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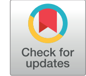
Evaluation of potentially probiotic attributes of certain dairy yeast isolated from buffalo sweetened Karish cheese



E.A. Helmy [a], S.A. Soliman [b], Tarek M. Abdel-Ghany [c,*], Magdah Ganash [d]

[a] The Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt
[b] Dairy Science Department, Faculty of Agriculture, Al-Azhar University, Cairo, Egypt
[c] Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Cairo, Egypt
[d] Biology Department, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia

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ABSTRACT

Egyptian traditional cheese has a long history and still represent an important part of the Egyptian diet. A lot of scientific studies in probiotic topic is usually related to bacteria, in particular lactic acid bacteria, and there is lack of information about potentially probiotic yeasts, except *Saccharomyces boulardii*. In the current study, 50 samples of traditional Egyptian buffalo sweetened cheese randomly were collected from five local Egyptian markets for yeast isolation. Isolated yeast species were identified using API20 kits techniques and the most frequently isolates were genotypically confirmed identified using the variability in the ITS rDNA. Appropriate *in vitro* assays have been conducted to examine their probiotic potentiality counting acid and bile salts tolerance, stimulated gastrointestinal tract tolerance, cell adhesion/hydrophobic characteristics, killer toxin productivity and antimicrobial activity against some clinical and food borne pathogens. The incidence of the obtained yeast taxa was found to be; *S. cerevisiae* (25%), *Wickerhamomyces anomalus* (23%), *Pichia kudriavzevii* (19%), *Kluyveromyces lactis* (17%), *Geotrichum candidum* (6%), *Debaryomyces hansenii* (4%), *Candida tropicalis* (3%), *Cryptococcus neoformans* (1%), *Rhodotorula glabrata* (1%) and *Trichosporon cutaneum* (1%). The most frequently isolates (*S. cerevisiae*, *W. anomalus* and *P. kudriavzevii*) exhibited high tolerance to bile salts elevated concentrations up to 2.0 %. *W. anomalus* could withstand the elevated bile salts concentrations and it was the most tolerable yeast isolate to intestinal juice environment. *W. anomalus* showed the lowest eradication from intestinal mucosa as indicated by the hydrophobicity average percentage 11.891% to xylene comparing to the *P. kudriavzevii* which showed the highest hydrophobicity average percentage of 46.185% to chloroform. Yeast isolates *S. cerevisiae*, *W. anomalus* and *P. kudriavzevii* (particularly *W. anomalus*) were recognized as ideal potentially probiotic model having *in vitro* properties that make them favorable candidates for probiotic applications.

1. Introduction

Egyptian customary cheese has a long history and keeps on being an imperative piece of the Egyptian eating regimen. There is proof of cheese making more than 5,000 years back in the season of the First Dynasty of Egypt. Interestingly with yogurt, cheese is an entrancing sustenance-based movement vehicle of probiotics to the gastrointestinal tract thinking about a higher of pH, fat substance and solid consistency [1]. The traditional system for Karish cheese creation deals with various open entryways for microbial corrupting. It is generally delivered utilizing unrefined drain every now and again of poor bacteriological quality and made under unsterilized conditions. In like manner, this thing is sold uncovered without a holder; thusly the risk of pollution is high. Therefore, it can be considered as a nice medium for the improvement of different sorts of pollution and pathogenic microorganisms [2, 3].

Probiotics are live microorganisms that beneficially affect the host by altering the microbial group related with the body by guaranteeing enhanced utilization of the sustain, or upgrading its healthful esteem, by upgrading the host reaction toward sickness, or by enhancing the nature of its surrounding condition [4]. As of late, probiotic nourishment items are the principal divisions of the worldwide exchange advertised and are conceivable to develop at a compound yearly development rate of 6.8% from 2013 to 2018 and unsurprising to achieve US$ 37.9 billion by only 2018 [5]. In the interim, probiotic dairy items constitute a standout amongst the most created sections and speak to a noteworthy branch of the practical nourishments industry [6].

* Corresponding author.
E-mail address: tabdelghany@yahoo.com (T.M. Abdel-Ghany).

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It is commonly apparent that yeasts can be a basic piece of the microflora of numerous cheese varieties thinking about the low pH, low moisture content, high salt concentration and refrigerated storing of these things [7]. To be sure, in some cheese composes they make a positive pledge to the change of flavor and surface during the period of advancement, while in various collections, yeasts crumbling living beings that is seen as an issue in a general sense in matured drain [8, 9]. The wellsprings of these yeast ailments are arranged along the whole chain of creation from the farm to the last thing. Yeasts in some cheese sorts can occasionally cause both fiscal and general medicinal issues. Yeasts themselves are not by and large the explanation behind absconds in cheese unless they age lactose. For this circumstance, they can grow rapidly and make a trademark yeasty or fruity flavor and clear gas [10, 11, 12]. There are different references concerning the significance of the closeness of yeasts in dairy foodstuffs, where they may contribute determinedly to the trademark taste and flavor headway among the period of advancement [13, 14].

In the human body, yeasts can grow rapidly in the stomach related tract and make extracellular proteases, siderophores, and killer harms. Therefore, they expect a section for threatening vibe to some pathogenic microorganisms, for instance, pathogenic parasites and entero-pathogens [15]. Bordering known bacterial probiotics, unmistakable yeast species, for instance, *D. hansenii, Torulaspora delbrueckii, K. lactis, K. marxianus,* and *K. lodderae* showed versatility to area through the gastro intestinal tract or restriction of entero-pathogens [16]. Most of the *in vitro* probiotic looks at have been mulled over in minute creatures just not in yeast. The most normally used bacterial probiotics consolidate *Lactobacillus* species, *Bifidobacterium* species, *Escherichia coli, Streptococcus* species, *Lactococcus lactis*, and some *Enterococcus* species. Mostly, the primary probiotic yeast found in literatures was the non-pathogenic *S. boulardii*. A vital good position of using probiotic yeast for this application is their ability as eukaryotes to make post-translational adjustments that may enable enunciation of a wide arrangement of remedial proteins in their host to goodness adjustment [17].

A lot of research in this topic is usually related to bacteria, in particular lactic acid bacteria, and there is lack of information about potentially probiotic yeasts, except *S. boulardii*. Several previous reports considered probiotics properties of *S. boulardii* counting for their killer toxins and antimicrobial activities [18, 19, 20], indicated that *S. boulardii* yeast was able to prevent intestinal infection caused by *E. coli, Salmonella typhimurium, Staphylococcus aureus, Pseudomonas aeruginosa, Proteus vulgaris, Yersinia enterocolitica* and *C. albicans* involving both *in vitro* and *in vivo* analyses. The present study focused on isolation and full identification of yeasts from Egyptian buffalo sweetened Karish cheese and evaluation of its potentially probiotic properties for its possible use in the medical field and most of the food industry.

2. Materials and methods

2.1. Sample collection and isolation of yeasts

Screening of a total of fifty samples of Egyptian buffalo sweetened Karish cheese (500 g of each sample) randomly collected from five local Egyptian markets in Cairo and El-Obour city Districts. All samples were transported to the laboratory under refrigeration and analyzed immediately on arrival for isolation and identification of yeast. Isolation of yeasts was achieved per the method of Van der Walt and Yarrow [21]. Ten grams of each sample were taken from the inner part of the cheese, emulsified in 90 ml of sterile solution of 2% (w/v) sodium citrate and homogenized in a sterile mortar for 30 s. For all samples, tenfold serial dilutions were prepared in a sterile solution of 2% (w/v) sodium citrate and the numbers of yeasts were determined by surface plating on the yeast malt agar (YMA) medium. After incubation at 25 °C for 5 days, all samples were prepared and analyzed in duplicate. Yeast colonies were streaked to single colonies on yeast potato dextrose agar (YPDA) media, incubated for 5 days at 25 °C and checked for purity. Counts for each individual type of colony were made to estimate the relative occurrence of the various yeasts present in the samples. Yeast species counts were calculated as number of colonies forming units (CFU) per gram of sample. The growing colonies were maintained on the yeast malt agar (YMA) medium for further analysis.

2.2. Morphological and physiological diagnosis of the isolated yeasts

Morphological and physiological characteristics as well as incidence of the most frequently isolated yeast species were achieved using conventional methods and APi 20 C Aux as described [22, 23]. Physiological characteristics were investigated by API 20 C Aux (Sigma, St. Louis, MO, USA).

2.3. Molecular identification of the isolated yeasts

Further confirmation of the most frequently isolated yeast species using variability in the nucleotide sequence of ITS rDNA region analysis according to Tamura *et al.* [24] were achieved. Cellular DNA was extracted and conducted using DNeasy kit (Qiagen-Germany) as per manufacturer procedure. PCR amplification of the ITS1-5.8S-ITS2 rDNA regions was carried out in a final volume of 50 μl, each reaction contains 1.5 μl of DNA template, 0.2 μM of each of the forward primer (ITS1: 5′-TCC GTA GGT GAA CCT GCG G-'3) and the reverse primer (ITS4: 5′-TCC TCC GCT TAT TGA TAT GC-'3). The PCR analysis has been were got according to Sambrook *et al.* [25]. After amplification, the PCR products were purified for sequencing using AccuPrep PCR purification kit (Bioneer, Daejeon, South Korea) and then sequenced with automated DNA sequencer (ABI prism® 310 genetic analyzers, Applied Biosystems, CA, USA). Molecular Evolutionary Genetic Analysis (MEGA version 6) software was used for phylogenetic analyses. The closets homologous to the sequences were selected and multiple sequence alignments were carried out using the Clustal W program in the MEGA6 software. Phylogenetic tree was constructed, using neighbor joining method with 1,000 bootstrap replicates based on ITS gene sequences, to show the phylogenetic relationships between the three tested yeast isolates and the closely related isolates retrieved from NCBI Gen Bank [26]. These three investigated taxa were then giving an accession number to be conserved at the Regional Center for Mycology and Biotechnology (RCMB) culture collection unit of Al-Azhar University, Cairo, Egypt.

2.4. Probiotic properties of the isolated yeasts

2.4.1. Low pH tolerance in simulated gastric juice (SGJ)

The SGJ was prepared by suspending pepsin (3 g l^{-1}) in saline solution (9 mg ml^{-1} of NaCl). The pH was adjusted at different values from 3, 2, to 1.5 using 12 *M* HCl. The prepared solution was sterilized by filtration using microbiological membrane filter of 0.22 μm pore diameter (Millipore, USA). Gastric juice was freshly prepared in the same day of the experiment procedure and yeast cell tolerance to this artificial gastric juice at different pH values was measured [27]. The results were got as the mean value of three replicates and expressed as percentage log viability calculated according to Eq. (1),

$$\text{Log Viability } (\%) = \left(\frac{\log N}{\log N0} \times 100\right) \quad (1)$$

where N is the count after incubation ($cfu.ml^{-1}$), N0 is the count at zero time ($cfu.ml^{-1}$) [28].

Effect of SGJ pH on the growth rate percentages (%) of investigated yeasts was estimated using UV–visible spectrophotometer (Spectronic Milton Roy 1201 UV). Optical density of yeasts growth increase percentage (%) was estimated everyone hour starting from the earliest point of zero time until the next three hours at the wavelength of 600 nm [27].

Table 1
Morphological characteristics and features of the yeast isolates.

Yeast Isolates	Colonies and cell Description						Sediment Formation	
	Color	Glistening	Margin	Budding	Cell Shape	Cell diameter (μm)	Appearance	Light turbidity
P. kudriavzevii QLB,	Wh/Cr	+	R	Multi	Ovoid/elongate	1.3–6 × 3.3–14	Sprightly	+
W. anomalus HN1	Wh/Cr	+	R	Multi	Ellipsoidal/Cylindrical	2–6 × 3–11	Sprightly	+
S. cerevisiae gbLKX237673.1	Wh/Cr	+	R	Multi	Round/ovoid	5–10	Sprightly	+

Wh/Cr, white/Cream; R, Regular;+,Present.

2.4.2. Bile salt tolerance

Bile salt solutions were freshly prepared in the same day of the experiment procedure as per the method of Lian *et al.* [27]. Sensitivity of yeast cells to different bile salt concentrations was estimated using Oxgal (Difco Lab., Detroit, MI, USA) which dissolved in yeast malt broth (YMB) medium to prepare bile salts concentrations ranged from 0.2 to 2.0%.The results were got as the mean value of three replicates and expressed as percentage log viability calculated according to the following formula as per Williamson and Johnson [28].

2.4.3. In vitro survival in intestinal environment (intestinal juice)

Survival intestinal environment was estimated *in vitro* [29]. Active yeast cells were harvested by centrifugation at 3000× g for 10 min, then inoculated at the level (10^6 cfu ml^{-1}) into medium reproducing human intestinal condition, constituted by an aqueous solution containing 1 $g.l^{-1}$ in saline NaCl (0.5%, w/v), the pH being adjusted to 8.0 with 0.1M NaOH before 2% glucose was added. The cells viability was determined by counting plates after 0, 1, 2, 3 and 4 hours of incubation at 37 °C. The results were got as the mean value of three replicates and expressed as percentage log viability calculated according to the following formula as per Williamson and Johnson [28].

2.4.4. Cell surface adhesion/hydrophobicity

Selected isolates were measured for cell surface hydrophobicity by measuring microbial adhesion to hydrocarbons assays [30]. After the activation of the three yeast isolates using yeast peptone dextrose (YPD) broth at 28 °C for 48 h, the yeast cultures were centrifuged at 1500 rpm for 15 min and yeast cells were twice washed in 50 mM phosphate buffer solution containing; (KH_2PO_4; 6.4 g and Na_2HPO_4; 21.8 g, pH 7.0), suspended in 0.1 M KNO_3 (pH 6.2) and the optical density was read at the value of 600 nm for determination of the absorbance values of the aqueous phase using UV-visible spectrophotometer (Spectronic Milton Roy 1201 UV). Four milliliters of the suspension were added to 1 ml of each of; xylene (a polar solvent), chloroform (acidic solvent) and ethyl acetate (basic solvent). After 5 minutes of mixing, phases were homogenized using a vortex for 2 minutes and the solvent phase could be completely separated by its incubation for one hour at 37 °C. Finally, the aqueous phase was removed carefully, and the optical density was read at the value of 600 nm again for determination of the absorbance values of the aqueous phase after reaction with solvents. The decrease in the absorbance value of the aqueous phase before and after addition of solvents was taken as a measure for the cell surface hydrophobicity (H) in different solvents according to Eq. (2),

$$H\ (\%) = \left(\frac{A0 - N0}{A0} \times 100\right) \tag{2}$$

where A_0 is the absorbance values before addition of solvents; N0 is the absorbance values of the aqueous phase after addition of solvents.

2.5. Antimicrobial activity and killer toxin potency

All tested microbial cultures were kindly provided by culture collection unit (CCU) of the RCMB. These cultures including some food borne pathogens and common intestinal flora of bacteria; *Salmonella typhimurium* ATTC 14028, *Bacillus subtilis* NRRL B-543, *Pseudomonas aeruginosa* ATTC 27853, Methicillin Resistant-*Staphylococcus aureus* (MRSA) RCMB011001, *S. aureus* ATTC25923, *Escherichia coli* ATTC 25955 and *Enterococcus faecalis* ATCC 29212 as well as some pathogenic filamentous

Table 2
Potentiality to ferment some compounds of the yeast isolates.

Biochemical tests		Yeast Isolates		
		P. kudriavzevii QLB	*W. anomalus* HN1	*S. cerevisiae* gbLKX237673.1
Sugar Fermentation	Lactose	–	–	–
	Glucose	+	+	+
	Raffinose	+	+	+
	Sucrose	+	+	+
	Galactose	+	-	–
	Maltose	–	–	–
	Starch	–	–	–
Assimilation of Carbon Compounds	Citrate	–	–	–
	Galactose	+	–	–
	Maltose	–	–	–
	Butane 2.3 diol	–	–	–
	propane-1,2-diol	–	–	–
Assimilation of Nitrogen Compounds	Tryptophan	–	–	–
	Ethylamine	–	–	–
	DL-L lysine	–	–	–
Complementary tests	5 % NaCl	+	+	+
	10 % NaCl	–	–	–
	16% NaCl	–	–	–
	50 % glucose	+	+	+
	60% glucose	–	–	–
	Acetic Acid Tolerance (1%)	–	–	–
	Hydrolysis of Urea	–	–	–
	Gelatin liquefaction	–	–	–

(+); Positive reaction (–); Negative reaction.

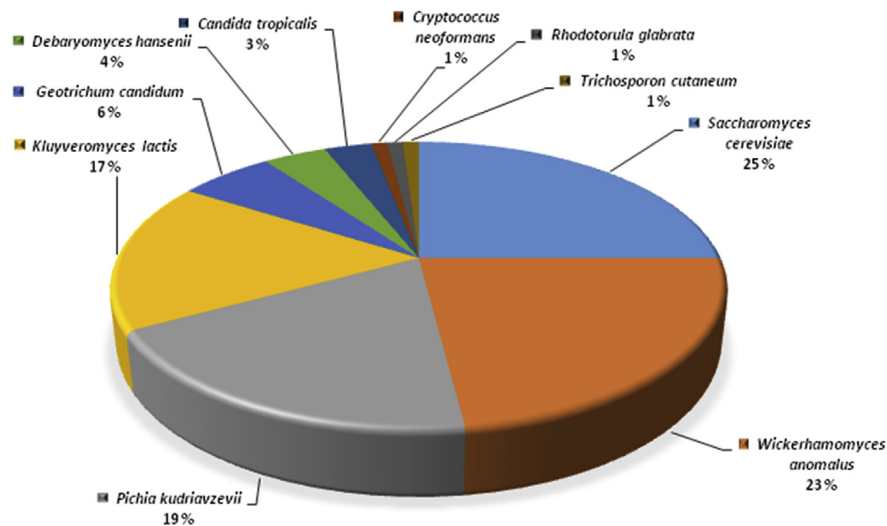

Fig. 1. Incidence % of the isolated yeasts using conventional phenotypic as well as biochemical identification protocols.

fungi; *Aspergillus niger* ATCC 16404, *A. flavus* RCMB 002002 (5), *A. fumigatus* RCMB 002008 (3), *Penicillium marnefeii* RCMB 001022 and unicellular fungi; *C. albicans* ATCC 10231 and *C. lipolytica* RCMB 005007. Furthermore, the assessed yeasts were tested also against a toxin-sensitive strain of *S. cerevisiae* RCMB 006001 for their antimicrobial and the killer toxin potential. Antimicrobial activity and the killer toxin potency of the screened yeast isolates were also tested using agar well diffusion technique [31]. The inhibition activity of each isolate was evaluated measuring the zone of inhibition around the potentially probiotic growth in wells.

2.6. Statistical analysis

All statistical calculations were done using computer programs Microsoftexcel version 10 and SPSS (Statistical package for the Social Science version 20.00) statistical program. At $p > 0.05$ level of

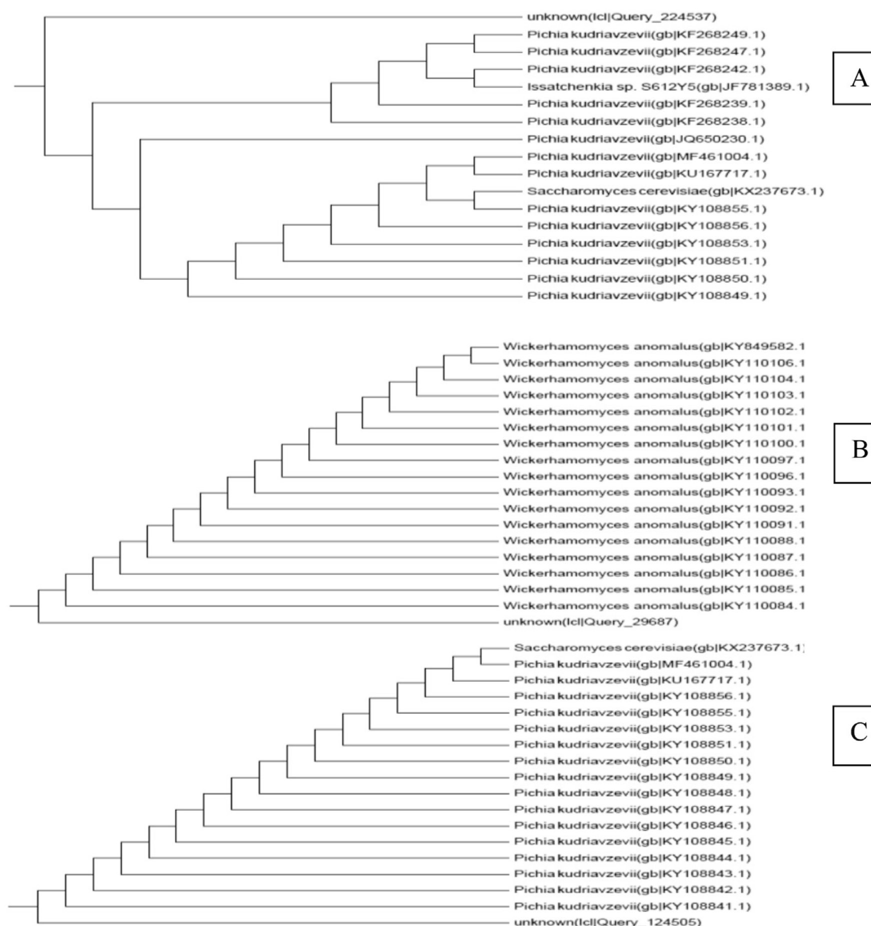

Fig. 2. Phylogenetic relationships between the three Probiotic yeasts A; *P. kudriavzevii* QLB, B; *W. anomalus* HN1; C; *S. cerevisiae* gbLKX237673.1 and the ITS sequences of closely related fungal strains retrieved from NCBI Gen Bank.

Table 3
Effect of simulated gastric juice at different pH values on the viability of investigated yeasts. Data represents the means of standard deviation of triplicate assays.

pH	Colony forming units per ml (CFU.ml^{-1}) of yeast isolates* at incubation time (hrs.)														
	0			1			2			3			4		
	S.c.	W.a.	P.k.	S.c.	W.a.	P.k.	S.c.	W.a.	P.k.	S.c.	W.a.	P.k.	S.c.	W.a.	P.k.
2.0	5.0×10^9	5.0×10^9	5.0×10^9	8.2×10^6	9.3×10^6	7.2×10^6	6.2×10^5	7.3×10^5	5.2×10^5	5.9×10^4	6.7×10^4	4.8×10^4	6.7×10^3	7.8×10^3	5.5×10^3
2.5	5.0×10^9	5.0×10^9	5.0×10^9	3.8×10^7	4.9×10^7	2.6×10^7	1.8×10^6	2.9×10^6	1.3×10^6	8.2×10^5	9.3×10^5	7.2×10^5	4.8×10^4	5.6×10^4	3.5×10^4
3.0	5.0×10^9	5.0×10^9	5.0×10^9	2.3×10^8	3.8×10^8	1.6×10^8	6.7×10^7	7.9×10^7	5.6×10^7	2.1×10^6	3.3×10^6	1.8×10^6	7.8×10^5	8.5×10^5	6.5×10^5
3.5	5.0×10^9	5.0×10^9	5.0×10^9	8.1×10^8	9.3×10^8	7.2×10^8	2.4×10^8	3.6×10^8	1.8×10^8	6.8×10^7	7.5×10^7	5.4×10^7	2.4×10^7	3.7×10^7	1.9×10^7
4.0	5.0×10^9	5.0×10^9	5.0×10^9	3.9×10^9	4.7×10^9	2.7×10^9	2.8×10^9	3.7×10^9	1.9×10^9	2.6×10^8	3.5×10^8	1.9×10^8	1.6×10^8	2.7×10^8	1.9×10^8
Control	5.0×10^9	5.0×10^9	5.0×10^9	5.0×10^9	5.0×10^9	5.0×10^9	4.9×10^9	4.9×10^9	4.9×10^9	4.9×10^9	4.9×10^9	4.9×10^9	4.8×10^9	4.8×10^9	4.8×10^9

S.c., *S. cerevisiae* gbLKX237673.1; W.a., *W. anomalus* HN1; P.k., *P. kudriavzevii* QLB. Control experiments were without any treatments.

probability, the One-way ANOVA results of the population viability were represented as the arithmetic mean of three assays with standard deviation not exceeding 0.2 logarithmic units [32]. The discernment, Pearson correlation and automatic linear models' analysis were estimated to show the relationship of the investigated parameter to each other as per Härdle and Simar [33].

3. Results and discussion

The samples of Egyptian buffalo sweetened Karish cheese were rich with yeast, where 78 yeast isolates were detected in it. Morphological and physiological characteristics of the isolated yeasts were detected (Tables 1 and 2). The incidence of the obtained yeast taxa was found to be; *S. cerevisiae* (25%), *W. anomalus* (23%), *P. kudriavzevii* (19%), *K. lactis* (17%), *G. candidum* (6%), *D. hansenii* (4%), *C. tropicalis* (3%), *Cryptococcus neoformans* (1%), *R. glabrata* (1%) and *T. cutaneum* (1%) (Fig. 1). Approximately the same isolates were isolated from Egyptian Karish cheese but with differed prevalence % [34]; where prevalent isolates were *T. cutaneum* (25%), *Candida catenulata* (23%), *Yarrowia lipolytica* (13%), *D. hansenii* (13%), *K. lactis* (6%), *G. candidum* (7%), *C. zeylanoides* (5%), *C. lambica* (3%), *C. albicans* (2%), *C. neoformans* (1%), *R. glabrata* (1%) and *S. cerevisiae* (1%).

3.1. Molecular identification of the isolated yeasts

In the current work, three yeast species were obtained as the most prevalent isolates namely; *P. kudriavzevii, W. anomalus* and *S. cerevisiae*. They were further genotypically confirmed identified using nucleotide sequence analysis of the specific amplification of theinter transcribed spacer region (ITS). A phylogenetic tree was constructed using the Neighbor Joining Algorithm with 1000 bootstrap replications for the identification of the yeast isolates using the sequence obtained from the molecular marker used. The 18 SrDNA- 28 SrDNA sequence of the yeast isolates was BLAST-searched on a database using the multiple sequence alignment in MEGA 6 software. From the alignment profile results, the constructed phylogenetic relatedness of the whole sequence of the 18S rDNA-28S rDNA genes of tested yeast isolates were compared with the closely related isolates from the BLAST. NCBI database at http://blast.ncbi.nlm.nih.gov/Blast.cg, which have revealed the molecular identity of the current isolates in comparison with the other stored isolates. Regarding the outcomes of this study, three taxa were obtained using the variability in the ITS rDNA region namely; *P. kudriavzevii* QLB*, W. anomalus* HN1 and *S. cerevisiae* gbLKX237673.1 as illustrated from the phylogenetic relationships between the three potentially probiotic yeasts (Fig. 2) and the ITS sequences of closely related fungal strains retrieved from NCBI Gen Bank. These three investigated taxa were then giving an accession number to be conserved at the RCMB culture collection unit of Al-Azhar University, Cairo, Egypt as *P. kudriavzevii* QLB, (RCMB 050001) *W. anomalus* HN1 and (RCMB056001) *S. cerevisiae* gbLKX237673.1 (RCMB006002).

3.2. Low pH tolerance in SGJ

In the current study, bile acids tolerance at low pH in SGJ was carried out to evaluate the potentially probiotic properties of the isolated yeasts. Previously, Chou and Weimer [35] stated that isolate used for the potentially probiotic applications should be able to tolerate acid for at least 90 minutes (time from gastric tract entrance to release from the stomach), tolerate to bile acids. In the present study, *W. anomalus* HN1 showed high viability when cultivated in pH 3.0 (Table 3). This is considered as the primary indication of suitability of this isolate as a potentially probiotic isolate benefit for human use. Cultivation at 37 °C and pH 3.0 the number of living cells was slightly decreased from 5×10^9 to 8.5×105 CFU ml^{-1} after 4 h. Moreover, *W. anomalus* HN1 cells showed high acid tolerance when cultivated in SGJ of different pH values. Cell survival of *W. anomalus* in terms of CFU. ml^{-1} after 2 h incubation at

Table 4
Effect of simulated gastric juice of different pH values on the growth rate % (OD at 600 nm) of investigated yeasts.

Time (h) and Increase (%)	pH	Yeast isolates growth (OD at 600 nm)		
		P. kudriavzevii QLB	*W. anomalus* HN1	*S. cerevisiae* gbLKX237673.1
0	1.5	0.209	0.088	0.207
	2.0	0.163	0.049	0.152
	3.0	0.199	0.070	0.187
1	1.5	0.214	0.174	0.203
	2.0	0.287	0.100	0.276
	3.0	0.204	0.089	0.203
Increase (%)	1.5	2.30	97.7	1.20
	2.0	76.0	104.0	65.0
	3.0	2.50	27.0	1.40
2	1.5	0.383	0.324	0.172
	2.0	0.460	0.151	0.350
	3.0	0.453	0.234	0.342
Increase (%)	1.5	35.4	268.2	24.3
	2.0	182.2	208.0	171.1
	3.0	127.6	234.3	116.5
3	1.5	0.443	0.279	0.332
	2.0	0.233	0.070	0.122
	3.0	0.568	0.211	0.457
Increase (%)	1.5	111.9	217.0	100.8
	2.0	43.0	42.0	32.0
	3.0	185.4	217.0	100.8

Table 5
Effect of different bile salt concentrations on the viability of investigated yeasts. Data represents the means of standard deviation of triplicate assays.

Bile salt (%)	Incubation time (hrs)	Colony forming units per ml (CFU. ml^{-1})		
		S. cerevisiae gbLKX237673.1	*W. anomalus* HN1	*P. kudriavzevii* QLB
Control	0	5.0×10^9	5.0×10^9	5.0×10^9
	1	5.0×10^9	5.0×10^9	5.0×10^9
	2	4.9×10^9	4.9×10^9	4.9×10^9
	3	4.9×10^9	4.9×10^9	4.9×10^9
	4	4.8×10^9	4.8×10^9	4.8×10^9
0.2	0	5.0×10^9	5.0×10^9	5.0×10^9
	1	2.6×10^7	3.4×10^7	1.8×10^7
	2	1.6×10^6	2.1×10^6	1.1×10^6
	3	7.5×10^5	8.2×10^5	6.8×10^5
	4	4.5×10^4	5.3×10^4	3.6×10^4
0.5	0	5.0×10^9	5.0×10^9	5.0×10^9
	1	1.6×10^7	3.2×10^7	1.2×10^7
	2	4.7×10^5	5.2×10^5	3.3×10^5
	3	8.4×10^4	9.2×10^4	7.5×10^4
	4	2.6×10^4	3.2×10^4	1.8×10^4
1.0	0	5.0×10^9	5.0×10^9	5.0×10^9
	1	5.6×10^6	6.3×10^6	4.6×10^6
	2	1.7×10^5	2.3×10^5	1.5×10^5
	3	2.5×10^4	3.4×10^4	1.8×10^4
	4	1.6×10^4	2.2×10^4	1.2×10^4
1.5	0	5.0×10^9	5.0×10^9	5.0×10^9
	1	2.5×10^6	3.1×10^6	1.5×10^6
	2	4.6×10^5	5.2×10^5	3.5×10^5
	3	8.4×10^4	9.1×10^4	7.6×10^4
	4	8.6×10^3	9.7×10^3	7.9×10^3
2.0	0	5.0×10^9	5.0×10^9	5.0×10^9
	1	5.7×10^5	6.4×10^5	4.6×10^5
	2	2.5×10^4	3.1×10^4	1.8×10^4
	3	5.8×10^3	6.1×10^3	4.3×10^3
	4	1.7×10^3	2.3×10^3	1.5×10^3

Control experiments were without any treatments.

different pH values of 2.0, 2.5, 3.0, 3.5 and 4.0 were 7.3×10^5, 2.9×10^6, 7.9×10^7, 3.6×10^8, $3.7\times10^9$and 4.9×10^9 CFU ml^{-1}, respectively (Table 3). While, Table 4 represented the effect of SGJ at different pH values on the growth rate % (OD at 600 nm) of investigated yeasts, where also the *W. anomalus* HN1 growth rate was exceeding the other investigated strains. These results indicated that *W. anomalus* HN1 could

Table 6
Effect of bile salts concentrations on the growth rate % of investigated yeasts. Data represents the means of standard deviation of triplicate assays.

Time (h) and Increase (%)	Bile salts concentrations (%)	Yeast isolates growth (OD at 600 nm)		
		P. kudriavzevii QLB	*W. anomalus* HN1	*S. cerevisiae* gbLKX237673.1
0	0.2	0.025	0.037	0.014
	0.5	0.078	0.064	0.067
	1.0	0. 115	0.031	0.104
1	0.2	0.038	0.047	0.027
	0.5	0.134	0.110	0.123
	1.0	0.127	0.047	0.116
Increase (%)	0.2	52.0	27.0	41.0
	0.5	71.70	71.8	60.60
	1.0	10.40	51.6	9.30
2	0.2	0.077	0.069	0.066
	0.5	0.167	0.154	0.156
	1.0	0.220	0.064	0.101
Increase (%)	0.2	208.0	86.5	104.0
	0.5	114.0	140.6	103.0
	1.0	91.30	106.4	80.20
3	0.2	0.118	0.249	0.107
	0.5	0.209	0.192	0.108
	1.0	0.243	0.113	0.132
Increase (%)	0.2	372.0	572.0	261.0
	0.5	168.0	200.0	154.0
	1.0	111.3	264.5	100.2

withstand the acidic stomach environment and reach the areas of beneficial activity and can be applied in human application and thus it could be suggested as potentially probiotic yeast for several applications. Our results were in agreement with previous studies [16, 36] observed the ability of the probiotic yeasts to grow at low pH. Thus, we can conclude that acidic tolerance of yeast is an isolate specific parameter.

3.3. Bile salts tolerance

Bile salts tolerance is required for cells to survive in the small intestine during passage in gastro intestinal tract (GIT). Optimum bile concentration of our human gut environment ranges from 0.3% to 0.6% [36]. As shown in Table 5, most yeast cells exhibited high tolerance to bile salts elevated concentrations up to 2.0 % and still viable in all applied concentrations. However, significant reduction in cell viability could be detected by increasing either bile salt concentrations or incubation time (Table 6). When cells cultivated in a solution of 2% bile salt, the number of viable cells (in CFU. ml^{-1}) in case of *W. anomalus* HN1 were reduced from 5×10^9 to 5.7×10^5, 2.5×10^4, 5.8×10^3, 1.7×10^3 CFU ml^{-1} after 1 h, 2 h, 3 h and 4 h, respectively. Accordingly, *W. anomalus* HN1cells could withstand the elevated bile salts concentrations (Tables 5 and 6). Correspondingly, this level of bile salt tolerance of this isolate is close to those reported for lactic acid bacteria [37]. Syal and Vohra [38] reported some yeast isolates that can survive in low pH and high bile salt including *S. cerevisiae, C. tropicalis, Aureobasidium* sp. and *P. manschuria* which possess tolerance to bile salt, high NaCl, SGJ, intestinal environment, α-amylase, trypsin and lysozyme.

3.4. In vitro survival in intestinal environment (intestinal juice)

Most probiotic tests were performed *in vitro* and for the conceivable advantages of human and creature wellbeing, probiotic living beings can be fused into dietary extras for the support of a sound gastrointestinal adjust. In this way, there is an open door for growing genius and probiotic mediations for regulation of the gut microbiota towards dependable wellbeing. During this investigation, survival intestinal environment assay was estimated *in vitro* for the determination of the capacity of the tested yeasts to overcome the stress conditions of the intestinal habitat by estimating their viability as well as the growth rate of them after a period with high concentrations of the intestinal juice. According to the obtained outcomes, *W. anomalus* HN1 was the most tolerable yeast isolate

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Table 7
Effect of Intestinal Juice on The Viability of Investigated Yeasts. Data represents the means of standard deviation of triplicate assays.

Bile salt (%)	Incubation time (hrs)	Colony forming units per ml (CFU ml^{-1})		
		S. cerevisiae gbLKX237673.1	*W. anomalus* HN1	*P. kudriavzevii* QLB
Control	0	5.0×10^9	5.0×10^9	5.0×10^9
	1	5.0×10^9	5.0×10^9	5.0×10^9
	2	4.9×10^9	4.9×10^9	4.9×10^9
	3	4.9×10^9	4.9×10^9	4.9×10^9
	4	4.8×10^9	4.8×10^9	4.8×10^9
Intestinal Juice	0	5.0×10^9	5.0×10^9	5.0×10^9
	1	3.1×10^6	3.1×10^6	3.1×10^6
	2	4.6×10^5	5.2×10^5	3.5×10^5
	3	8.4×10^4	9.1×10^4	7.6×10^4
	4	8.6×10^3	9.7×10^3	7.9×10^3

Control experiments were without any treatments.

Table 8
Effect of intestinal juice on the growth rate % of investigated yeasts. Data represents the means of standard deviation of triplicate assays.

Time (h) and Increase (%)	Yeast isolates growth (OD at 600 nm)		
	P. kudriavzevii QLB	*W. anomalus* HN1	*S. cerevisiae* gbLKX237673.1
0	0.043	0.052	0.031
1	0.069	0.081	0.051
Increase (%)	13.20	14.90	12.90
2	0.078	0.093	0.060
Increase (%)	40.60	61.50	33.30
3	0.024	0.073	0.045
Increase (%)	−44.0	−12.90	−45.20

to intestinal juice environment as explained by the 5.2 × 10^5 CFU ml^{-1} viability as well as the 61.5 % increase in growth rate after 2 h of incubation under these harsh conditions of intestinal environment (Tables 7 and 8). In early life, the colonization of the gut microbiota and its digestion are associated with the etiology of gastrointestinal conditions, for example, childish colic [39] and necrotizing entero-colitis [40].

3.5. Cell surface adhesion/hydrophobicity

As shown in Fig. 3, the three investigated yeast isolates adhesion to chloroform was the highest, followed by ethyl acetate and finally xylene indicating its capability to adhere to the cell surface with low kinetics elimination. Consequently, the three investigated yeasts can stick to the gastric mucosa and thus offering the prospective function as a potentially probiotic complement for human being and live-stock well-being. Our results are in consistently agreement with Ragavan and Das [17]. Consequently, results got represented in Table 9 concerning the hydrophobicity average percentage (%) of investigated yeasts to different solvents declared that the *W. anomalus* yeast isolate showed the lowest eradication from intestinal mucosa as indicated by the hydrophobicity average percentage (%) of 11.891 to xylene comparing to the *P. kudriavzevii* yeast isolate which showed the highest hydrophobicity average percentage (%) of 46.185 to Chloroform indicating the hydrophilic adhesion capacity to the intestinal mucosa. These results agreed with those obtained by Yang *et al.* [41] who found that some marine red yeast *Rhodosporidium paludigenum* can adhere to the intestinal mucosa of *Litopenaeus vannamei* and thus providing the credible application as a potentially probiotic supplement for human and animal health.

3.6. Antimicrobial activity and killer toxin productivity

One of the promising trends in the antimicrobial screening probiotic yeast protocols is the usage of produced antagonistic metabolites by the

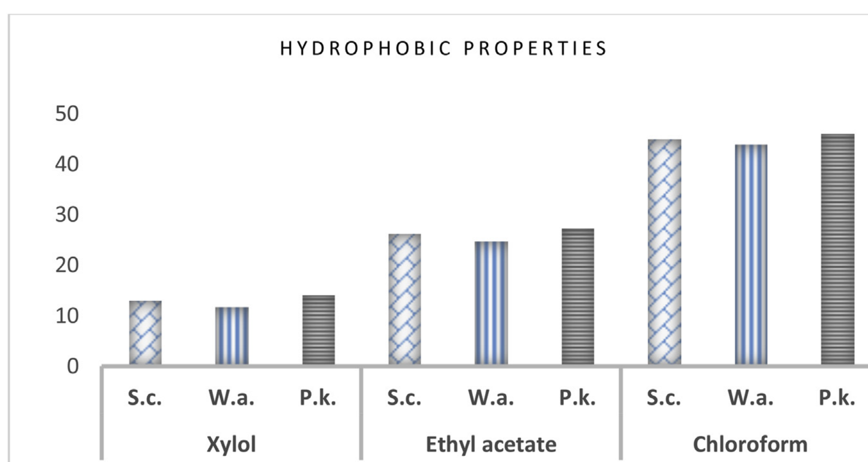

Fig. 3. Hydrophobic Behavior of Investigated Yeasts Towards Different Solvent Systems. S. c., *S. cerevisiae* gbLKX237673.1; W. a., *W. anomalus* HN1; P.k., *P. kudriavzevii* QLB.

Table 9
Hydrophobicity percentage (%) of investigated yeasts to different solvents.

Time (h)	Xylol			Ethyl acetate			Chloroform		
	S.c.	W.a.	P.k.	S.c.	W.a.	P.k.	S.c.	W.a.	P.k.
1	12.23	11.13	13.33	15.35	14.25	16.45	26.29	25.19	27.39
2	12.87	11.77	13.97	22.31	21.21	23.41	39.19	38.18	40.29
3	13.45	12.35	14.55	30.67	29.57	31.77	51.33	50.23	52.43
4	13.87	12.67	14.97	36.49	34.39	37.59	63.53	62.43	64.63
Average	13.105	11.891	14.205	26.205	24.855	27.305	45.085	44.075	46.185

S.c., *S. cerevisiae* gbLKX237673.1; W.a., *W. anomalus* HN1; P.k., *P. kudriavzevii* QLB.

Table 10
Antimicrobial activity of investigated yeasts against different clinical and food borne pathogens.

Yeast isolate	Inhibition Zone Diameter (cm) of Bacteria & Normal Flora						
	St. aureus	*B. Subtilis*	*E. coli*	*Sal. typhimurium*	*MRSA*	*Ps. aeruginosa*	*Ent. faecalis*
P. kudriavzevii	1.9	1.4	0.9	2.4	0.0	2.5	0.4
S. cerevisiae	2.1	1.9	0.3	2.1	0.0	2.1	0.2
W. anomalus	2.1	1.9	0.7	2.2	0.0	2.3	0.9
Yeast isolate	Inhibition Zone Diameter (cm) of Filamentous Fungi & Yeasts						
	A. niger	*A. flavus*	*A. fumigatus*	*P. marnefeii*	*C. albicans*	*C. lipolytica*	*[*]S. cerevisiae*
P. kudriavzevii	1.3	1.5	1.2	1.1	1.5	1.3	1.5
S. cerevisiae	0.8	0.0	0.7	0.0	0.0	0.9	1.2
W. anomalus	1.4	0.7	1.2	0.6	0.6	1.4	1.2

[*] Toxin-sensitive yeast.

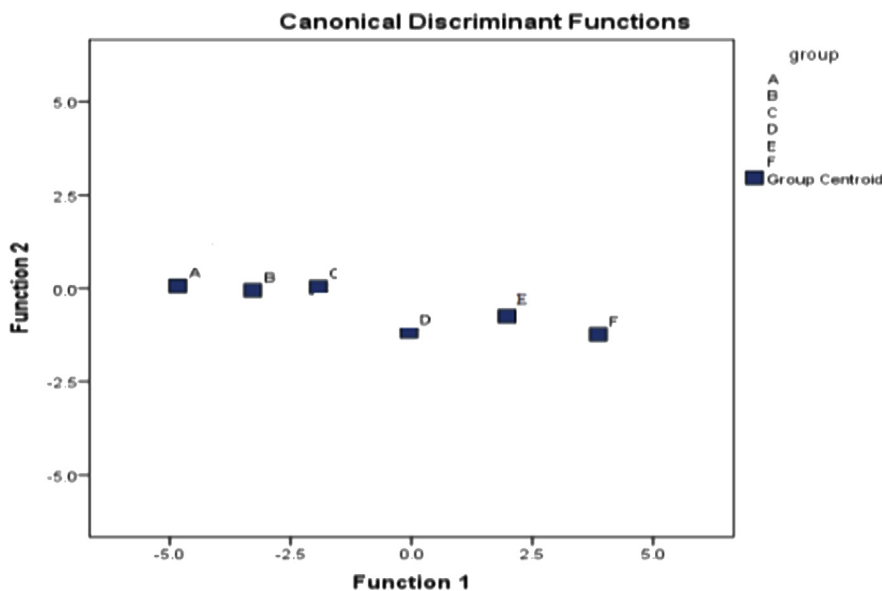

Fig. 4. Canonical Discriminant Function of viable population analyses represented by the growth rate % of investigated three yeasts as potential probiotics under all tested conditions. Where; the growth rate % of *P. kudriavzevii* QLB at zero time treatment (A) the growth rate % of *W. anomalus* HN1 at zero time treatment (B) the growth rate % of *S. cerevisiae* gbLKX237673.1 at zero time treatment (C) the growth rate % of *P. kudriavzevii* QLB at end time treatment (D) the growth rate % of *W. anomalus* HN1 at end time treatment (E) the growth rate % of *S. cerevisiae* gbLKX237673.1 at end time treatment (F).

tested yeast cells. Although this killer behavior of yeast strains has been thoroughly studied *in vitro*, our familiarity of the antagonistic specificity of killer impacts in environment stays restricted. In this study, three potentially probiotic yeasts were screened for their antagonistic activity against a toxin-sensitive strain of *S. cerevisiae*, some clinical and food borne pathogens of filamentous fungi, yeasts and bacteria (Table 10). Killer activity by some yeast against *C. albicans* was first reported by Middelbeek *et al.* [42]. According to the study of Banjara *et al.* [43], a cell-free mycocins preparation showed transient killer activity against *C. albicans* at 35 °C and a cheese sample containing a killer *D. hansenii* strain demonstrated sustained killer activity against both *C. albicans* and *C. tropicalis*. Together, these observations raise the possibility that *D. hansenii* could influence *Candida* populations in the gut. Among the most three frequently tested potentially probiotic yeast isolates, *P. kudriavzevii* QLB yeast isolate showed the highest antimicrobial activity against *S. typhimurium* and *P. aeruginosa* with 2.4 and 2.5 cm of growth clear zone diameter, respectively. While *W. anomalus* HN1 yeast isolate showed a moderate antimicrobial activity against most tested pathogenic fungi and bacteria as well as the toxin-sensitive strain of *S. cerevisiae* and normal flora of the intestine compared to the remaining investigated potentially probiotic yeast *S. cerevisiae* gbLKX237673.1. Coherently, the antifungal impact of the same yeast strain *P. kudriavzevii* QLB yeast isolate was proven by the study of Helmy [44] using it alone or in combination with biogenic selenium nano particlesas for achieving the antifungal, detoxification and/or anti-mycotoxigenic impact on Ochratoxin-A production. Canonical discriminate function of viable population analyses represented by the growth rate % of investigated three yeasts as potential probiotics under all tested conditions (Fig. 4) has been showed that the growth rate % of *W. anomalus* HN1 at end time treatment was the highest compared with *P. kudriavzevii* QLB and *S. cerevisiae* gbLKX237673.1 The two potential probiotic strains showed also higher significant amount of viability under the same stress conditions of investigation. However, the three control experiments with any treatment were showed almost constant growth rate % at the end of treatment for all the three investigated yeasts.

4. Conclusions

The three investigated yeasts isolated in this study exhibited advantageous potentially probiotic attributes having *in vitro* characteristics that make them promising candidates for dairy business. *W. anomalus* HN1 revealed interesting potentially probiotic properties such as excellent pH and biles tolerance, suppression of pathogen growth under *in vitro* conditions which suggest their possible use in the medical field and in food industry for the development of functional foods as novel additives for preserved food products. The results indicated that the antagonistic activity of these yeasts has a narrow or limited effect against normal flora of the intestine such as *E. coli* and *Enterococcus faecalis* indicating that the antagonistic activity of some species is strain-dependent. The moderate antimicrobial activity as well as the potential killer toxin productivity of the *W. anomalus* HN1 could also assume its application for biological control in agricultural and aquaculture.

Declarations

Author contribution statement

Helmy E. A.: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Soliman S.A.: Performed the experiments; Wrote the paper.

Tarek M. Abdel-Ghany: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Magdah Ganash: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Conflict of interest

The authors declare that they have no conflict of interest.

Additional information

No additional information is available for this paper.

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