การศึกษาคุณสมบัติโพรไบโอติกและฤทธิ์การทำงานของยีสต์ที่แยกได้เพื่อใช้เป็นอาหารเสริมในสัตว์

Study of Probiotic Properties and Functional Activities of Yeasts Isolated for Feed Supplement

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บทคัดย่อ

การศึกษาคุณสมบัติความเป็นโพรไบโอติกของยีสต์จำนวน 18 ไอโซเลท ซึ่งคัดแยกได้จากข้าวหมักและตัวอย่างต่าง ๆ ในธรรมชาติ พบว่าทุกสายพันธุ์ไม่ย่อยเม็ดเลือดแดงและอยู่รอดได้ในสภาวะคล้ายน้ำย่อยในระบบทางเดินอาหารที่มีความเป็นกรดและเกลือน้ำดีที่อุณหภูมิ 37 องศาเซลเซียส สายพันธุ์ต่าง ๆ ดังกล่าวถูกพิสูจน์เอกลักษณ์ได้ 7 สายพันธุ์ ได้แก่ *Meyerozyma caribbica Saccharomyces cerevisiae Pichia kudriavzevii Starmerella sorbosivorans Kodamaea ohmeri Ambrosiozyma kamigamensis* และ *Metschnikowia koreensis* โดยทุกสายพันธุ์มีความสามารถในการยึดเกาะเซลล์เยื่อบุผนังลำไส้ได้ดี ไม่มีคุณสมบัติในการดื้อต่อยาปฏิชีวนะที่ใช้ในการยับยั้งการเจริญของกลุ่มเชื้อราและยีสต์ จึงยอมรับได้ว่าเป็นสายพันธุ์ที่มีความปลอดภัย ที่สำคัญมีเพียง 2 สายพันธุ์ คือ *M. caribbica* TISTR 6026 และ *M. koreensis* TISTR 6044 ที่สามารถสร้างไซลาเนส (xylanase) นอกจากนี้มีเชื้อยีสต์ 4 สายพันธุ์ คือ *M. caribbica* TISTR 6017 *S. cerevisiae* TISTR 5104 *S. cerevisiae* TISTR 5328 และ *P. kudriavzevii* TISTR 6028 ที่สามารถยับยั้งการเจริญของเชื้อแบคทีเรียก่อโรคที่ใช้ทดสอบได้ จึงถูกนำไปศึกษาคุณสมบัติในการปรับระดับภูมิคุ้มกัน โดยตรวจสอบการกระตุ้นให้เกิดกิจกรรมฟาโกไซโตซิส การสร้างไนตริกออกไซด์ และการยับยั้งการสร้าง

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ไซโตไคน์ชนิด TNF-α และ IL-6 ดังนั้นจากผลการทดลองดังกล่าวได้แสดงถึงความสำคัญของยีสต์ทั้ง 4 สายพันธุ์ที่มีคุณสมบัติโพรไบโอติกช่วยปรับระบบภูมิคุ้มกันและจัดการกับเชื้อโรคได้ดี

คำสำคัญ: ยีสต์ โพรไบโอติก ไซลาเนส การปรับสมดุลระบบภูมิคุ้มกัน อาหารเสริมสำหรับสัตว์

Abstract

Eighteen isolated yeasts from rice and natural sources are characterized as probiotics. All strains, at 37°C, lack hemolysis and withstand simulated gastrointestinal conditions and bile salts. Strains were identified as *Meyerozyma caribbica*, *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, *Starmerella sorbosivorans*, *Kodamaea ohmeri*, *Ambrosiozyma kamigamensis*, and *Metschnikowia koreensis*. Strong adherence to intestinal cells was noted, plus sensitivity to certain antifungals, confirming safety. Notably, *M. caribbica* TISTR 6026 and *M. koreensis* TISTR 6044 demonstrated xylanase activity. Also, *M. caribbica* TISTR 6017, *S. cerevisiae* TISTR 5104, TISTR 5328, and *P. kudriavzevii* TISTR 6028 exhibited antimicrobial action against pathogens. These four strains showed immunomodulatory potential, impacting phagocytosis, nitric oxide, TNF-α, and IL-6. These findings underscore their promise as versatile probiotics for immunomodulation and addressing pathogens, showcasing their broad potential.

Keywords: Yeast, Probiotic, Xylanase, Immunomodulation, Feed supplement

Introduction

In recent times, the European Union has prohibited the utilization of antibiotic feed additives. This regulatory measure stems from concerns regarding the emergence of antibiotic resistance in pathogenic microorganisms, the presence of chemical residues in animal-derived products, and the release of antibiotics into the environment (Retta, 2016). Consequently, as a viable alternative to antibiotics, the application of probiotic additives has gained prominence in efforts to enhance animal health and productivity. Among ruminant animals, including cattle, sheep, and goats, the process of microbial degradation of ingested feed is pivotal. These microorganisms provide essential components such as proteins, vitamins, and short-chain organic acids to the host animals, augmenting their nutrition through intricate fermentation processes (Pinloche *et al.*, 2013). Probiotics, constituting live microorganisms, hold the potential to restore equilibrium within the intestinal microflora. In ruminants, commonly used strains of probiotics encompass lactic acid bacteria and yeast. The integration of probiotics within the rumen ecosystem yields significant implications for animal health and performance.

As mentioned earlier, yeasts can prevent pathogenic infection. In intricate ecosystems, microorganisms typically develop strategies to secure access to nutrients and space, thereby ensuring their survival. These strategies typically involve inhibiting other coexisting microorganisms within the same ecosystem. This inhibition is achieved through several mechanisms, including pH changes, release of Inhibitory metabolites, direct nutrient competition, and production of killer toxins (i.e., mycocins). Mycocins can interfere with cell division by inhibiting DNA synthesis, cleaving tRNA, blocking calcium uptake, hydrolyzing β-glucans, disrupting plasma membrane permeability, and causing ion leakage (Gil-Rodríguez and Garcia-Gutierrez, 2021). This enhancement subsequently manifests in a range of multifaceted benefits, including heightened nutrient intake, improved nutrient digestibility, enhanced growth rates, augmented milk yield and composition, elevated meat production, attenuation of pathogenic load, fortification of immune responses, stabilization of ruminal pH, and acceleration of the establishment of cellulolytic populations within the rumen (Retta, 2016).

The present study explores these promising avenues further by subjecting 18 strains of probiotic yeasts to rigorous screening and comprehensive characterization, thus contributing to our understanding of their functional properties and potential applications.

Materials and Experiment

1. Isolation and identification

Samples from northeastern Thailand were collected and stored at 4°C. Ten-gram samples were suspended in 90 milliliters (ml) of 0.1% peptone solution and mixed using a stomacher. Ten-fold serial dilution was conducted. 0.1 ml of each diluted sample was spread on yeast malt agar and incubated aerobically at 30°C for 24-48 hours. Isolates were purified, identified, and preserved in 15% glycerol for further studies, and the isolates were deposited in TISTR culture collection.

Genomic DNA was extracted following the protocol by Lõoke *et al.* (2011). The D1/D2 sequence was amplified, purified, and analyzed using the method described by Lõoke *et al.* (2011). Sequences were aligned with GenBank/EMBL/DDBJ, and the NCBI BLAST program was used. Alignment was edited, and gaps were removed before constructing the phylogenetic tree. The neighbor-joining method was employed for the phylogenetic tree in MEGA version 11 software (Tamura *et al.*, 2021). Bootstrap resampling (1,000 replications) was used to evaluate the confidence values of nodes for a reliable tree topology (Felsenstein, 1985).

2. Hemolysis activity

Hemolysis activity was assessed using 5% sheep blood agar, as described by Lad *et al*. (2022). Isolates were streaked on 5% sheep blood agar, and incubated aerobically at 30°C for 48 hours.

3. Antibiotic susceptibility

The antibiotic susceptibility of the chosen yeast isolates was assessed using the Method of Antifungal Disc Diffusion Susceptibility Testing of Yeasts, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (Weinstein and Lewis, 2020). The commercial antibiotic test discs, such as Fluconazole (25 micrograms (µg)), Ketoconazole (15 µg), and Caspofungin (5 µg), were used to determine antibiotic susceptibility.

Yeast isolates were cultivated on Yeast Malt Agar (YM Agar) for 24 hours. An inoculum was prepared by adjusting the yeast culture turbidity to match McFarland standard No. 1 and was then carefully applied to the YM agar. Each antibiotic disc was placed on the inoculated agar under aseptic conditions, with Candida albicans used as a positive control. After 24-hour incubation at 30°C, the results were compared with the breakpoint values provided by Espinel-Ingroff (2022). Antibiotic susceptibility was interpreted and reported as "S" (sensitive) or "R" (resistant).

4. Acid and bile salt tolerance

The preparation of simulated gastrointestinal fluid followed the method described by Hyronimus *et al*. (2000). Briefly, 0.1% pepsin was dissolved in 0.85% Sodium Chloride (NaCl), and the pH was adjusted to 2 using 1 molar (M) hydrochloric acid (HCl). Gilliland *et al.* (1984) employed the method to investigate bile salt tolerance. Bile salts were dissolved in 0.85% NaCl at a concentration of 0.3%, with a pH of 8. One milliliter of overnight yeast culture in YM broth was inoculated into 9.0 ml of SGF (pH 2) and incubated anaerobically at 37°C for 3 hours. Then, selected isolates were transferred to a 0.3% bile salt solution (pH 8) and incubated at 37°C for 5 hours. After incubation, viable cells were assessed using the pour-plate method and then incubated at 37°C for 48 hours.

5. HT-29 and Caco-2 cell adhesion assay

The adhesion ability of the yeast strain to human epithelial cells (HT-29 and Caco-2) was assessed following the method outlined by Jacobsen *et al*. (1999). Epithelial cells were seeded in a 24-well culture plate at 1×105 cells/well density and incubated for 7 days at 37°C in a 5% CO_2 humidified incubator. After incubation, the cells were washed with phosphate buffer (PBS) (pH 7.2), followed by the addition of 2 ml of serum-free Dulbecco's Modified Eagle Medium (DMEM) and further incubation at 37°C for 30 minutes. Yeast cultures ($1x10^9$ Colony forming unit per milliliter (CFU/ml)

were prepared in DMEM, followed by a 1-hour incubation at 37°C in a 5% CO_2. Afterward, non-adherent yeast cells were washed away, and monolayer cells were detached using 1% Triton-X-100, following the method described by Roselli *et al*. (2006). Yeast adhesion was quantified through pour-plating on YM agar using serial dilutions, followed by incubation at 30°C for 24-48 hours.

6. Antimicrobial activity of isolated yeast strains

Antimicrobial activity was assessed using the spot-on-lawn method (Hudzicki, 2009) against six pathogenic bacteria: TISTR *Escherichia coli* 780, *Pseudomonas aeruginosa* TISTR 1995, *Listeria monocytogenes* TISTR 2196, *Salmonella enteritidis* TISTR 2202, *S.* typhimurium TISTR 2519, and *Staphylococcus aureus* TISTR 1466. To conduct the test, 10 microliters (µl) of yeast suspensions were placed on 15 ml of Yeast Malt agar and incubated at 37°C for 5 days to create yeast spots. The pathogens were cultured under their optimal conditions. The turbidity of the bacterial culture was adjusted to match McFarland standard No. 1 using 0.85% sterile normal saline. Then, 5 ml of the adjusted bacterial culture was overlaid on the yeast spots and incubated at 37°C for 24 hours. Inhibition zones around the yeast indicated antimicrobial activity. The zone diameter was measured and categorized as follows: - (No inhibition zone), + (Inhibition zone diameter ≥ 10-14 millimeter (mm)), ++ (Inhibition zone diameter ≥ 15-20 mm), +++ (Inhibition zone diameter ≥ 20 mm).

7. Cellulase and Xylanase activity

The cellulase and xylanase activity of the yeast strain was screened qualitatively following the method described by Sridevi and Charya (2011). To induce enzyme activity, 0.5% cellulose or Xylan was added as a carbon source in a basal medium. The yeast was then inoculated and incubated at 37°C for 5 days. Colonies that exhibited clear zones around them were selected as cellulase and xylanase-producing strains, indicating their ability to degrade cellulose and Xylan.

8. Cytotoxicity test

The RAW 264.7 mouse macrophage cell line (ATCC® TIB-71™, Virginia, USA) was cultured in DMEM with 10% Fetal Bovine Serum (FBS) and 1% volume/volume (v/v) antimycotic at 37°C in a 5% CO_2 humidified incubator. For the experiment, RAW 264.7 cells were seeded into each well of a 96-well plate at $2x10^5$ cells/well. After 24 hours, cells were pre-incubated with various sample concentrations. 10% of Dimethyl Sulfoxide (DMSO) was used as a positive control, while DMEM supplemented with 10% of FBS was used as a negative control. Cytotoxicity was determined by measuring cell viability. The optical density (OD) was measured at 570 nm. The percentage of cell viability was calculated using the following formula:

$$\text{Cell viability (\%)} = (OD_s/OD_m) \times 100$$

Here, OD_s represents the OD values of the stimulated wells, and OD_m represents the OD values of the medium wells.

9. Phagocytic activity

RAW-264.7 cells ($1x10^5$ cells/ml) were cultured in a 96-well plate using DMEM medium with 10% FBS and incubated at 37°C for 24 hours. After changing the medium, samples were added at 3.13% v/v and incubated for another 24 hours at 37°C. The negative control was DMEM, and the positive control was beta-glucan. Stimulated RAW 264.7 cells were washed twice with PBS to assess phagocytic activity. Then, 100 µl of 0.075% neutral red in PBS was added to each well, followed by 3 hours of incubation at 37°C. After washing, a mixture of 2% acetic acid and ethanol (1:1) was used to de-stain cells, keeping the plate at 4°C for 1 hour. OD was measured at 540 nanometers (nm). Phagocytic activity (%) was calculated using the following formula:

$$\text{Phagocytic activity (\%)} = (OD_s/OD_{pc}) \times 100$$

where OD_s is the OD of stimulated wells, and OD_{pc} is the OD of positive control wells. The untreated cell's phagocytic activity is set as 100%.

10. Nitric oxide production

RAW-264.7 cells ($2x10^5$ cells/well) were plated in a 96-well culture plate. After 24 hours, cells were pre-incubated with 3.13% (v/v) of the test sample. Lipopolysaccharide (LPS) at 1 microgram per milliliter (µg/ml) was served as the positive control, and 40 µg/ml diclofenac (100 µl) was used as the negative control. Nitric oxide concentration was assessed by using a Griess reagent. The OD was determined at 570 nm and calculated by comparing it with the nitric oxide standard curve.

11. Immunomodulation effects (tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) productions)

TNF-α and IL-6 levels were assessed using ELISA kits (AlphaLISA Research reagent). Samples or standards (5 µl) were mixed with freshly prepared MIX reagent (2.5 µl) and incubated at 23°C for 1 hour. Next, 2X SA-Donor beads were added and incubated at 23°C for 30 minutes in the dark. The OD and the amounts of TNF-α and IL-6 were measured by comparing OD values with the standard curve.

12. Statistical analysis

All experiments were conducted in triplicate. Results were presented as mean ± standard deviation (SD). All results were subjected to one-way analysis of variance (ANOVA) using SPSS version 22.0 software. For pairwise comparisons, Duncan's Multiple Range Test was applied to mean values with a significance threshold of $p < 0.05$.

Results

1. Isolation and identification

This study obtained 18 diverse yeast isolates from flowers, mushrooms, fermented rice, and natural environments. After purification and initial identification based on morphological and genotypic characteristics, their taxonomic classification was determined through 26S rDNA gene sequencing analysis. The resulting species included *Meyerozyma caribbica* TISTR 6026 and TISTR 6017, *Saccharomyces cerevisiae* TISTR 5092, TISTR 5104, TISTR 5161, TISTR 5328, and TISTR 5872, *Pichia kudriavzevii* TISTR 6028, TISTR 6025, TISTR 6022, and TISTR 6030, *Starmerella sorbosivorans* TISTR 6018, TISTR 6027, and TISTR 6019, *Kodamaea ohmeri* TISTR 6020, *Ambrosiozyma kamigamensis* TISTR 6029, and TISTR 6021, and *Metschnikowia koreensis* TISTR 6044. The sequencing analysis revealed a high degree of homology among these species, ranging from 99.43% to 100.00%. For further insights and a visual representation of evolutionary relationships, refer to Figure 1 for detailed information and Table 1 for the comprehensive phylogenetic tree.

2. Hemolysis activity

All eighteen yeast isolates displayed gamma-hemolysis, as seen by the lack of zones around their colonies on the sheep blood agar plate.

3. Antibiotic susceptibility

The susceptibility of all yeast isolates to three antifungal antibiotics, Fluconazole (25 µg), Ketoconazole (15 µg), and Caspofungin (5 µg), was assessed following the Clinical and Laboratory Standards Institute (CLSI). These strains demonstrated susceptibility to antifungal antibiotics.

4. Acid and bile salt tolerance

The results demonstrated that the yeast strains exhibited robust survivability, with a survival rate of over 80.00% in acidic conditions and 70.00% in intestinal conditions.

5. HT-29 and Caco-2 cell adhesion assay

The *in vitro* adhesion assay on human colon adenocarcinoma cell lines HT-29 and Caco-2 revealed robust adhesive properties of all yeast strains, with adhesion rates surpassing 60.00% (data not shown).

6. Antimicrobial activity

A coveted trait of probiotic yeasts is their ability to combat human pathogens. The antimicrobial potential of 18 yeast strains was tested via the spot-on-lawn method against six pathogenic bacteria: *E. coli* TISTR 780, *S. aureus* TISTR 1466, *P. aeruginosa* TISTR 1995, *S.* typhimurium TISTR 2519, *S. enteritidis* TISTR 2202, and *L. monocytogenes* TISTR 2196. Table 2 highlights that only four yeast strains, *M. caribbica* TISTR 6017, *S. cerevisiae* TISTR 5104, TISTR 5328, and *P. kudriavzevii* TISTR 6028, exhibited significant antimicrobial activity against these pathogens.

Table 1 Isolation source, and identification of isolates based on 26s rDNA gene sequencing analysis.

No.	Isolated No.	Source	Province	Identification	Similarity (%)
1	YK9/1	Mushroom	Nakhon Ratchasima	*M. caribbica* TISTR 6026	100.00
2	YE22/1	*Great morinda*	Nakhon Ratchasima	*M. caribbica* TISTR 6017	100.00
3	P4	Coconut toddy	Phetchaburi	*S. cerevisiae* TISTR 5092	100.00
4	P5	Fermented soybean pastes	Pathum thani	*S. cerevisiae* TISTR 5104	100.00
5	P6	Fermented rice	Phetchaburi	*S. cerevisiae* TISTR 5161	100.00
6	P8	Fermented rice	Surin	*S. cerevisiae* TISTR 5328	100.00
7	P9	Fermented rice	Surin	*S. cerevisiae* TISTR 5872	100.00
8	Y17-1	Yellow elder flower	Chaiyabhumi	*S. sorbosivorans* TISTR 6018	100.00
9	Y17-2	Yellow elder flower	Chaiyabhumi	*S. sorbosivorans* TISTR 6027	100.00
10	Y17-3	Yellow elder flower	Chaiyabhumi	*S. sorbosivorans* TISTR 6019	100.00
11	Y19-1	*Kaempferia pulchra Ridl.*	Suphan Buri	*K. ohmeri* TISTR 6020	99.80
12	Y83-2	Tapioca fiber	Chaiyabhumi	*P. kudriavzevii* TISTR 6028	100.00
13	Y101-2/1	Flower	Nakhon Ratchasima	*P. kudriavzevii* TISTR 6025	100.00
14	Y101-3	Flower	Nakhon Ratchasima	*P. kudriavzevii* TISTR 6022	100.00
15	Y180-3	*Dioscora alata*	Nakhon Ratchasima	*A. kamigamensis* TISTR 6029	100.00
16	SK83-4	*Pavetta indica* L.	Nakhon Ratchasima	*P. kudriavzevii* TISTR 6030	100.00
17	SK180-2	*D. alata*	Nakhon Ratchasima	*A. kamigamensis* TISTR 6021	100.00
18	SK114-2	*P. indica* L.	Nakhon Ratchasima	*M. koreensis* TISTR 6044	99.43

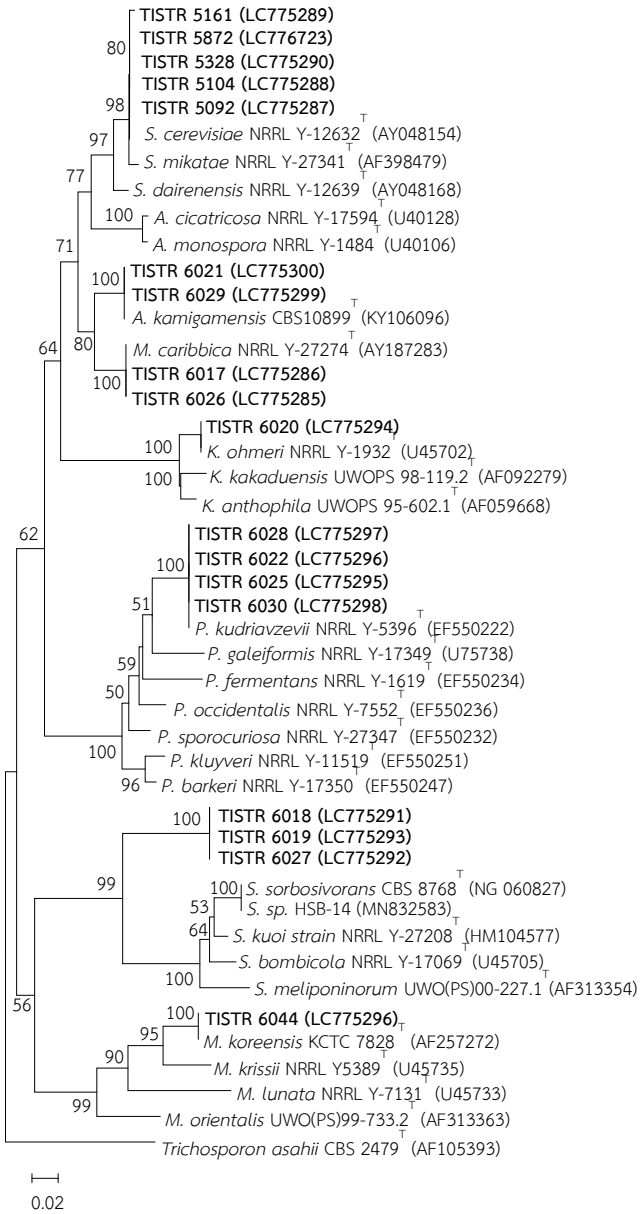

Figure 1 The phylogenetic tree of gene sequences of 26S rRNA was generated by Neighbor-Joining method. Bootstrap values (>50%) based on 1,000 replications are given at branch nodes. Accession of each organism used is given in parentheses.

7. Cellulase and Xylanase activity

Among the 18 yeast strains, *M. caribbica* TISTR 6026 and *M. koreensis* TISTR 6044 exhibited xylanase activity, which was proved by the clear zone around their colonies. However, none of them displayed cellulase activity.

8. Cytotoxicity test

These strains, including *S. cerevisiae* TISTR 5104, TISTR 5328, *M. caribbica* TISTR 6017, and *P. kudriavzevii* TISTR 6028, were tested using the MTT assay at concentrations of 0.00%, 3.13%, 6.25%, 12.50%, 25.00%, and 50.00% (v/v). A key safety criterion was a RAW-264.7 cell survival rate exceeding 80% at each concentration. At 3.13% (v/v), all selected showed survival rates over than 80% (data not shown).

9. Phagocytic activity

The assessment of phagocytic activity revealed significant phagocytic activity in four yeast strains: *S. cerevisiae* TISTR 5328, *M. caribbica* TISTR 6017, *S. cerevisiae* TISTR 5104, and *P. kudriavzevii* TISTR 6028. The uptake percentages for these strains were 60.64±3.45, 68.86±3.58, 73.48±3.53, and 100.00±2.00, respectively, at a concentration of 3.13% (v/v). These values were compared to the positive control, 100 µg/ml Beta-glucan, as illustrated in Table 3.

10. Nitric oxide production

The NO assay revealed that selected strains triggered and slightly suppressed NO production, differing significantly from the control (Table 3). These strains effectively influenced RAW 264.7 macrophages to generate nitric oxide. Notably, strains TISTR 5104, TISTR 5328, TISTR 6017, and TISTR 6028 induced substantial nitric oxide production in RAW 264.7 cells. At 3.13% (v/v) concentration, nitric oxide values were 37.68±3.68, 24.65±0.75, 28.77±0.65, and 27.42±0.31, respectively.

11. Immunomodulatory effects

In the TNF-**α** and IL-6 production assay involving four yeast strains (*S. cerevisiae* TISTR 5104, TISTR 5328, *M. caribbica* TISTR 6017, and *P. kudriavzevii* TISTR 6028) on RAW-264.7 cells at 3.13% (v/v) concentration, strains TISTR 5328 and TISTR 6017 stimulated TNF-**α** production with values of 1,136.21±297.18 picograms per milliliter (pg/ml) and 1,419.10±814.12 pg/ml, respectively. Notably, these values were lower than the positive control producing TNF-**α** at 1,669.00±1.99 pg/ml. Conversely, strain TISTR 5104 and strain TISTR 6028 induced TNF-**α** production with values of 4,478.41±567.44 and 1,725.58±86.92 pg/ml, respectively, significantly exceeding the LPS positive control (Figure 2). Among these strains, strain TISTR 5104 exhibited the highest capacity to stimulate IL-6 production (67,455.56±2,411.11 pg/ml), followed by strain TISTR 5328 (42,500.00±11.11 pg/ml), strain TISTR 6017 (32,916.67±1661.11 pg/ml), and strain TISTR 6028 (6,421.00±412.22 pg/ml) (Figure 2).

Table 2 Antimicrobial activity of yeast strains against six pathogenic bacteria.

Strain	Antimicrobial activity					
	E. coli TISTR 780	*S. aureus* TISTR 1466	*P. aeruginosa* TISTR 1995	*S.* Typhimurium TISTR 2519	*S. enteritidis* TISTR 2202	*L. monocytogenes* TISTR 2196
M. caribbica TISTR 6017	++	+++	+++	-	+++	-
S. cerevisiae TISTR 5104	-	-	-	++	++	++
S. cerevisiae TISTR 5328	-	-	-	++	+	++
P. kudriavzevii TISTR 6028	-	+++	-	-	-	+++

Note: - Diameter of inhibition clear zone (mm): - (no inhibition zone), + (diameter ≥10-14 mm), ++ (diameter ≥15-20 mm), +++ (diameter ≥20 mm)

Table 3 Phagocytosis and nitic oxide modulating effects of selected yeast strains on RAW 264.7 cell.

Treatments	Phagocytosis (%)	Nitric oxide production (μM)
S. cerevisiae TISTR 5104	73.48±3.53^{b}	37.68±3.68^{a}
S. cerevisiae TISTR 5328	60.64±3.45^{b}	24.65±0.75^{c}
M. caribbica TISTR 6017	68.86±3.58^{b}	28.77±0.65^{b}
P. kudriavzevii TISTR 6028	104.54±2.06^{a}	27.42±0.31^{b,c}
Negative control	**Medium** 0.05±2.10^{c}	**Diclofenac (40 μg/ml)** 19.05±0.53^{d}
Positive control	**Beta-glucan (100 μg/ml)** 100.35±0.70^{a}	**LPS (1 μg/ml)** 28.41±0.70^{b}

Note: - Significantly different results were indicated by superscripts, employing Duncan's method for multiple comparisons ($p < 0.05$). The highest value is denoted by the letter 'a,' ordered in descending sequence.

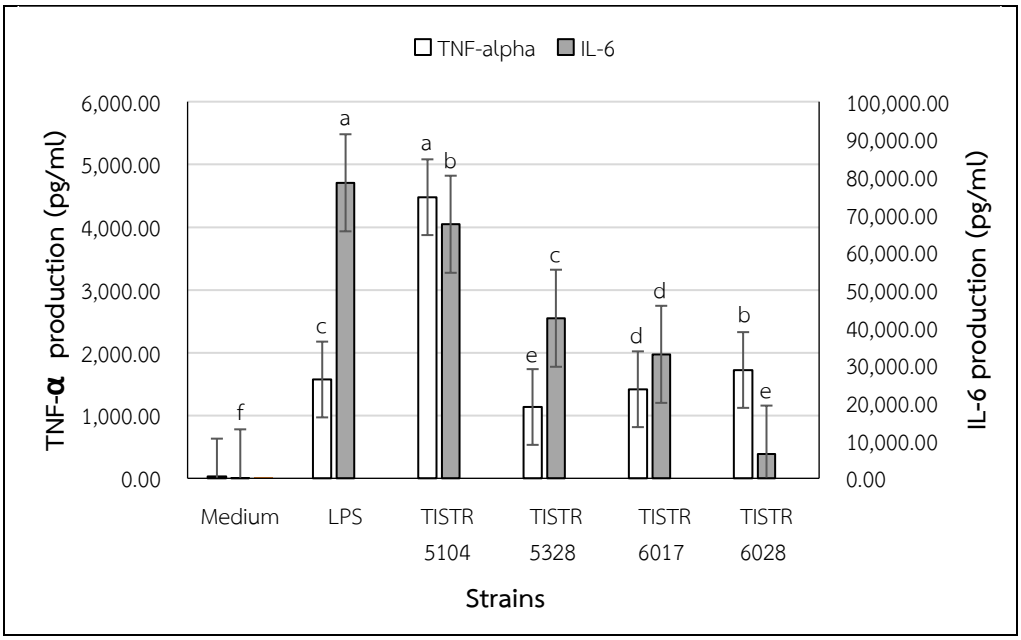

Figure 2 TNF-α and IL-6 productions (pg/ml) in RAW-264.7 cell induced by yeast strains, *S. cerevisiae* TISTR 5104, *S. cerevisiae* TISTR 5328, *M. caribbica* TISTR 6017 and *P. kudriavzevii* TISTR 6028. LPS and medium were used as positive control and negative control. Significantly different results were indicated by superscripts, employing Duncan's method for multiple comparisons ($p < 0.05$). The highest value is denoted by the letter 'a,' ordered in descending sequence. Letters above the bars coded by different colors represent TNF-α and IL-6 productions, respectively. According to Duncan's test ($p < 0.05$), the levels not connected by the same letter are significantly different. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Discussion

This study evaluates the probiotic properties and potential benefits of 18 yeast strains isolated from natural and fermented sources. The results show that these yeast strains are safe, survive well in gastrointestinal conditions, have antimicrobial properties, and can support immune function, making them strong candidates for probiotics. None of the strains showed hemolytic activity (gamma-hemolysis), confirming they are non-pathogenic and safe. Gamma-hemolysis, which indicates no breakdown of blood cells, minimizes health risks. Additionally, the strains were sensitive to antifungal antibiotics, reducing the risk of drug resistance and ensuring they could work well with medical treatments. This aligns with studies by Abulreesh *et al*. (2019) and Maciel *et al*.

(2019), which reported similar antifungal sensitivity in yeast species. The yeast strains also survived in harsh conditions, with over 80% surviving in acidic environments and 70% in bile salt conditions, simulating the human digestive system. This strong survival rate suggests they could thrive in the gut. All strains tested showed survival rates above 70%, making them reliable options for probiotic use. Furthermore, these yeasts showed good adhesion to intestinal cells (HT-29 and Caco-2), with adhesion rates exceeding 60%. This means they can effectively colonize the gut and interact with intestinal cells, a crucial factor for probiotics to function well.

Four strains—*M. caribbica* TISTR 6017, *S. cerevisiae* TISTR 5104 and TISTR 5328, and *P. kudriavzevii* TISTR 6028 stood out for their antimicrobial properties. They inhibited harmful bacteria like *E. coli* and *S. aureus*, showing their potential to support gut health by controlling pathogens. Studies by Rajkowska and Kunicka-Styczyńska (2012) and Syal and Vohra (2013) reported antimicrobial effects in yeasts. Other research supports these findings: *S. cerevisiae* IFST062013 inhibits bacteria and fungi (Fakruddin *et al*., 2017), *M. caribbica* can control *Penicillium expansum* (Qiu *et al*., 2022), and *P. kudriavzevii* has antibacterial properties (Choińska *et al*., 2020). Additionally, two strains—*M. caribbica* TISTR 6026 and *M. koreensis* TISTR 6044—produced xylanase, an enzyme that helps break down dietary fibers. This activity could improve animal digestion, although the lack of cellulase activity limits their role in breaking down cellulose.

Safety testing confirmed that these strains are non-toxic, with over 80% of RAW-264.7 cells remaining viable at all tested concentrations. This demonstrates their safety for further applications. The selected strains also showed strong immune-boosting effects. They enhanced the activity of immune cells and increased the production of key immune signals like TNF-α and IL-6. *S. cerevisiae* TISTR 5104 was particularly effective, showing the highest immune stimulation. Controlled immune responses triggered by these strains could help manage infections or inflammation (Rocha-Ramírez *et al*., 2020). Yeasts activate defenses by increasing IL-6 and TNF-α production (Fidan *et al*., 2009). Beta-glucans, a key component of yeast cell walls, likely contribute to these effects (Wang *et al*., 2020), while other cell components may influence nitric oxide production and immune responses (Kingkaew *et al*., 2022).

In summary, *S. cerevisiae* TISTR 5104, TISTR 5328, *M. caribbica* TISTR 6017, and *P. kudriavzevii* TISTR 6028 show the most promise as probiotics due to their safety, ability to survive in the gut, antimicrobial activity, and immune-boosting effects. These strains have significant potential for functional foods, animal feed supplements, and therapies to improve gut health and immunity. Further studies are needed to confirm these findings and explore how these strains work, especially in living organisms.

Conclusions

This study aimed to identify and assess 18 yeast isolates for potential probiotic use from various sources. Using 26S rDNA gene sequencing, species such as *M. caribbica*, *S. cerevisiae, P. kudriavzevii, S. sorbosivorans, K. ohmeri, A. kamigamensis*, and *M. koreensis* were identified. These strains demonstrated safety, tolerance to gastrointestinal conditions, adhesive properties to human cells, and antimicrobial potential against pathogenic bacteria. Notably, *M. caribbica* and *M. koreensis* exhibited xylanase activity. Certain strains (*S. cerevisiae, M. caribbica*, and *P. kudriavzevii*) were non-cytotoxic and showed immune-modulating effects in macrophages. This study highlights these yeast strains' taxonomy, safety, antimicrobial properties, survival capabilities, and immune-modulating potential, suggesting their value as probiotics for diverse applications, including immune therapies and combating bacterial pathogens. Further exploration of these strains is warranted.

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