEQ® D2 LSU rDNA Fungal Identification Kit was

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

219 **2.5. Antibacterial properties**
220
221 **2.5.1. Growth inhibition**

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

228 Production of antibacterial molecules or pH effects analysis was carried out as
229 reported (Bajaj, Raina, & Singh, 2013) with modification. Fermentation was performed
230 by growing yeast isolates and controls in killer toxin medium (KTM) consisting of YEPD
231 plus glycerol (50 g/L, Sigma, St. Louis, USA), buffered at pH 5 using 50 mM citrate-
232 phosphate buffer, and fermented at 30 °C under shaking (180 rpm) for 24–72 hrs.
233 Fermented broth was centrifuged (Eppendorf AG, Hamburg, Germany) at 4,000 g for
234 30 minutes at 4 °C. Supernatant from KTM was used for well diffusion assay as
235 described previously (Bajaj, Raina, & Singh, 2013).

236 **2.5.2. Sedimentation and adhesion assay.**

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

252 To visualize the adherence of bacteria onto yeast cells, 10 µL of the sediments were
253 smeared onto microscopic slides. Gram stain was performed as described (Claus,
254 1992) and analyzed under optical Motic microscope (Motic, Melbourne Australia).

255 **2.6. Statistical analysis**

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

261 **3.0. Results and discussions**

262 **3.1. Isolation and enumeration of yeast isolates**

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

276 **3.2. Survival in simulated GIT**

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

285 kefir is an acidic and low alcohol beverage (Prado et al., 2015) and therefore have
286 likely developed resistance to harsh conditions. HSK and TVR kefir pH were 4.7 and
287 4.5 respectively after 48 hours of incubation as described above. Resistance of *S.*
288 *boulardii* MYA 797 to GIT conditions has been postulated to be due to thicker cell walls
289 compared to other *Saccharomyces cerevisiae* strains including W303 and BY4741
290 (Hudson et al., 2016). However, exposure of *S. boulardii* MYA 797 to Caspofungin, an
291 antifungal drug that interferes with synthesis of cell wall, was found to significantly
292 reduce resistance of these strains to GIT simulated environment (Hudson et al., 2016).
293 Role of cell wall thickness of kefir yeast isolates in GIT survival in this study needs
294 further investigation. Survival in the GI tract is an important criteria for microorganisms
295 to be classified as probiotic, and involves being able to resist acidic gastric juice such
296 as pepsin, basic pancreatic enzymes lysozyme, and bile salts at physiological
297 temperature (Gut et al., 2018). Survival of these yeast strains in simulated GIT showed
298 their resistance to digestive enzymes including pepsin, pancreatins as well as bile salts
299 and low pH. *S. cerevisiae* CIDCA8112 and *Kluyveromyces marxianus* were reported
300 to exhibit immunomodulatory properties which depended on viability of the yeast
301 species (Romanin et al., 2010). Furthermore, viability of yeast probiotics is associated
302 with several antagonistic properties towards enteropathogenic bacteria including
303 competition for nutrients, binding sites and production of antibacterial molecules
304 (Revolledo, Ferreira, & Ferreira, 2009).

305 **3.3. Growth at human body temperature**

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

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

314 **3.4. Auto-aggregation**

315 Auto-aggregation of kefir isolates and controls are shown in table 2. It is defined as
316 aggregation among yeast cells to form flocs or flor which provides competitive
317 advantage over other microorganisms including enteric bacterial pathogens in a harsh
318 environment such as human GIT (Brückner & Mösch, 2012). Auto-aggregations of
319 kefir isolates were slightly comparable to that of *S. boulardii* strains used as controls.
320 The percentage auto-aggregations of isolates and controls were consistent with
321 previous studies (Fadda et al., 2017a; Gil-Rodríguez , Carrascosa, & Requena, 2015).
322 Formation of cell aggregates provides shielding to cells in the center against harmful
323 environmental conditions (Suvarna, Dsouza, Ragavan, & Das, 2018). *In vitro* auto-
324 aggregation can be influenced by duration of incubation used during analysis to
325 separate aqueous and n-hexadecane phase (Gil-Rodríguez, Carrascosa, & Requena,
326 2015). On the other hand, yeasts auto-aggregation has been reported to be strain-
327 specific (Gil-Rodríguez et al., 2015; Suvarna et al., 2018). Therefore, variations of
328 auto-aggregation in the current study was likely due to these factors which have
329 similarly been reported by Fadda et al. (2017) using similar strains.

330 **3.5. Hydrophobicity**

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

334 this study, TVR18099-12 showed significantly higher hydrophobicity and therefore
335 was capable of interacting with other cell bodies compared to HSK18099-11 and
336 controls. Similar findings showed significantly lower hydrophobicity for control strains
337 compared to other strains (Fadda et al., 2017a). Hydrophobicity is crucial in adhesion
338 of probiotic microorganisms onto GIT epithelial cells where they may provide
339 prophylactic and therapeutic benefits (Fadda et al., 2017a). Hydrophobicity is species
340 and strain specific as demonstrated in this study (Fadda, Mossa, Deplano, Pisano, &
341 Cosentino, 2017b; Suvarna et al., 2018). Furthermore, similar to a previous study,
342 there was no correlation between auto-aggregation and hydrophobicity (Fadda et al.,
343 2017a).

344 **3.6. Hydrolytic enzymes screening**

345 **3.6.1. Phospholipase activity**

346 The kefir yeast isolates and probiotic controls did not produce these enzymes (Figure
347 3). Only positive control (C*. albicans*) produced phospholipase hence the zone of
348 precipitation around the colony shown in Figure 3 (Mayer, Wilson, & Hube, 2013; Park,
349 Do, & Jung, 2013). This activity was in agreement with previous studies in which C*.*
350 *albicans* produced this enzyme (Deorukhkar et al., 2014; Ramesh et al., 2011; Yang,
351 2003). Lack of phospholipase production by kefir grain yeasts isolates make them safe
352 in respect to this enzyme as it is associated with yeast virulence (Ramesh et al., 2011).

353 **3.6.2. Hemolytic activity**

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

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

372 **3.6.3. Proteolytic activity**

373 All kefir yeast isolates and controls showed very weak proteolytic activity shown in
374 Figure 5. Proteolytic enzymes are associated with active entry of pathogens into the
375 host tissue (Mayer et al., 2013; Sacristan et al., 2011). However, production of
376 proteolytic enzymes by yeasts may provide prophylactic and therapeutic benefits to
377 the host as *S. boulardii* serine protease has been reported to break down *Clostridium*
378 *difficile* and *Clostridiums perfringens* toxins (Czerucka et al., 2007; Hudson et al.,
379 2016; Palma et al., 2015). Therefore, production of proteolytic enzymes may protect
380 host against infections by toxins produced by enteropathogenic bacteria such as
381 *Salmonella, Vibrio, Clostridiums, E.coli* and *Bacillus* species (Gut et al., 2018).

382 **3.7. Identification of yeast isolates**

383 The yeast isolates from two traditional kefir grains showed potential probiotic
384 properties and after identification and characterization, two phylogenetic trees were
385 obtained as shown in Figure 6. A phylogenetic tree by definition shows evolutionary
386 relationships among species (Mooers & Heard, 1997). Isolates HSK18099-11 and
387 TVR18099-12 were identified as *Saccharomyces unisporus* ATCC 10612 (*S.*
388 *unisporus*) and *Kluyveromyces lactis* var. *lactis* ATCC 56498 *(K. lactis)*
389 */Kluyveromyces marxinus ATCC16045* (*K. marxinus)* respectively. The first part of
390 the figure shows a good separation of *S. unisporus* from its evolutionarily related
391 species including *Kazachstania africana*, *Saccharomyces cerevisiae* and
392 *Saccharomyces bayanus*. Therefore, further differentiation identification method was
393 not required. However in a previous study *S. unisporus* isolated from Tibetan kefir
394 grains could not be differentiated from Kazachstania unisporus and Kazachstania
395 exigua using 26s rDNA (Zhou, Liu, Jiang, & Dong, 2009). On the other hand, the
396 second part of the figure showed close similarity between *K. lactis* and *K. marxinus,*
397 which were not effectively differentiated using 26s ribosomal DNA. This was in
398 agreement with a previous study in which two *Kluyveromyces* species could not be
399 differentiated on the basis of their amino acid sequence (Lertwattanasakul et al.,
400 2015). However, in another study, it was reported that *K. lactis* and *K. marxinus* were
401 clearly separated and identified from kefir grain using 26s rDNA (Zhou et al., 2009).
402 The TVR18099-12 colonies labelled as *K. lactis*/*K. marxinus* by 26s rDNA (Figure 6)
403 were further analyzed using ITS sequencing, and identified as *K. lactis* ATCC 56498.
404 ITS sequencing was used successfully to identify *Kluyveromyces* species and strain
405 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.

430 Specific binding using type 1 fimbriae on bacteria such as *E.coli*, *E. aerogenes* and
431 *Salmonella* cell with mannan oligosaccharides on yeast cells, and non-specific binding
432 such as electrostatic and hydrophobic (Adegbola & Old, 1985; Pérez-Sotelo et al.,
433 2005; Tiago et al., 2012). These results are consistent with previous results where
434 *E.coli* was reported to bind both viable and non-viable *S. boulardii* and *Saccharomyces*
435 *cerevisiae* UFMG 905. Binding of enteric bacteria onto yeast cells is reported to be
436 irreversible leading to transient passage of bacteria through GIT. *S. boulardii* does not
437 bind to GIT (Gut et al., 2018). The adherence of enteric bacterial pathogen is
438 postulated to be responsible for probiotic effects such as inhibition of signalling
439 transduction pathway activation and subsequent translocation (Tiago et al., 2012) and
440 hence their prophylactic and therapeutic application in human (Gut et al., 2018), and
441 animal husbandry to promote health possibly through reduction of infection (Perez-
442 Sotelo et al., 2005). Survival in GIT of these yeast isolates as shown in this study may
443 lead to increased numbers, and hence increase capacities to scavenge (adhered
444 bacterial cells) potential pathogenic gram negative enteric bacteria from the gut and
445 subsequent flushing out in the feces. Moreover, since these yeasts survive in GIT and
446 are not affected by antibiotics (for example, not affected by Chloramphenicol in this
447 study, data not shown) aimed at bacteria, their use as complementary therapy with
448 antibiotics during enteric bacterial infection may also improve treatment through
449 increased numbers and subsequent mopping out enteric bacteria from the GIT.
450 Furthermore, the binding of opportunistic enteric bacteria onto non-viable yeasts is of
451 great significance since consumption of viable yeast probiotics is associated with
452 fungemia especially in immunocompromised individual or those with GIT issues (Gut
453 et al., 2018).
454

455

456 ## 4. Conclusion and future perspective

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

465 **Acknowledgment**

466 The authors acknowledge Victoria University of Technology for funding this work.

467 **Conflicts of interest**

468 The authors declare that they have no conflicts of interest.

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Figure 1. *HSK18099-11* and TVR18099-12 colonies morphology appearance on Rose Bengal Chloramphenicol Agar incubated at 25°C for 5 days.

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Figure 4. Hemolytic activity in SDA supplemented with 7% defibrinated sheep blood and incubated at 30 °C for 5 days. A= HSK18099-11, B = MYA 797™, C = MYA 796™, D = TVR18099-12, E = *C. albicans*.

Figure 5. Proteolytic activity in 16.25 g SDA, 100 mL UHT skim milk, and 100 mL deionised sterile water and incubated at 30 °C for 5 days. A= *HSK18099-11*, B = MYA 797™, C = MYA 796™, D = *TVR18099-12*, E = *C. albicans*

Figure 6. Phylogenetic tree analysis of 26s rDNA *K. lactis/K. marxinus* and *S. unisporus* obtained by MicroSEQ® D2 LSU rDNA Fungal Identification Kit following manufacturers protocols. The tree shows evolutionary closeness of the traditional kefir grain yeasts isolates to the other yeast species.

Figure 7. Gram stain showing adherence of *E.coli* and *E. aerogenes* onto yeasts cell wall observed under optical Motic microscope at 100x magnification. A = *E. aerogenes* plus *K. lactis,* B = *E.aerogenes* plus *S.unisporus,* C = *E. aerogenes* plus MYA 796™,

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

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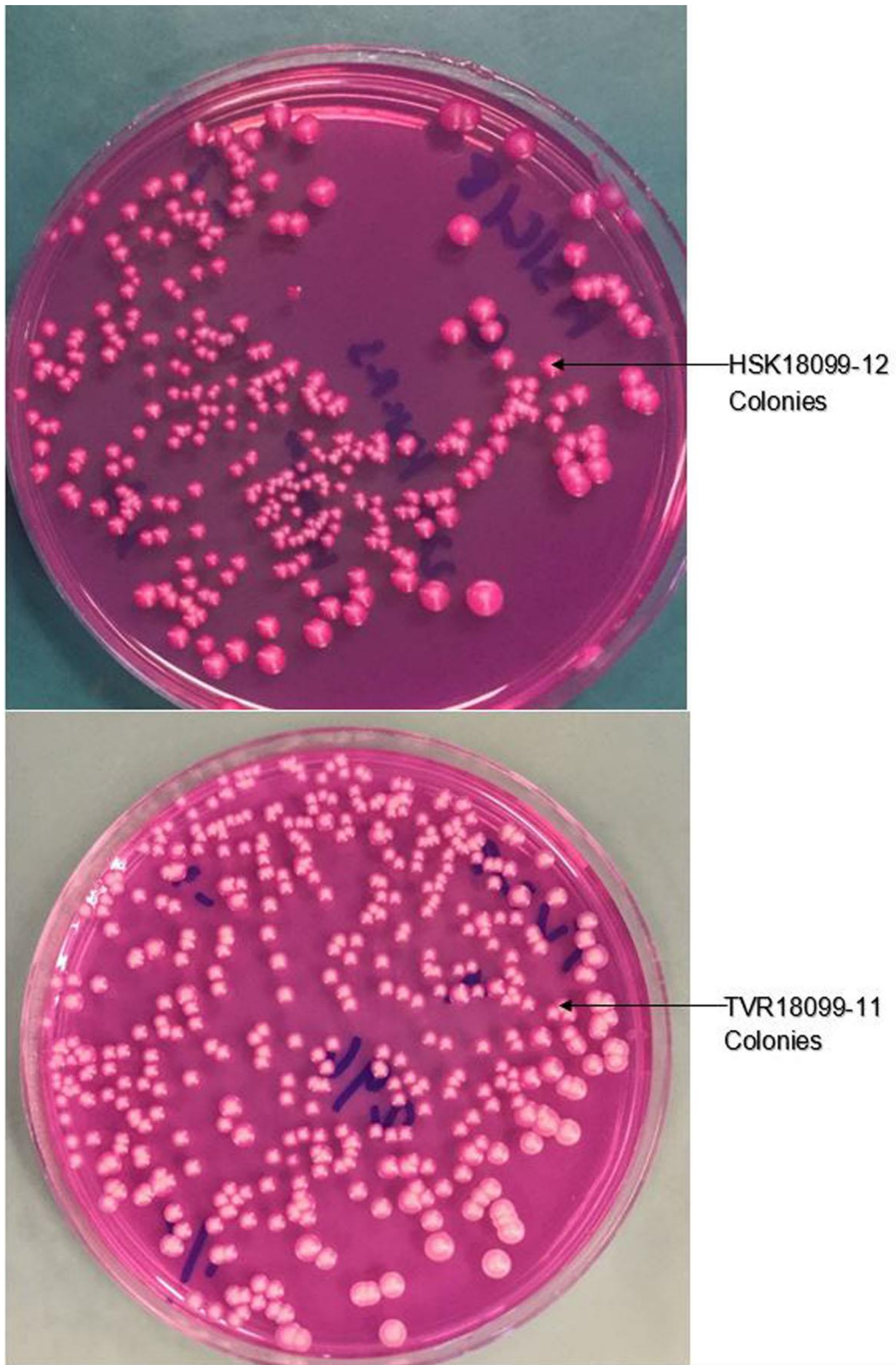
711 **Figure**



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713 Figure 1

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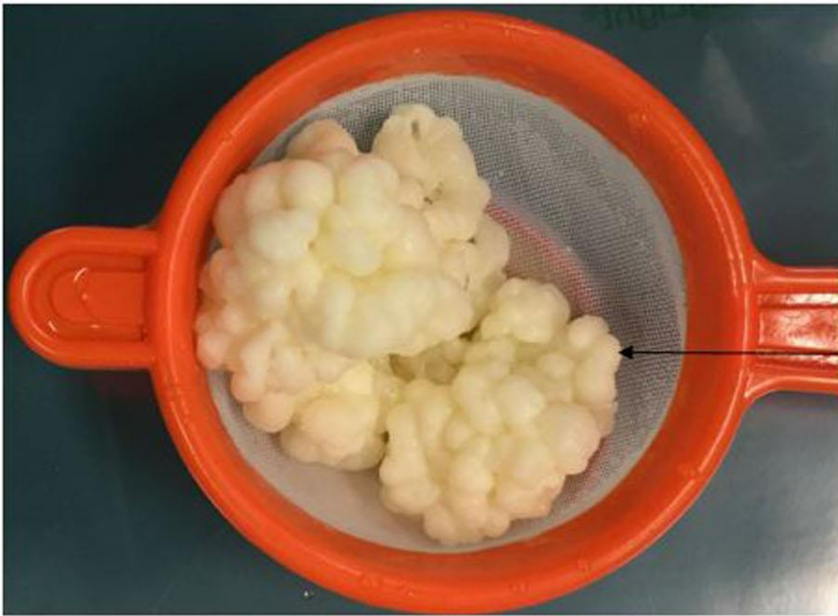

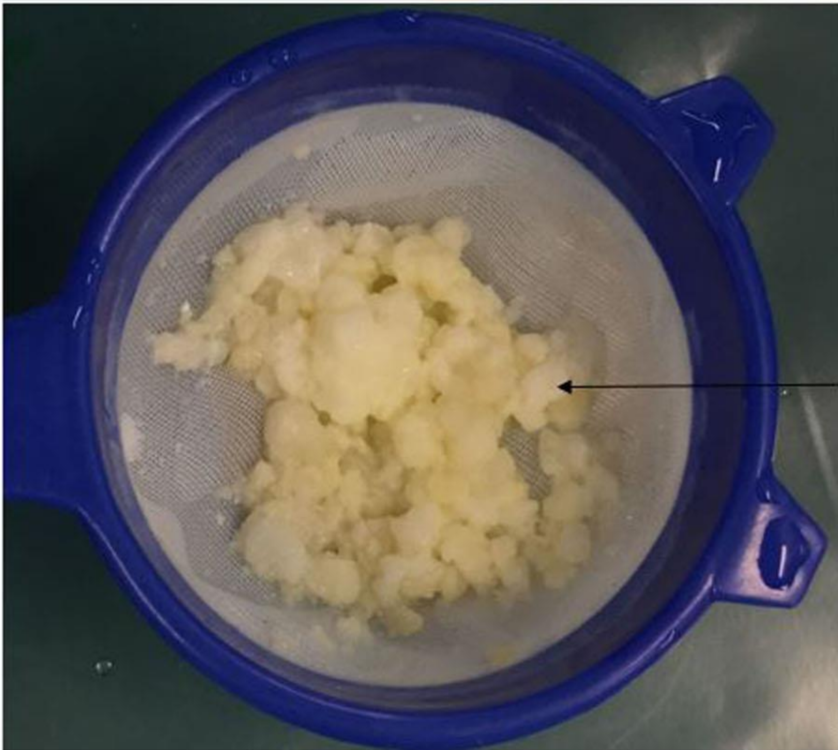

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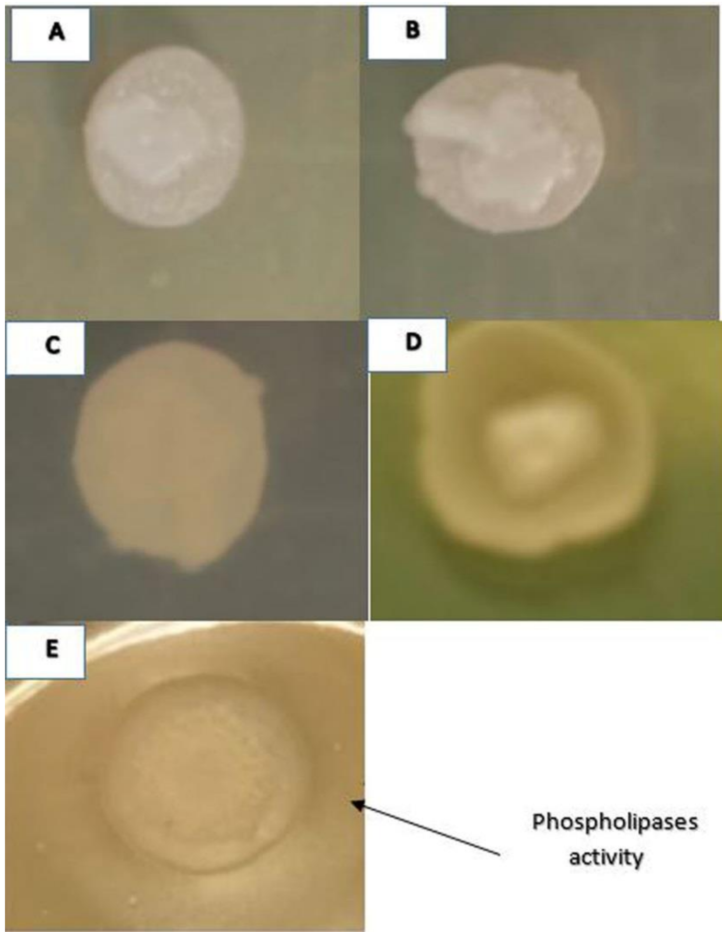

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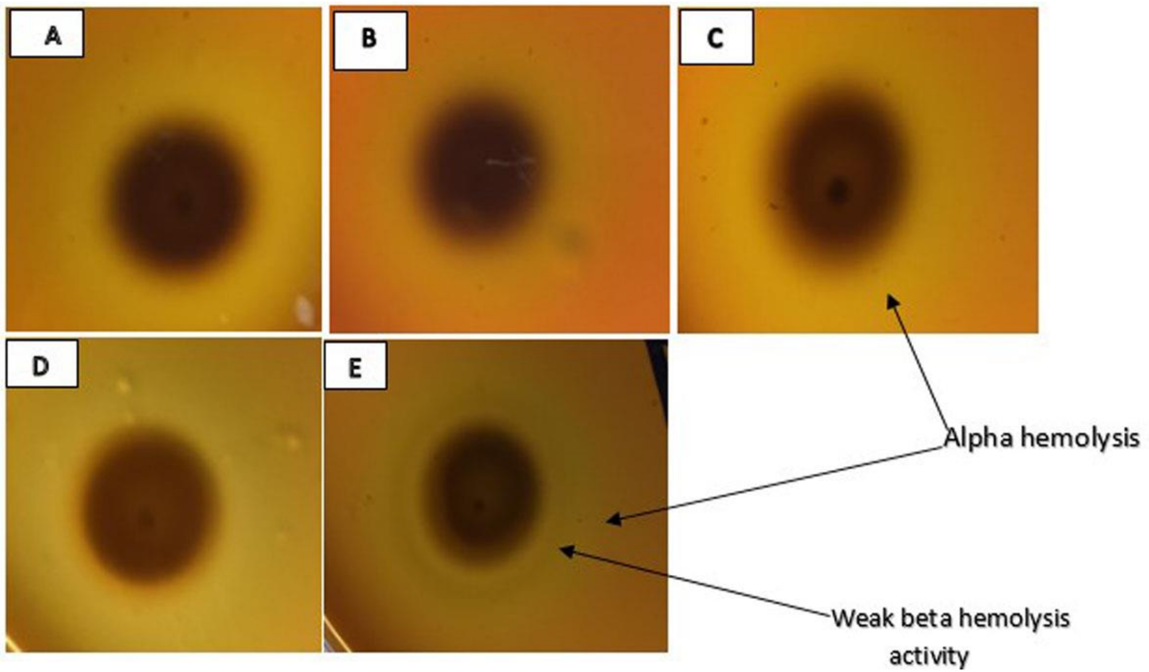

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725 Figure 4

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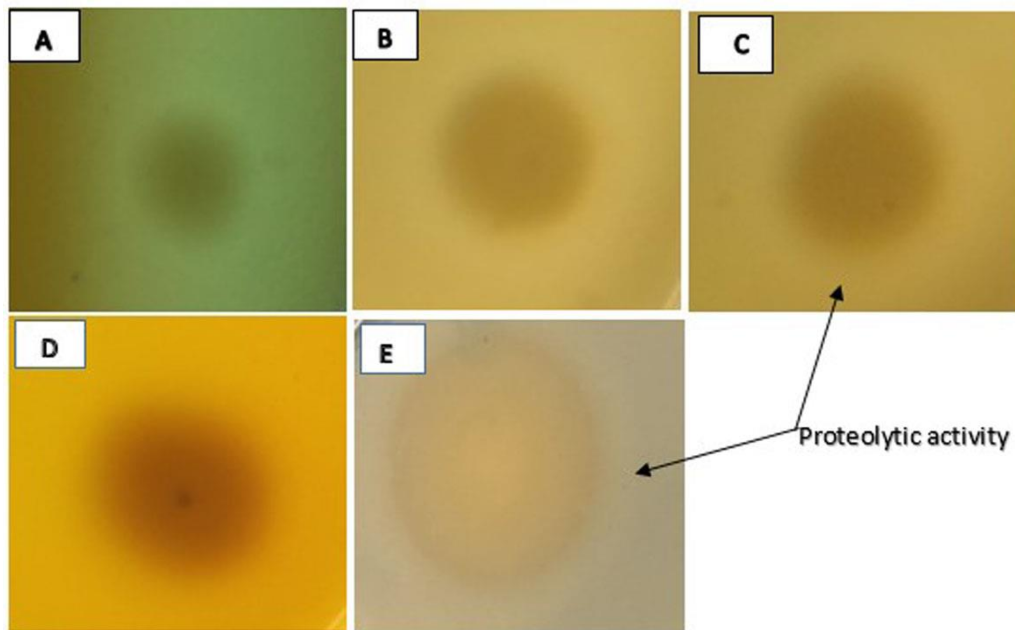

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728 Figure 5

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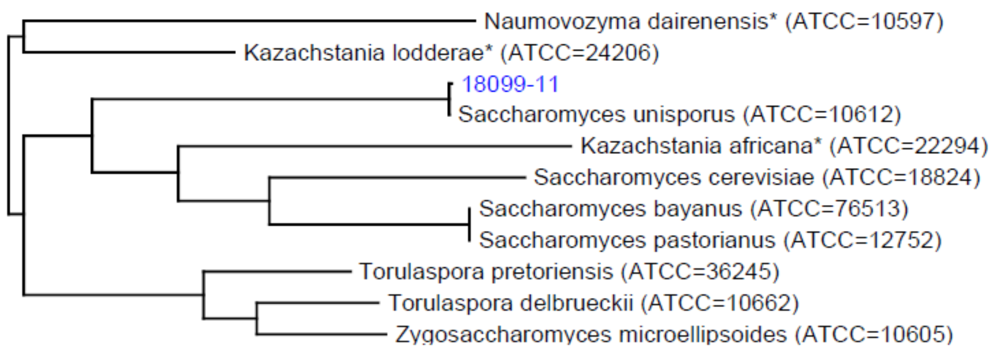

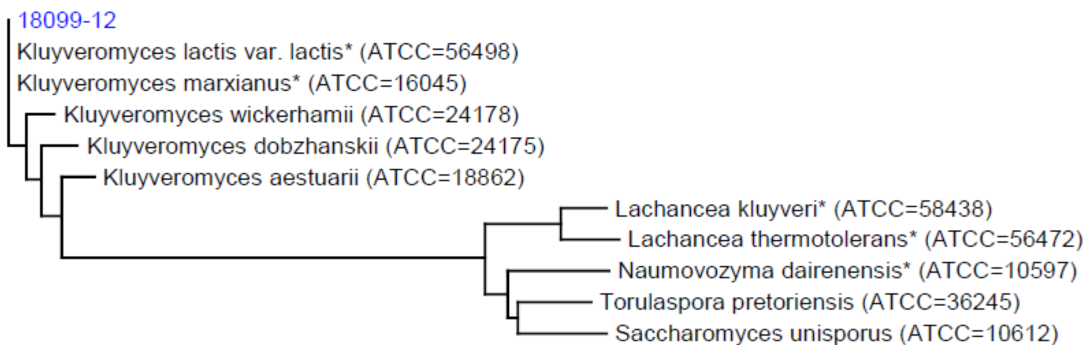

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731 Figure 6

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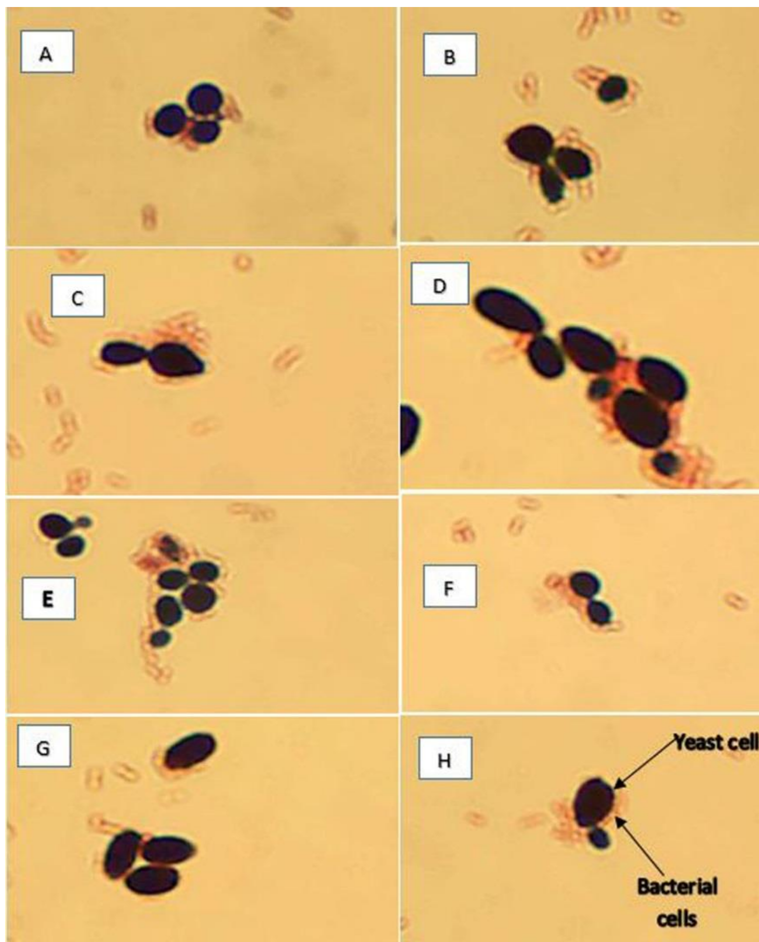

733 Figure 7

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746 **Tables**

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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

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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^{TM}	7.04	6.95	0.09	+++	15.58	42.86
MYA 797^{TM}	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-

754 aggregation= 0.03. MYA 796™ and MYA 797™ are two strains of *S. boulardii*
755 corresponding to SB48 and SB49 respectively. The difference between GIT treated
756 and untreated counts for both isolates and controls are statistically significant.
757

758 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™	0.77	1	0.78	1
MYA 797^T	0.84	1	0.82	1
Calb	0.78	0.94	0.76	0.72

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