Characterization of Probiotics From Water Kefir Grains

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ABSTRACT The probiotic potential of lactic acid bacteria (*Lactobacillus mali*, *Lactobacillus casei*, and *Oenococcus oeni*), acetic acid bacteria (*Gluconobacter maltaceti*), and yeast (*Saccharomyces cerevisiae*) strains isolated from water kefir (WK) grains were identified and evaluated. All five isolates were acid- and bile-salts tolerant. These strains appeared to be non-hemolytic, susceptible to antibiotics, adhered to porcine gastric mucin, and exhibited high antimicrobial activities against enteric pathogens. All identified five strains have *in vitro* probiotic properties. The detected genera in the WK grains used in this study included *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Acetobacter*, and *Gluconobacter*.

KEYWORDS: 16S rRNA; Acid tolerance; Bile tolerance; Probiotic potential; Water kefir
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INTRODUCTION

Probiotics are live microorganisms that confer beneficial effects on human health by maintaining a balanced gut microbial community when administered in adequate amounts (Swanson *et al.*, 2020). They must be non-pathogenic, antimicrobial, antibiotic-resistant, could adapt to high bile salts and low pH conditions, and show intestinal mucosa binding ability (Liu *et al.*, 2020). Traditionally, dairy is the most common probiotic carrier. The growing popularity of vegetarianism, the increasing prevalence of lactose intolerance, and the detrimental effects of cholesterol in dairy have encouraged the development of probiotic products using dairy-free alternatives (Muncey & Hekmat, 2021).

Water kefir (WK) is a non-dairy fermented beverage cultivated by WK grains, which has an acidic, yeasty, sweet, fizzy, slightly alcoholic taste, and fruity aroma (Lynch *et al.*, 2021). WK grains are soft, shapeless gelatinous masses that harbor lactic acid bacteria (LAB), acetic acid bacteria (AAB), and yeast (Luang-In *et al.*, 2018). Due to their health-promoting properties and increasing consumption of WK in widespread countries, WK represents a promising cultural option for developing new, non-dairy probiotic products (Fiorda *et al.*, 2017; Tu *et al.*, 2019).

WK grains have been used as starter cultures to ferment fruits and vegetable juices, whey-based, and molasses solutions (Fiorda *et al.*, 2017). However, limited reports have been published on the probiotic potential of the microbial strains in WK (Lynch *et al.*, 2021). Understanding the microbial composition and the probiotic properties of WK grains are of utmost importance. Thus, the present research was undertaken to extract and identify the LAB, AAB, and yeast strains present in WK grains through their *in vitro* probiotic properties.

METHODOLOGY

Activation of water kefir (WK) grains

The WK grains (10 g) purchased from My Kefir World, Kuala Lumpur, Malaysia was activated and cultured (25 °C, 3 days) in a brown sugar solution (100 ml, 5% w/v). After incubation, the grains were filtered. The fermented medium was discarded, and the process was repeated until the mass of grains increased by 12-18% and the medium pH reached pH 4.0-4.3.

Enumerations of LAB, AAB, and yeast

The serial diluted homogenized kefir samples (10 g WK grains, 90 ml 1/4-strength Ringer's solution) were streaked on the de Man, Rogosa, and Sharpe agar (MRS) (Merck, Germany), Acetobacter agar (AM) (Himedia, India), and yeast extract-glucose agar (YPG) (Himedia). Cycloheximide (150 μg ml^{-1}, Sigma-Aldrich, USA) and chloramphenicol (100 mg l^{-1}, Sigma-Aldrich) were added into these agars to restrain the growth of fungi and bacteria. After aerobically incubated for 72 h (MRS and AM: 37 °C; YPG: 28 °C), the colonies were counted and expressed as CFU per gram of grains (CFU g^{-1}).

Isolation and purification of kefir-isolated strains

Microbial strains were isolated after being aerobically incubated on agar plates for 24, 48, and 72 h. Colonies were chosen randomly to observe their colonies and cell morphologies. A total of 5 colonies (3 from MRS, 1 from YPG, and 1 from AM agar) were selected according to their morphologies, re-cultured (streak plate method) until uniform-grew colonies appeared (Park *et al.*, 2016) and gram stained. Among the 5 isolates, 3 from MRS agar plate were Gram-positive, rod- or coccus-shaped, 1 from YPG agar plate was Gram-positive, globose, ellipsoid to elongate in shape, and 1 from AM agar plate was Gram-negative, oval-shaped. They were identified as LAB, yeast, and AAB strains, respectively. All the isolates were preserved at -80 °C in their corresponding broth containing 30% (v/v) glycerol. The frozen isolates were sub-cultured at least once before each experiment and every 6 months.

Identification of isolates

A genomic extraction kit (NucleoSpin Tissue, Machery-Nagel, Duren, Germany) was utilized to extract the genomic DNA from the strains isolated, using the manufacturer's specified procedure. Both the quantity and purity of extracted DNA were determined by using a UV spectrophotometer (UV mini-1240, Shimadzu, Kyoto, Japan). The pure genomic DNA was polymerase chain reaction-amplified the 16S and 18S rRNA sequencing and subsequently identified using the DNA sequencing method.

Acid and bile salt tolerance

The acid and bile salt tolerances of the strains isolated were determined according to the method described by Anandharaj and Sivasankari (2014). After incubation (24 h), the cultures of each of the isolated strain (1.5 x 10^8 CFU ml^{-1}) were inoculated into their respective pH-adjusted (pH 1, 2, and 3, using 1N NaOH and 1N HCl) and bile (0, 0.3, 0.5, and 1% w/v; Sigma-Aldrich) added broth (MRS, AM, and YPD broth, respectively). After incubation (0, 3, 5 h, 37 °C), the survivability of these strains in both conditions was assessed (plate count method). The results were expressed as the survival rates (%): $\log_{10} N_1/\log_{10} N_0 \times 100\%$, where N_0, and N_1 represented the total viable count (in CFU g^{-1}) of the microbial strain before and after treatment, respectively.

Adhesion to Mucin

The mucin adhesion capabilities of the isolated strains were tested using the technique described by Azcarate-peril *et al.* (2009) with some modifications. Type III porcine gastric mucin solution (100 ul, 10 mg ml-1, Sigma-Aldrich) was first immobilized in a 96-well microplate (Fisher Scientific, USA). After incubation (4 °C, 4 h), the excess mucin was washed away with phosphate-buffered saline (PBS, 200 ul). Subsequently, overnight-grown strains (100 μl, 1.5 x 10^8 CFU ml^{-1}) were added to the well and incubated (37 °C, 1 h). Free bacteria were washed away with PBS (200 ul) and the bound bacteria were released by treating with Triton X-100 solution (200 ul, 0.05% v/v), transferred (100 ul) into PBS, plated, incubated, and quantified. The adherence was expressed as the percentage of viable counts adherent to the mucin to the total number of bacteria added.

Susceptibility of pathogens

The inhibitory activity of isolated strains against pathogens was assessed by the agar well diffusion assay (Davoodabadi *et al.*, 2015). Diluted (0.5 McFarland turbidity) overnight-grown (37 °C, 24 h) pathogenic cultures (*Shigella flexneri* ATCC 12022, *Shigella sonnei* ATCC 9290, *Escherichia coli* ATCC 43889, *Yersinia enterocolitica* ATCC 23715, and *Salmonella enteritidis* ATCC 49221) were transferred to nutrient agar plate (spread plate method) and the cell-free culture supernatants (100 µl) of the isolated strains (overnight-grown, 24 h) were added into the wells of the nutrient agar. After incubation (37 °C, 24 h), the antibacterial effects of strains were investigated by measuring the diameter (mm) of the inhibition (clear) zone surrounding the well.

Antibiotic susceptibility

The agar overlay disc diffusion method as described by CLSI (Clinical & Laboratory Standards Institute) was used to determine the antibiotic susceptibility of the isolated strains. The antimicrobial agents used in this study included chloramphenicol (30 µg), ciprofloxacin (5 µg), oxacillin (1 µg), penicillin G (20 units), tetracycline (30 µg), and vancomycin (30 µg. After the turbidity was adjusted to 0.5 McFarland standard, the overnight-grown cultures were spread onto their corresponding antibiotic-containing media followed by incubation (37 °C, 24 h). After incubation, the inhibition zone was measured.

Hemolysis

Diluted overnight-grown microbial cultures (0.5 McFarland turbidity) were streaked onto defibrinated sheep blood agar (5%, Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 37 °C for 24 h (Park *et al.*, 2016).

16S metagenomic sequencing of WKGs

The metagenomic DNA extraction, 16S metagenomic sequencing, raw sequence reads processing and analysis were conducted on the supernatant of lyzed (Nalbantoglu *et al.*, 2014) homogenate samples (2 ml, 25 g activated WKGs in 0.9% NaCl solution, 10,000 x g, 15 min, 4 °C).

Statistical analysis

All the data were analyzed using SPSS Statistics 21.0 (IBM, Armonk, NY, USA) and presented as mean ± standard deviation (n = 3). The means were compared using one-way ANOVA followed by Tukey's post-hoc test (α = 5%).

RESULT AND DISCUSSION

Although there has been increased research interest in the WK grains, the in *vitro* probiotic potential of the LAB, AAB, and yeast strains in grains have not been well studied (Lynch *et al.*, 2021). Their microbial composition was also varied based on their origin, the steps, raw materials, and the conditions used in cultivation (Fiorda *et al.*, 2017). The cells viabilities of LAB, AAB, and yeast were 10^9, 10^6, and 10^7 CFU g^{-1}, respectively, and were in compliance with the criteria established by Codex Alimentarius (kefir: ≥10^7 CFU g^{-1} of viable probiotics, ≥10^4 CFU g^{-1} of yeast).

The LAB, AAB, and yeast strains isolated were identified as *Lactobacillus mali* (GenBank ID: FJ157230.1), *Lactobacillus casei* (AY699577.1), *Oenococcus oeni* (HM013942.1), *Saccharomyces cerevisiae* (KU131579.1), and *Gluconobacter maltaceti* (HE861938.1). All five isolated strains survived in acidic conditions (pH ≤ 3 after 5 h, Fig. 1 A-C) and bile concentrations (≤1% after 5 h, Fig. 1 D-F) indicating that the probiotic strains showed very strong acid- and bile-tolerance. Hence, they could survive the

adverse conditions of the gastrointestinal (GI) tract to exert their beneficial effects on the host (Anandharaj & Sivasankari, 2014).

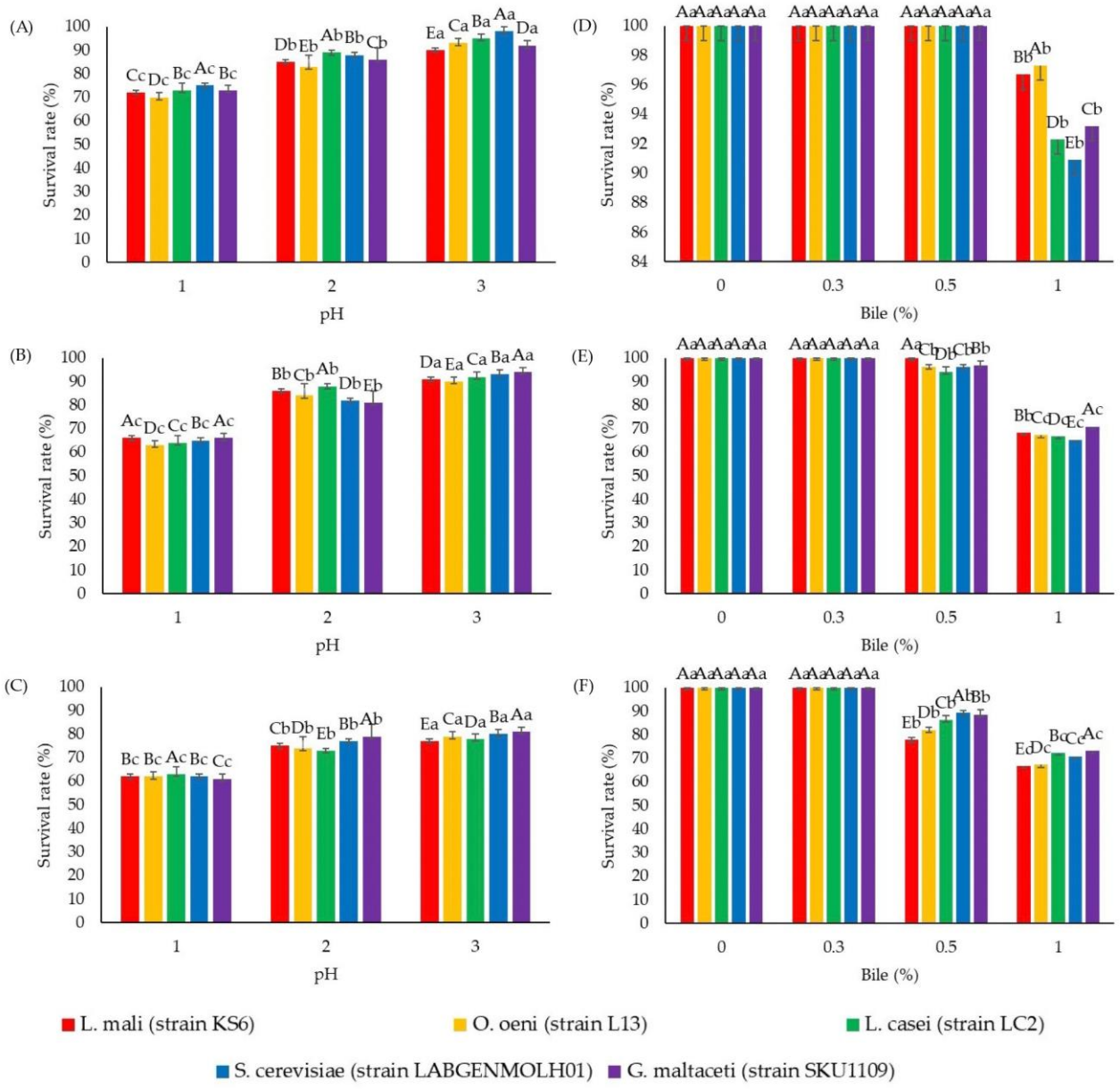

Figure 1. The effects of pH and bile concentrations on the survivability of the strains after incubated for 0 (A,D), 1 (B,E), and 3h (C,F). Bars with different alphabets are statistically significant (^{ABC} isolated strains, ^{abc} pH or bile salt concentrations).

All five isolated strains demonstrated good adherence to mucin (Fig. 2). The adherence is essential for the microorganism to colonize and persist in the GI tract (Azcarate-Peril *et al.*, 2009). Probiotics bind to the mucus layer of epithelial cells in a strain-specific manner, but their adherence is affected by the cell entrapment within the mucus, the mucus adhesion gene expression, and the excretion of surface-layer proteins (Campana *et al.*, 2017; Celebioglu & Svensson, 2018).

All five isolated strains inhibited the growth of all five tested enteric pathogens (Table 1). These inhibitory effects were mainly attributed to the H_2O_2, organic acids or bacteriocins generated by the WK strains (Davoodabadi *et al.*, 2015). All isolated strains were also susceptible to all targeted antibiotics, except vancomycin (results not shown). There was also no clear lysis zone that could be seen surrounding the cell colonies of all the strains. Hence, they are safe for consumption.

The genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Acetobacter*, and *Gluconobacter* were the predominant microbiota of the WK grains used in this study (results not shown), which were similar to the findings obtained by Luang-In *et al.* (2018) and Kumar *et al.* (2021). However, two potential pathogens (*Klebsiella oxytoca* and *Enterobacter aerogenes*) were also detected, which was most probably due to improper food-handling practices. This can be resolved by using isolated strains as starter cultures.

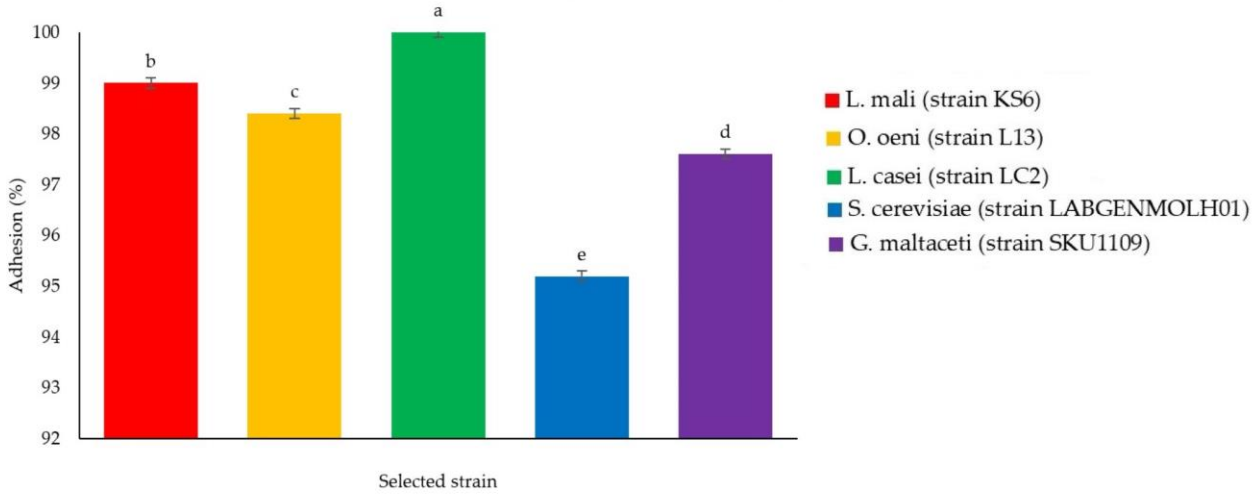

Figure 2. Adhesion (%) of the strains. Bars with different alphabets are statistically significant.

Table 1. Antimicrobial activity of the isolated strains against the enteric pathogenic bacteria.

	L. mali	*O. oeni*	*L. casei*	*S. cerevisiae*	*G. maltaceti*
Escherichia coli ATCC 43889	+	+	+	+	+
Shigella flexneri ATCC 12022	++	+++	++	++	++
Shigella sonnei ATCC 9290	+	+	+	+	+
Yersinia enterocolitica ATCC 23715	++	++	+	+	+
Salmonella enteritidis ATCC 49221	+	+	+	+	+

Inhibition zone diameter: <11 mm: negative (–), 11-16 mm: mild (+), 17-22 mm: strong (++), and >23 mm: very strong (+++).

CONCLUSION

Five strains isolated from WK grains could survive the low pH condition, resistant to bile salts, adhered to porcine gastric mucin, non-hemolytic, possessed antagonistic activity against enteric pathogens, and resistant to antibiotics. Therefore, these strains were considered as potential probiotic candidates for their application as starter cultures in novel WK manufacture.

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