Original article

Screening for the potential probiotic yeast strains from raw milk to assimilate cholesterol

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Abstract – Consumption of dairy products containing probiotics with cholesterol-lowering activity has been proposed as a means to lower serum cholesterol. In the present work, 19 yeast strains, isolated from raw milk, were tested to obtain potential probiotic yeasts for assimilating cholesterol. During in vitro tests, 17 yeast strains were capable of growth in bile salt solutions, and most of the yeast strains tolerated low pH, surviving in gastric juice. Among the 19 strains assessed, *Geotrichum* sp. BY2 and *Pichia kudriavzevii* BY10 showed highest adhesive ability to HT-29 cells. All yeast strains were able to assimilate cholesterol in the range of 3.6–44.4% over a 72 h incubation, and seven of the yeast strains were significantly higher at assimilating cholesterol ($P < 0.05$). According to these results, the yeast strains *P. fermentans* BY5, *P. kudriavzevii* BY10, *P. kudriavzevii* BY15 and *Yarrowia lipolytica* HY4 may serve as potential probiotics to assimilate cholesterol in the human intestine.

yeast / probiotic / raw milk / cholesterol

摘要 – 原料乳中降胆固醇益生酵母菌的筛选。食用含有降胆固醇益生菌的乳制品能降低人体血清胆醇。本文研究了 19 株来源于原料乳酵母菌潜在降胆固醇的益生菌特性。体外实验发现 17 株酵母菌能够在胆盐溶液中生长，大多数菌株能耐受低 pH 值，可以在胃液中存活。在 19 株菌中，*Geotrichum* sp. BY2 和 *Pichia kudriavzevii* BY10 对 HT-29 细胞有最强的黏附性。培养 72 h 后，所有菌株都能降低胆固醇含量，范围从 3.6 到44.4% 不等，其中有 7 株菌明显高于其它菌株 ($P < 0.05$)。实验结果表明酵母菌 *P. fermentans* BY5、*P. kudriavzevii* BY10、*P. kudriavzevii* BY15 和 *Yarrowia lipolytica* HY4 能作为潜在降胆固醇的益生菌株进行深入研究。

酵母 / 益生菌 / 原料乳 / 同化胆固醇

Résumé – Cribable du potentiel d'assimilation du cholestérol de souches probiotiques de levure isolées à partir de lait cru. La consommation de produits laitiers contenant des probiotiques ayant une activité hypo-cholestérolémiante a été proposée comme moyen d'abaisser le cholestérol sanguin. Dans la présente étude, 19 souches de levure isolées à partir de lait cru ont été testées comme probiotiques potentiels pour assimiler le cholestérol. Au cours des tests in vitro,

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17 souches étaient capables de croissance dans des solutions de sels biliaires, et la plupart des souches tolérait bien les faibles pH, en survivant dans le jus gastrique. Parmi les 19 souches testées, *Geotrichum* sp. BY2 et *Pichia kudriavzenii* BY10 montraient la plus grande capacité d'adhésion aux cellules HT-29. Toutes les souches de levure étaient capables d'assimiler le cholestérol à hauteur de 3,6 à 44,4 % après une incubation de 72 h et 7 souches avaient des taux d'assimilation significativement plus élevés (*P* < 0,05). D'après ces résultats, les souches de levure *Pichia fermentans* BY5, *P. kudriavzenii* BY10, *P. kudriavzenii* BY15 et *Yarrowia lipolytica* HY4 pourraient être utilisées comme probiotiques potentiels pour assimiler le cholestérol au niveau de l'intestin chez l'Homme.

levure / probiotique / lait cru / cholestérol

1. INTRODUCTION

Probiotics have been defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [7]. To benefit human health, probiotic microorganisms have been increasingly incorporated into food as dietary adjuncts. The usual microbial inhabitants of the intestinal tracts of human beings and other vertebrates are typically lactobacilli or bifidobacteria [1, 12]. The use of yeasts, as potential probiotics, has also been reviewed [9, 11]. It is well recognized that yeasts play a significant role in some cheeses and fermented milks [9, 19]. They have the ability to decrease the acidity and facilitate the growth of acido-sensitive bacterial biota by metabolizing lactic acid. In addition, they generate the precursors of aroma compounds, such as free amino acids and free fatty acids, which contribute significantly to the final flavor [8, 26]. Due to the positive microbial interaction that yeasts have with other species as well as their inhibitory effects against spoilage microorganisms in fermented milk, yeasts are worth studying for use as starter cultures for the development of new dairy products [28].

Several published studies have indicated that some yeast strains isolated from dairy products or human feces have been considered as potential probiotics, as they can survive in the low pH and bile environment of the gastrointestinal tract [11, 14, 20, 21]. It has been reported that *Saccharomyces boulardii* was effective as a biotherapeutic agent in the prevention or treatment of antibiotic-associated diarrhea and human colitis, especially for the treatment of acute diarrhea in children [25]. Recently, many researchers have focused on the antioxidant ability and cholesterol assimilation of yeasts [12, 16, 17, 21]. Some reports indicated that yeasts can remove cholesterol in vitro [21]. However, except for *S. cerevisiae*, *Kluyveromyces lactis* and *Pichia kudriavzevii* (former name: *Issatchienka orientalis*), a limited number of yeasts have been investigated for their ability to assimilate cholesterol [21]. Milk is a suitable growth medium for yeast and some cheeses, such as Cantal cheese in France and Toma Piemontese cheese in Italy, are made from raw milk. Yeast strains, originally isolated from raw milk, impart flavor characteristics and health-promoting properties to the cheese. Therefore, raw milk may be an excellent origin for some probiotic yeasts. The aim of the present work was to identify novel potential probiotic yeasts isolated from raw milk by testing their probiotic potential for assimilating cholesterol.

2. MATERIALS AND METHODS

2.1. Yeast isolates and growth media

Yeasts were isolated from the raw milk of healthy cattle from various farmhouses in Beijing and Heilongjiang province in China. The somatic cell counts in the raw milk were lower than 500 000. Three samples were

collected from each region in autumn, winter and summer. All strains were obtained from fresh raw milk or the retentate from a 1.4 μm MF membrane (Type MSF 1, Tetra Alcross®, Tetra Pak, Denmark) [23]. Strains were identified using an integrated approach that included phenotypic (morphological, biochemical and physiological characterization) and genotypic (RAPD-PCR, sequencing of the D1/D2 domain of 26S rDNA encoding gene) methods as well as a commercial API system (API 20C Aux) [3]. The control strain *S. cerevisiae* var. *boulardii* (CICC1850) was purchased from the China Center of Industrial Culture Collection (CICC).

Yeast strains were cultured at 25 °C for 24 h in yeast-peptone-dextrose (YPD) broth containing 0.5% (w/v) yeast extract, 1% peptone and 2% glucose. YPD agar (0.5% (w/v) yeast extract, 1% peptone, 0.5% glucose and 1.8% agar) was used to maintain the cultures. Prior to the experiment, the cultures were activated for 72 h at 25 °C and subcultured at least three times. The total viable cells were adjusted to about 10^8 cfu·mL^{-1}.

2.2. Bile tolerance

Freshly prepared active yeast cells (1% v/v) were added into YPD broth with different concentrations (0.1%, 0.3% and 0.5% w/v) of bile salts (Sigma Chemical Co.). The bile tolerance of strains was evaluated based on the decimal increase of yeasts by estimating the number of viable cells after 0 and 72 h of incubation on YPD agar plates [20]. The bile tolerance was expressed as a percentage (%) = (increment of cells in broth with bile/increment of cells in broth without bile) × 100.

2.3. Growth at low pH

The growth at low pH was evaluated by inoculating (1% v/v) activated strains into YPD tubes adjusted to pH 1.5, 2.0, 3.0 and 5.0 with 0.1 mol·L^{-1} HCl and 3 mol·L^{-1} NaOH. The tubes were maintained at 37 °C and growth was determined visually after five days.

2.4. In vitro survival in gastric juice

To determine the transit tolerance of yeast strains through simulated gastric juice, the method of Psomas was used with slight modifications [20]. Thirty milliliters of the selected yeast strains were centrifuged at 3000× *g* (10 min, 4 °C). The cell pellet was washed with sterile phosphate-buffered saline (PBS; 0.1 mol·L^{-1}, pH 7.2) and 0.8% NaCl, and re-suspended in the culture at a ratio of 1:9. Then, 0.1 mL of the suspension was added to 1.0 mL simulated gastric juice that was made up of a solution of pepsin (0.3% w/v; 1000 U·g^{-1}, Amresco, USA) and NaCl (0.5% w/v), to achieve pH 1.2. The yeast cells were enumerated on YPD agar plates after 0 and 90 min of incubation at 37 °C. The results were expressed as the decrease in viability % = $[(\text{cfu·mL}_{0\,h}^{-1} - \text{cfu·mL}_{1.5\,h}^{-1})/\text{cfu·mL}_{0\,h}^{-1}] \times 100$.

2.5. Adhesion assay

The adhesion ability of the strains to human cells was investigated using HT-29 cells that were routinely grown at 37 °C in a 5% CO$_2$–95% air atmosphere in PRMI 1640 Medium (Gibco-BRL, USA). L-glutamine with 10% inactivated (30 min, 56 °C) fetal calf serum (FCS, Sigma), 100 U·mL^{-1} penicillin and 0.1 mg·mL^{-1} streptomycin was added to the culture. The method of Delgado et al. [6] was used with some modifications. Monolayers of the HT-29 cells were prepared in six-well tissue culture plates (Corning Inco., USA) and then incubated with ~ 1 × 10^7 cfu·mL^{-1}of the strains to be tested. After 2 h of incubation at 37 °C in 5% CO$_2$–95% air, monolayers were washed four times with sterile PBS, fixed with 10% formaldehyde solution and stained with Giemsa solution, followed

by microscopic examination under oil immersion. The number of adherent yeast and HT-29 cells was counted in 20 random microscopic areas. Adhesion capacity of the strains was expressed as the number of yeast cells adhering to 100 HT-29 cells.

2.6. Assimilation of cholesterol

Activated yeast cells (1%) were inoculated in 12 mL of YPD broth containing 224.2 μg·mL^{-1} of cholesterol (Sigma Chemical Co.) micelles (YPD-CHOL) prepared according to the method of Razin et al. [22]. The mixture was supplemented with 0.3% bile salt and incubated at 37 °C for 12–72 h. All experiments were carried out at 37 °C in order to simulate the temperature of the human gastrointestinal tract. The cells were harvested by centrifugation (12 000× g, 15 min, 4 °C), and 1.0 mL supernatant fluid was prepared for further analysis of residual cholesterol. Meantime, the pellet was re-suspended in 12 mL sterile distilled water, and 1.0 mL of suspension was used to analyze the cholesterol content by GC-MS. To investigate the mechanism of cholesterol assimilation of yeasts, each pellet sample was mixed with 2 mL distilled water and 0.3 g acid-washed glass beads (0.5 mm diameter). The mixture was then homogenized three times in a bead beater for 30 s at ambient temperature and maximum speed. The mixture was centrifuged as mentioned above, and the suspension was analyzed.

Cholesterol in the samples was detected according to the method developed by Fletouris et al. [10]. An aliquot (1.0 mL) of the sample and 5.0 mL of methanolic KOH solution (0.5 mol·L^{-1}) were added into a tightly capped sample tube and vortexed for 15 s, and then kept in an 80 °C water bath. During heating, the tube was blended for a quick 10 s every 5 min. After 15 min, the sample was cooled, and 1 mL water and 5 mL hexane were added. The contents were blended vigorously for an additional 1 min. An aliquot of the upper phase was transferred into the autosampler vial and analyzed by a GC/MS system (Model 6890N; Agilent Tech., New York). Analysis was carried out on a gas chromatograph equipped with a flame ionization detector, an automatic sampler (Model AS3000) and a chromatography data system (Model Class-VP). A fused silica capillary column (30 m × 0.32 mm i.d. × 0.25 μm) (Hp-413, Hewlett-Packard Company, USA) was used in this work. The oven temperature was set at 285 °C, injection port temperature at 300 °C and flame ionization detector temperature at 300 °C. The flow rates were 2.0 mL·min^{-1} for helium, 30 mL·min^{-1} for hydrogen and 300 mL·min^{-1} for air. The injection volume was 1 μL with a split ratio of 20:1. Mass spectrometer conditions were as follows: interface temperature 220 °C, ion source temperature 200 °C and quadrupole temperature 100 °C. Acquisition was performed in electron impact mode (70 eV) with 2.0 min solvent delay.

A 7-point calibration curve was generated by injecting 1 μL from each working standard solution of cholesterol (purity > 99.5%, Sigma Co.), plotting the recorded peak area versus the corresponding mass of the injected analyte. The content of cholesterol was computed from the standard curve.

2.7. Statistical analysis

Data analyses were carried out with SPSS software (version 12.0; SPSS Inc., Chicago, USA). One-way analysis of variance was used to identify significant differences between means, with the significance level at $\alpha = 0.05$. Duncan's test was used to perform multiple comparisons between means. All assays were repeated in triplicate.

Table I. Yeast strains used in this study.

Strains	Species name	Accession no.	Base pair[1]
BY1	*Galactomyces* sp.	FJ219589	2 (539)
BY10	*P. kudriavzevii*	FJ219593	0 (373)
BY15	*P. kudriavzevii*	FJ357144	0 (573)
BY31	*P. guilliermondii*	FJ219594	0 (160)
HY1	*P. guilliermondii*	Identical to BY31	–
HY18	*P. guilliermondii*	Identical to BY31	–
BY6	*Trichosporon* sp.	FJ219592	9 (585)
HY4	*Y. lipolytica*	FJ357148	9 (500)
HY15	*Y. lipolytica*	FJ357147	3 (512)
HJ6	*Y. lipolytica*	FJ219597	0 (651)
HJ8	*Y. lipolytica*	Identical to HJ6	–
HJ10	*Y. lipolytica*	FJ357146	4 (508)
BY2	*Geotrichum* sp.	FJ219590	3 (563)
BY5	*P. fermentans*	FJ219591	3 (561)
HJ15	*P. fermentans*	FJ219598	0 (529)
HJ16	*P. fermentans*	Identical to HJ15	–
HJ22	*R. mucilaginosa*	FJ219600	0 (371)
HJ1	*T. gracile*	FJ219596	4 (588)
HJ2	*T. gracile*	Identical to HJ1	–
CICC1850	*S. boulardii*	Reference culture	–

[1] Number of base pair difference with the corresponding type strain and the overall length of the match are shown in parentheses.

3. RESULTS AND DISCUSSION

3.1. Effects of bile salt on viability

A set of 19 strains isolated from raw milk from Beijing and Heilongjiang province in China were studied in this work (Tab. I). According to the suggestion of FAO/WHO, it is mandatory to perform a preliminary in vitro assessment before assessing the probiotic properties of bacterial strains [7], such as the capacity for bile tolerance to ensure survival during passage through the gastrointestinal tract [15, 27]. Regarding the ability of yeasts to withstand bile salts, all yeast strains were incubated in bile salt solutions of 0.1%, 0.3% and 0.5% for 72 h, respectively. As shown in Table II, the growth of most strains was inhibited by the bile salt solutions, but four

strains, *P. guilliermondii* HY18, *Trichosporon gracile* HJ2, *Geotrichum* sp. BY2 and *Yarrowia lipolytica* HY4, showed good bile resistance, even at a concentration of 0.5%. In the presence of 0.3% bile, 17 strains showed growth, with the exception of *Galactomyces* sp. BY1 and *T. gracile* HJ1. Among the 17 strains, only the growth of *T. gracile* HJ2 was not affected by the addition of bile salt as the control.

3.2. Effects of low pH and gastric juice on the viability

Another critical factor influencing the viability of microorganisms in the stomach is the presence of gastric acid. The degree of inhibition is related to pH, which can vary widely among individuals. It is an important selection criterion that probiotic

Table II. Effect of bile salt concentration on the growth of selected yeast strains cultured in YPD at 25 °C after 72 h incubation.

Strains	Increment[1] in the presence of bile salt % (w/v)[2]		
	0.1	0.3	0.5
Galactomyces sp.			
BY1	–	–	–
Geotrichum sp.			
BY2	43.9 ± 1.3	45.0 ± 3.0	55.3 ± 4.1
P. fermentans			
BY5	30.9 ± 2.1	10.3 ± 1.3	9.5 ± 2.3
HJ15	56.6 ± 2.7	79.0 ± 2.5	9.2 ± 3.7
HJ16	15.5 ± 2.3	46.5 ± 1.6	54.9 ± 2.4
Trichosporon sp.			
BY6	12.4 ± 3.6	10.7 ± 0.6	8.7 ± 0.4
P. kudriavzevii			
BY10	60.8 ± 1.2	25.9 ± 1.2	16.1 ± 2.5
BY15	41.8 ± 2.4	21.9 ± 2.3	18.9 ± 3.1
P. guilliermondii			
BY31	15.6 ± 0.3	59.3 ± 2.6	81.4 ± 2.7
HY1	24.4 ± 2.4	35.2 ± 2.0	21.9 ± 1.4
HY18	92.8 ± 1.4	90.5 ± 5.0	100.0
R. mucilaginosa			
HJ22	8.5 ± 2.6	15.8 ± 1.8	100.0
Y. lipolytica			
HY4	13.7 ± 2.6	12.7 ± 1.8	15.9 ± 1.0
HY15	11.7 ± 3.8	18.1 ± 3.3	1.2 ± 0.4
HJ6	64.6 ± 2.4	62.9 ± 2.4	56.6 ± 1.0
HJ8	15.7 ± 3.2	36.8 ± 4.3	43.3 ± 2.9
HJ10	11.9 ± 0.2	11.9 ± 0.8	4.8 ± 1.3
T. gracile			
HJ1	–	–	–
HJ2	100.0	100.0	100.0
S. boulardii			
CICC1850	100.0	100.0	100.0

[1] Bile tolerance (%) = (increment of cells in broth with bile/increment of cells in broth without bile) × 100; – means cannot tolerate bile; and 100% means that the growth rate of yeasts is not affected by the added bile salt at all.
[2] All results are expressed as mean ± standard deviation.

microorganisms can transit the human gastric environment. In this work, the abilities of yeast strains to endure low pH (1.5, 2.0, 3.0 and 5.0) were investigated. All yeast strains survived at pH 3.0 and 5.0 after five days of incubation. However, at pH 1.5 and 2.0 only *P. kudriavzevii* BY10, *Y. lipolytica* HJ6 and *Y. lipolytica* HY15 displayed viability (data not shown).

It has been reported that the abilities of probiotic bacteria to survive transit through the gastrointestinal tract are variable and strain dependent. The yeast strains displayed variability in surviving in simulated

Table III. In vitro survival of selected yeast strains at 37 °C in simulated gastric juice.

Strains	Viable counts $(\log_{10}$ cfu·mL$^{-1})$[2]		
	0 h	1.5 h	Decrease in viability (%)[1]
Galactomyces sp.			
BY1	7.02 ± 0.03	6.65 ± 0.07	57.12 ± 9.30[ef]
Geotrichum sp.			
BY2	7.93 ± 0.04	7.94 ± 0.03	0.00[a]
P. fermentans			
BY5	7.48 ± 0.01	6.40 ± 0.03	91.81 ± 4.67[ghi]
HJ15	7.58 ± 0.03	7.36 ± 0.05	39.52 ± 1.44[cde]
HJ16	7.56 ± 0.04	7.27 ± 0.01	48.56 ± 1.79[def]
Trichosporon sp.			
BY6	7.98 ± 0.03	7.13 ± 0.03	85.83 ± 3.32[ghi]
P. kudriavzevii			
BY10	8.27 ± 0.03	8.36 ± 0.02	0.00[a]
BY15	8.29 ± 0.01	8.21 ± 0.02	16.71 ± 0.30[b]
P. guilliermondii			
BY31	8.34 ± 0.03	8.17 ± 0.01	35.11 ± 1.58[cd]
HY1	7.88 ± 0.04	7.00 ± 0.01	87.12 ± 1.69[ghi]
HY18	7.18 ± 0.03	6.54 ± 0.08	76.78 ± 4.04[gh]
R. mucilaginosa			
HJ22	7.30 ± 0.06	7.10 ± 0.01	37.03 ± 1.41[cd]
Y. lipolytica			
HY4	8.02 ± 0.09	7.75 ± 0.04	46.21 ± 2.53[def]
HY15	8.36 ± 0.05	7.97 ± 0.07	59.15 ± 1.27[ef]
HJ6	6.72 ± 0.09	5.76 ± 0.01	89.18 ± 4.48[ghi]
HJ8	7.93 ± 0.04	6.39 ± 0.12	97.17 ± 0.35[i]
HJ10	7.62 ± 0.01	7.41 ± 0.02	36.72 ± 2.19[cd]
T. gracile			
HJ1	7.51 ± 0.05	6.09 ± 0.12	96.21 ± 0.45[hi]
HJ2	7.11 ± 0.05	6.47 ± 0.16	77.32 ± 3.27[g]
S. boulardii			
CICC1850	7.44 ± 0.03	7.32 ± 0.01	24.12 ± 5.37[b]

[1] Decrease in viability % = $[(\text{cfu·mL}_{0\,h}^{-1} - \text{cfu·mL}_{1.5\,h}^{-1})/\text{cfu·mL}_{0\,h}^{-1}] \times 100$.
[2] All results are expressed as mean ± standard deviation.
[a–i] Means with the same letter within a column are not significantly different ($P > 0.05$).

gastric juice at pH 1.2 (Tab. III). After incubation in the simulated gastric juice for 1.5 h, cell counts of all yeast strains were reduced by 16.7–97.1% with the exception of *Geotrichum* sp. BY2 and *P. kudriavzevii* BY10 which continued to increase in the juice. Under the conditions used in this study, strains *P. kudriavzevii* BY10 and *P. kudriavzevii* BY15 (which decreased by 16.7%) exhibited strong resistance to gastric juice, in a manner similar to a strain of *P. kudriavzevii* isolated from infant faces previously reported by Psomas et al. [21]. In comparison to the control strain, there was no significant difference between the control and *P. kudriavzevii* BY15 ($P > 0.05$). Gastric juice resistance of *Geotrichum* sp. BY2 and *P. kudriavzevii* BY10
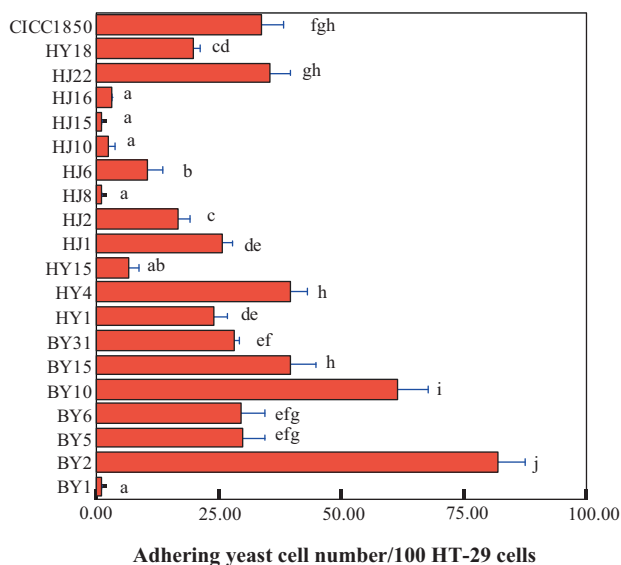
Figure 1. Adhesion to HT-29 cells of yeast strains isolated from raw milk. Number of yeast adhering to the cell monolayer per 100 cells is shown. See Table I for the names of species. CICC1850: the control strain. Standard deviation is shown. [a–j] Values with the same letters are not significantly different ($P < 0.05$).

was significantly higher than the control CICC1850 ($P < 0.05$), while the other isolates were significantly lower than the control ($P < 0.05$).

3.3. Adhesion ability to HT-29 cells

Adhesion of probiotic bacteria to the intestinal mucosa is an effective way to exclude pathogens and undesirable bacteria [2, 12]. Figure 1 shows the number of yeast cells that adhered to 100 HT-29 cells. The adhesion ability of BY2 (80 cells/ 100 HT-29 cells) was highest followed by BY10 (62 cells/100 HT-29 cells) compared to all other strains ($P < 0.05$) tested. The adhesion abilities of both strains were significantly higher than the control (CICC1850) ($P < 0.05$). Strains BY15, HY4, HJ22, BY31, BY5 and BY6 had similar adhesion abilities to the control strain. In contrast to the strains referred to above, the

yeasts *P. guilliermondii* HY1 and *T. gracile* HJ1 showed lower adhesion ability; with the other yeasts, including BY1, HJ10, HJ15 and HJ8, being poorly adhesive to HT-29 cells, at < 5 cells/100 HT-29 cells. The results indicated that the adhesion ability of yeasts to HT-29 cells was lower than those of bifidobacterium strains (e.g. *B. breve* ATCC 15698 and *B. longum* BB536) [5]. It may be considered that a single yeast cell is larger than a bacterium cell and hence requires a larger surface with which to adhere to the surface of an intestinal cell than does a bacterium. The adhesive values of HT-29 cells ranging from 2 to 80 were similar to values reported by Kumura et al. [12] regarding yeast adhesion to Caco-2 cells.

To date, there is no selection criterion for the screening of probiotic yeasts for adhesion abilities. However, in vitro experiments have confirmed that the adhesion of
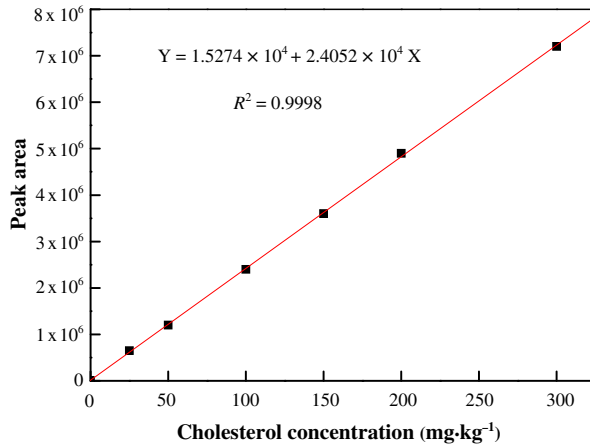
Figure 2. Calibration curve of the cholesterol concentration and peak area.

probiotics can inhibit the adhesion of pathogenic *E. coli*, *Salmonella* and other pathogens due to space hindrance [12]. Besides, adhesion is related to the proteins expressed on the surface of the cell walls combined with the cell surface glycolipids of the intestinal bacterial polysaccharide components which are located on the surface of microorganisms and can play an important role in affecting mucosal immune modulation [12]. Furthermore, the doubling time will undoubtedly be extended if a probiotic strain can adhere to the intestinal wall, while nonadhesive microorganisms only remain in the small intestine for several hours [4]. Simply put, the higher the adhesion ability of a strain the greater the chance of protecting the host. Consequently, strains with high adhesive abilities, such as *Geotrichum* sp. BY2, *P. kudriavzevii* BY10, *P. kudriavzevii* BY15, *Rhodotorula mucilaginosa* HJ22 and *Y. lipolytica* HY4, are the first to be selected as probiotics.

3.4. Ability to assimilate cholesterol

High levels of serum cholesterol have been associated with the risk of coronary heart disease, and the use of probiotic bacteria to reduce serum cholesterol levels has attracted much attention [13]. *S. boulardii*, *P. kudriavzevii* and *S. cerevisiae* have previously been evaluated as potential probiotics for assimilating cholesterol over the past few years [21]. According to the cholesterol concentration standard curve (Fig. 2), the abilities of 19 yeasts to assimilate cholesterol in the presence of bile salts were calculated as shown in Table IV. All yeast strains could assimilate cholesterol from YPD-CHOL broth containing bile salt and cholesterol after 72 h growth at 37 °C. After 12 h, seven yeast strains (BY1, BY2, BY5, BY10, BY15, BY31 and HJ10) had cholesterol assimilation abilities ranging from 2.3% to 17.5%. After 24 h, 10 strains had cholesterol assimilating abilities that ranged from 6.7% to 27%. Among the 10 yeast strains, *P. kudriavzevii* BY10 had the highest cholesterol assimilation ability. After 48 h incubation, *P. kudriavzevii* BY15 and *P. guilliermondii* BY31 displayed the best cholesterol assimilation ability of 44.3–45.6%. After 72 h incubation, seven strains, namely *Galactomyces* sp. BY1, *P. fermentans* BY5, *P. kudriavzevii* BY10, *P. kudriavzevii* BY15, *P. guilliermondii* BY31, *Y. lipolytica* HY4 and *Y. lipolytica*

Table IV. Cholesterol assimilation of various yeast strains inoculated in YPD broth, supplemented with bile salt (0.3%) and cholesterol (224.2 $\mu g \cdot mL^{-1}$), and incubated at 37 °C from 12 to 72 h.

Strains[1]	Cholesterol assimilation (%)[2]			
	12 h	24 h	48 h	72 h
BY1	7.5 ± 0.8^{de}	23.1 ± 1.9^{f}	36.3 ± 1.1^{de}	44.0 ± 2.4^{d}
BY2	2.3 ± 1.6^{b}	7.0 ± 0.4^{b}	16.7 ± 1.6^{b}	23.8 ± 1.5^{c}
BY5	5.7 ± 1.5^{cde}	17.9 ± 2.0^{e}	35.6 ± 1.8^{d}	40.3 ± 2.1^{d}
BY6	0.0^{a}	6.8 ± 0.4^{b}	17.5 ± 0.3^{b}	23.5 ± 2.3^{c}
BY10	17.5 ± 1.3^{f}	27.0 ± 2.6^{g}	37.4 ± 2.8^{f}	43.2 ± 1.9^{d}
BY15	6.2 ± 0.1^{c}	13.1 ± 1.1^{c}	45.7 ± 2.6^{g}	44.4 ± 3.0^{d}
BY31	8.7 ± 0.4^{e}	13.6 ± 1.3^{c}	44.4 ± 2.4^{g}	44.4 ± 2.3^{d}
HY1	0.0^{a}	0.0^{a}	0.0^{a}	5.6 ± 0.2^{ab}
HY4	0.0^{a}	13.7 ± 2.1^{c}	37.4 ± 1.8^{f}	39.8 ± 2.3^{d}
HY15	0.0^{a}	0.0^{a}	0.0^{a}	7.2 ± 1.1^{b}
HJ1	0.0^{a}	0.0^{a}	0.0^{a}	6.5 ± 0.2^{ab}
HJ8	0.0^{a}	0.0^{a}	0.0^{a}	3.6 ± 0.3^{a}
HJ2	0.0^{a}	0.0^{a}	0.0^{a}	6.2 ± 0.5^{ab}
HJ6	0.0^{a}	0.0^{a}	0.0^{a}	5.5 ± 0.4^{ab}
HJ10	6.2 ± 1.8^{cde}	22.3 ± 1.6^{f}	30.9 ± 1.1^{c}	45.5 ± 2.3^{d}
HJ15	0.0^{a}	0.0^{a}	0.0^{a}	4.6 ± 0.3^{ab}
HJ16	0.0^{a}	0.0^{a}	0.0^{a}	6.8 ± 1.1^{ab}
HJ22	0.0^{a}	0.0^{a}	0.0^{a}	5.4 ± 0.4^{ab}
HY18	0.0^{a}	0.0^{a}	0.0^{a}	3.7 ± 0.2^{ab}
CICC1850	5.2 ± 1.5^{cd}	16.8 ± 2.1^{d}	36.5 ± 1.4^{e}	41.5 ± 2.3^{d}

[1] The names of species are given in Table I.
[2] Results are expressed as mean ± standard deviation.
[a–g] Means within a column with the same letter are not significantly different ($P > 0.05$).

HJ10, showed high cholesterol assimilation abilities ranging from 39.8% to 45.5%, which were not significantly different ($P > 0.05$) than the control.

In vitro, significant differences were detected between yeast strains and incubation time ($P < 0.05$) for the cholesterol assimilation ability. In particular, a significant correlation was detected between the assimilation ability and the incubation time at $\alpha = 0.01$ level ($R^2 = 0.458$). Due to their strain specificity, *P. kudriavzevii* BY10 and *P. kudriavzevii* BY15 showed lower cholesterol assimilating abilities (43.2% and 44.4%) after 72 h incubation compared to that of *P. kudriavzevii* KK5.Y.1 (93.6%) reported by Psomas et al. [21]. However, strains *P. kudriavzevii* BY5 and *P. kudriavzevii* BY15

were capable of assimilating cholesterol after 12 h incubation in contrast to KK5.Y.1.

For lactobacilli, three possible mechanisms for the assimilation of cholesterol from media are proposed: assimilation of cholesterol during growth, incorporation of cholesterol into the membranes of the cells and binding cholesterol to the cell's surface [13]. In the present study, the mechanism of cholesterol reduction during growth was investigated using mass spectroscopy to detect the product formed by the metabolism of cholesterol by yeasts during growth. No metabolic products other than cholesterol were detected in the re-suspension, and only a little cholesterol was found in pellets homogenized in a bead beater (data not shown). Thus, the cholesterol removed

by the yeast strains tested was considered not to be metabolically degraded but absorbed by the growing yeast cells. This might be attributed to cell wall lysis by lysozymes [21]. As a result, yeast cells inhibit the absorption of cholesterol by the body.

It is well known that significant numbers of cells need to survive and grow in the bile of the human intestinal tract to influence the microbial balance of the intestine. As a result, *Galactomyces* sp. BY1 is not suitable to be used as a probiotic due to its poor tolerance to bile. *Y. lipolytica* HJ10 could not be chosen as a potential probiotic because of its low adhesion ability, although it has similar cholesterol assimilation ability as the control. In addition, *P. guilliermondii* has rarely been reported for use in the fermented milk products. Considering food safety, *P. guilliermondii* BY31 is not suitable for use in the dairy industry as a probiotic strain, even though it had good cholesterol assimilation ability. From the above-mentioned results, *P. kudriavzevii* BY10 and *P. kudriavzevii* BY15 are considered as ideal probiotic candidates. They have been isolated from human infant feces [21] and from cheese [24] which will accelerate their promising use as probiotics. Also, *P. fermentans* and *Y. lipolytica* have also been frequently isolated from cheese and kefir [18, 24]. Therefore, *P. fermentans* BY5 and *Y. lipolytica* HY4 could also be considered as potential probiotic yeasts for their good cholesterol assimilation ability, adhesive ability and better survival in simulated gastric acid.

4. CONCLUSIONS

Based on the tolerance to bile salt, survival under low pH and gastric juice and the assimilation of cholesterol, four yeast strains (*P. fermentans* BY5, *P. kudriavzevii* BY10, *P. kudriavzevii* BY15 and *Y. lipolytica* HY4) were selected as potential probiotics to assimilate cholesterol in the human intestine.

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