

## PROBIOTIC POTENTIAL AND ANTIMICROBIAL ACTIVITIES OF MICRO-ORGANISMS ISOLATED FROM AN INDIGENOUS FISH SAUCE

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**ABSTRACT.** *This study assessed the potential of probiotic and antimicrobial activity of strains isolated from an indigenous fish sauce in Malaysia. A total of 150 isolates were evaluated for their resistance toward low pH and bile salts as well as the production of inhibitory substances against four selected foodborne pathogens namely Listeria monocytogenes, Staphylococcus aureus, Salmonella typhimurium and Escherichia coli O157:H7. Lactobacillus plantarum (LP1, LP2), Saccharomyces cerevisiae (SC3), Candida glabrata (CG2), Lactococcus lactis subsp. lactis (LL2) and Staphylococcus arlettae (SA) strains showed resistance to low pH and bile salt at various concentrations. The LP1(86.3%), LP2 (86.2%), LL2 (84.4%) and CG2 (79.7%) strains exhibited higher survival rates than SA (66.7%) strain at extremely low pH concentration (pH 1.5) compared to other tested strains; while most of the strains tolerate bile salt at low concentrations (0.3%) which mimic the human small intestine environment. The growth rate of the tested strains decreased in proportion to the increase of bile salt concentrations. All the strains elicited different levels of antimicrobial activities against selected pathogens. Only the LP1, SC3 and SA strains showed greater inhibitory effect against Staphylococcus aureus and Listeria monocytogenes. The result suggested that LP1, LP2, SC3, CG2, LL2 and SA were technologically interesting and could be developed as starter cultures for the manufacturing of novel functional fermented foods.*

**KEYWORDS.** Fish sauce, probiotic, bile salt tolerance, antimicrobial activity, starter cultures

### INTRODUCTION

The association between functional foods and their health promoting effects has been widely studied for the past decades. The functional foods range from the food additives to the prebiotics and probiotics which exert a healthy effect on the host after consumption for a certain period of time. Probiotics are recognized as live microorganisms which when administered in sufficient amounts that can confer a healthy effect on the host by improving the properties of the indigenous micro flora in the intestinal tract (Saarela *et al.*, 2000). The probiotics are mostly comprised of lactic acid bacteria (LAB), particularly from the genera of *Lactobacillus*, *Bifidobacterium* or *Propionibacterium* which are commonly found in dairy products (Bao *et al.*, 2010), plants and vegetables (Wang *et al.*, 2010; Silva *et al.*, 2011) as well as fermented meat products (Erkilla & Petaja, 2000).

In searching for novel strains with probiotic potential, many studies demonstrated that fermented foods may serve as a pool for screening and selecting new isolates with probiotic properties before developing them into potential starter cultures for controlled fermentation. This is because fermentation involves the interaction between the micro-organisms and the natural substrates before further undergoing biodegradation and assimilation of the substrates under extreme conditions. Hence, only dominant micro-organisms with unique technological properties will survive and adapt to the fermentation environment. A probiotics culture can only be used if it

fulfills the following criteria: (i) recognition as safe (Generally Recognized as Safe, GRAS); (ii) viability during processing and storage; (iii) antagonistic effect on pathogens; (iv) tolerance to bile acid challenge and (v) adherence to the intestinal epithelium of the host among others (Vesterlund *et al.*, 2005).

Many scientists have proposed the use of probiotics as starter culture for controlled fermentation as well as to add value to the fermented foods since there are no significant technological or sensorial differences between fermented foods prepared with probiotics or non-probiotics (Incze, 1998; Tyopponen *et al.*, 2003; Angelov *et al.*, 2005). The functionality and metabolic features of the advantageous strains in most fermented foods such as fermented meat, vegetables or dairy products have been well documented. However, the understanding of the culture obtained from this indigenous fermentation process remains unexplored. Therefore, the objectives of this study were to screen and characterize the potential probiotic and antimicrobial activity of the strains isolated from an indigenous fish sauce of Malaysia.

## MATERIALS AND METHODS

### ***Bacterial strains and culture condition***

A total of 150 isolates of microorganisms which were phenol typically identified using BIOLOG Microlog Identification Software (Biolog Inc.) in the previous study (Sim *et al.*, 2009) were obtained and kept in the laboratory. The non LAB bacteria (*Micrococcus* and *Staphylococcus* sp) were grown overnight at 37°C in Nutrient broth in order to achieve the cell concentration of 10<sup>8</sup>CFU/ml, while the LAB (*Lactobacillus*, *Lactococcus* and *Pediococcus* sp) and yeasts (*Saccharomyces* and *Candida* sp) were maintained in the MRSB and yeast extract glucose peptone (YEGPB) broth at 37°C respectively prior to analysis. Meanwhile, the food pathogens used in this study were *Salmonella typhimurium* S1000, *Listeria monocytogenes* L55, *Escherichia coli* O157: H7 and *Staphylococcus aureus* S277. All bacterial pathogens were cultured in tryptone soy broth (TSB) in aerobic condition at 37°C.

### ***Acid tolerance test***

The method was according to Psomas *et al.* (2001) with minor modifications. The respective overnight cultures (LAB, bacteria and yeast) with a cell concentration of 10<sup>8</sup> CFU/ml were prepared respectively in MRSB, NB and YEGPB broth tubes with pH adjusted to 1.5, 2.0, 3.0 and 5.0 (with 3N HCl). The tubes were then incubated at 37°C for 3 hours. About 20µl of the sample solution was spread onto the surface of MRS, nutrient agar and yeast extract agar plates and the colony counts were measured as CFU/ml. Survival rate was calculated according to the following equation:

$$\text{Survival rate (\%)} = (\log \text{CFU } N_1 / \log \text{CFU } N_0) \times 100\%$$

Where N<sub>1</sub> represents the total viable count of tested strains after treatment, N<sub>0</sub> represents the total viable count before treatment.

### ***Bile tolerance test***

The ability of the isolates to grow in the presence of bile was determined using the method recommended by Vinderola & Reinheimer (2003) with minor modifications. The bile salt solutions were prepared using oxygall powder (Sigma) at final concentrations of 0.1, 0.3, 0.5 and 1.0%. In addition, sterile distilled water without oxygall was used as control. All solutions were autoclaved and the solutions (10 ml) were transferred into sterile test tubes. The cell suspension containing 10<sup>8</sup> CFU/ml was added to the solutions and incubated at 37°C for 12 hours. A total of 1ml cultures were serially diluted and incubated on the respective agar plates at 37°C for another 24 hours in order to determine their CFU/ml. The survival rate was calculated using the above equation.

***Antimicrobial activity of the isolates***

The antimicrobial activity of the tested strains was induced using agar spot method described by Uhlman *et al.* (1992). Bacterial pathogens namely *Salmonella typhimurium* S1000, *Listeria monocytogenes* L55, *Staphylococcus aureus* S277 and *Escherichia coli* O157: H7 were used. The overnight cultures were grown in MRSB (LAB), NB (bacteria) and YEGPB (yeast) at 37°C and the cultures were subsequently centrifuged at 2400 x g for 15 min. The supernatants were then neutralized with sterile 5 M NaOH and heated for 5 min to inactivate residual viable cells. A sterilized paper disc (6 mm) was immersed in these cell-free microbial supernatants and imprint them on the agar's surface containing selected pathogens before incubating overnight at 37°C under aerobic condition. The diameter of the inhibition zone surrounding the paper discs was then measured.

***Statistical analysis***

All data were expressed as mean  $\pm$  SD of three replicates. Student's t test was used to determine the differences in means. All statistics were performed using Statistical Package for the Social Sciences (SPSS) 11.0 software (SPSS, Inc).

**RESULTS AND DISCUSSION*****Acid and bile tolerance test***

A total of 82 isolates (52.7%) exhibited positive results in the preliminary screening test for both pH and bile resistance effect. The majority of the isolates were from *Lactobacillus plantarum* (LP), *Lactococcus lactis* subsp. *lactis* (LL), *Staphylococcus arlettae* (SA), *Saccharomyces cerevisiae* (SC) and *Candida glabrata* (CG). All the tested strains, except the SA, had residual microbial counts greater than  $10^7$ CFU/ml after 3 hours of incubation. The survival rate of the SA strain was 66.3%, which is relatively lower than the other tested strains used in this study (Table 1). Meanwhile, the LP1 and LP2 strains exhibited different survival rates when treated with different pH. This clearly indicated that the tolerance of the tested strains to low pH was strain-specific. The exposure to low pH used in this study was selective enough to differentiate the candidate for acid tolerance under extreme conditions (pH 2-3) that usually retard the growth of most microorganisms. Nevertheless, the microbial count increased once the pH level was increased. The results are in agreement with Wang *et al.*, (2010), who found *Lactobacilli* strains remained viable after exposure to pH of 2.5-4.0. In addition, it was observed that the LAB strains grew better at low pH as compared to non-LAB (SA) and yeasts (SC and CG) strains. This was owing to the ability of the *Lactobacillus* to withstand stressful conditions and survive for longer periods in highly acidic environments. Therefore, the acid tolerance of these bacteria (*Lactobacilli*) was a prerequisite for their use as dietary adjuncts in most fermented foods.

In the bile salts test, all the tested strains were able to grow at 0.3% bile salt, a concentration which mimics the human gastrointestinal tract (GI). It was observed that the microbial counts of the tested strains decreased when the concentration of the bile increased (Table 1). Bile usually exhibits specific and non-specific defense mechanisms of the gut against intestinal micro flora, the magnitude of its inhibitory effects is however determined by the concentration of the bile salts (Chateris *et al.*, 1998). In addition, most of the tested strains were resistant to bile even at concentrations higher than 0.3%. This phenomenon was related to the ability of the tested strains to hydrolyze the combined bile salt and reduce the toxic effect of the bile salt via bile salt hydrolase (BSH) activity (De Smet *et al.*, 1995).

**Table 1. Effect of acid and bile salt at different concentrations on tested microbial strains (log CFU/ml).**

Strains	Initial mean counts <sup>a</sup> (log CFU/ml)	Resistance to pH				Bile tolerance (%)			
		1.5	2.0	3.0	5.0	0.1	0.3	0.5	1.0
LP1	9.21±0.12	7.95±0.21 (86.3%) <sup>b</sup>	8.22±0.17 (89.2)	8.73±0.11 (94.8)	8.95±0.13 (97.2)	9.17±0.12 (99.6)	9.05±0.16 (98.3)	8.54±0.13 (92.7)	8.07±0.05 (87.6)
LP2	8.97±0.15	7.74±0.18 (86.2)	8.05±0.26 (89.7)	8.45±0.23 (94.2)	9.03±0.19 (97.3)	9.08±0.21 (98.9)	8.67±0.19 (96.6)	8.35±0.34 (93.1)	8.16±0.14 (90.9)
LL2	9.04±0.13	7.63±0.20 (84.4)	8.25±0.22 (91.3)	8.81±0.10 (97.5)	9.12±0.15 (99.3)	9.16±0.25 (99.1)	8.90±0.28 (98.5)	8.46±0.22 (93.6)	8.23±0.15 (91.0)
SC3	8.93±0.06	7.16±0.24 (80.2)	7.97±0.08 (89.2)	8.40±0.30 (94.1)	8.84±0.27 (98.9)	8.87±0.07 (99.3)	8.56±0.22 (95.9)	8.33±0.08 (93.3)	8.05±0.09 (90.1)
CG2	9.07±0.14	7.23±0.26 (79.7)	7.56±0.31 (83.4)	8.42±0.05 (92.8)	8.96±0.16 (98.8)	9.14±0.13 (99.0)	9.08±0.04 (96.8)	8.87±0.18 (94.5)	8.21±0.26 (89.4)
SA	8.86±0.11	5.87±0.05 (66.3)	6.04±0.2 (68.2)	7.36±0.29 (83.1)	8.15±0.22 (91.2)	8.95±0.03 (98.8)	8.72±0.23 (97.3)	8.56±0.1 (95.5)	7.08±0.17 (79.9)

<sup>a</sup> – Each value represents the mean value ± standard deviation (SD) from three trials

<sup>b</sup> - Figures in brackets represent the survival rate of each strain

LP1 – *Lactobacillus plantarum* 1; LP2- *L. plantarum* 2; LL2- *Lactococcus lactis* subp *lactis* 2; SC3 – *Saccharomyces cerevisiae* 3; CG2 – *Candida glabrata* 2; SA – *Staphylococcus arlettae*

**Antimicrobial activity of the tested isolates**

The use of LAB as natural bioprotective agents has been well documented in several studies due to their effectiveness in treating various food borne pathogens, especially *Staphylococcus aureus*, *E. coli*, and *Listeria monocytogenes*. (Vesterlund *et al.*, 2005; Vinderola & Reinheimer, 2003). In this study, the antimicrobial effects of the tested isolates against selected food borne pathogens were determined (Table 2). All the cell-free supernatants were shown to inhibit the growth of both Gram positive and Gram negative pathogens used in this study. The LP1, SC3 and SA strains exhibited the greatest inhibitory effect against *Listeria monocytogenes* L55 and *Staphylococcus aureus* S277 as compared to the other tested strains. This could be due to the production of bio-substances with bactericidal or bacteriostatic activities, such as bacteriocin, organic acids, and low molecular weight peptides that are inhibitory to the pathogens (Lefteris *et al.*, 2006). The effect of LAB especially *Lactobacillus* sp in eliminating the pathogenic bacteria in fermented dairy products has been well-documented (Mathara *et al.*, 2008). However, this study revealed that the yeast strains, SC3 and CG2, showed good inhibitory effects against indicator pathogens. This indicates that the yeasts may serve as bio control agents, as most of them were non-pathogenic and did not produce mycotoxins or allergenic spores. The inhibitory effect of the yeast strains might be due to the cell wall proteins, which could be proteases since the presence of the serine protease inhibitor (PmSF) abolished the inhibitory action (Tasteyre *et al.*, 2002).

**Table 2. Antimicrobial activity<sup>a</sup> of the selected probiotic strains against food pathogens.**

Strains	<i>Listeria monocytogenes</i> L55	<i>Staphylococcus aureus</i> S277	<i>Salmonella typhimurium</i> S1000	<i>Escherichia coli</i> O157:H7
LP1	+++	++	+	+
LP2	++	++	+	+
LL2	++	++	+	+
SC3	+++	++	+	+
CG2	++	++	+	+
SA	+++	++	++	+

Zone of inhibition: + 7-9mm; ++ 10-12mm; +++ >13mm ; a – Antibacterial zone: Diffusion diameter formed by colony –diameter of paper disc (6mm)

LP1 – *Lactobacillus plantarum* 1; LP2- *L. plantarum* 2; LL2- *Lactococcus lactis* subsp *lactis* 2; SC3 – *Saccharomyces cerevisiae* 3; CG2 – *Candida glabrata* 2; SA – *Staphylococcus arlettae*

**CONCLUSION**

The present study revealed that the LP1, LP2, LL2, SC3, CG2 and SA strains were identified as potential probiotic strains as they were tolerant to acid, bile salt and exhibited broad inhibitory effect against selected food borne pathogens. The results obtained via this *in vitro* study serve to select potential strains to be incorporated as starter cultures for food fermentation in a controlled environment. Further study should focus on the characterisation of the antimicrobial activities and adhesion properties of the pre-selected probiotic candidates prior to *in vivo* investigation using cell lines and animal models.

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