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Screening of *Lactobacillus* Strains Against *Salmonella* Both Isolated from Malaysian Free-Range Chicken Intestine for Use as Probiotic

(Penyaringan Strain *Lactobacillus* Melawan *Salmonella* yang Kedua-duanya Dipencilkan daripada Usus Ayam Kampung Malaysia untuk Kegunaan Sebagai Probiotik)

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ABSTRACT

A total of eight strains of Lactobacillus *and two strains of* Salmonella *were isolated from free-range Malaysian chickens intestine. Evaluation based on* in vitro *studies included aggregation, co-aggregation, growth with bile salts, tolerance to acidic pH, and inhibitory activity were carried out. The isolated* Lactobacillus *were* Lactobacillus fermentum *IA,* Lactobacillus fermentum *IB,* Lactobacillus fermentum *IC,* Lactobacillus fermentum *ID,* Lactobacillus salivarius *subsp. salicinus IE,* Lactobacillus salivarius *subsp.* salicinus *IF,* Lactobacillus salivarius *subsp.* salivarius *IG, and* Lactobacillus *spp. IH. The corresponding isolated* Salmonella *were* Salmonella *spp. 3B21 and* Salmonella *spp. 1A12. The ability of aggregation and also tolerance to pH 2.5 are found in* Lactobacillus fermentum *ID,* Lactobacillus salivarius *subsp.* salicinus *IF,* Lactobacillus salivarius *subsp. salivarius IG, and* Lactobacillus *spp. IH. The isolate most resistance to 1% bile salts is* Lactobacillus fermentum *ID but observed to be weak in inhibitory activity against* Salmonella *spp. The best co-aggregation and strongest inhibitory activity against* Salmonella *spp. was observed in* Lactobacillus salivarius *subsp.* salivarius *IG. Despite being not so resistant in the presence of bile salts 0.5 and 1% (w/v), the lag time in the presence of bile salts 0.3% (w/v) of* Lactobacillus salivarius *subsp.* salivarius *IG and also for* Lactobacillus *spp. IH are the shortest. Based on good aggregation properties, the best co-aggregation, tolerance to acidic pH 2.5 and bile salts 0.3% (w/v) and strongest inhibitory activity against* Salmonella *spp.,* Lactobacillus salivarius *subsp.* salivarius *IG comes out as the best candidate as probiotic for chicken.*

Keywords: Inhibitory activity; Lactobacillus*; Malaysian free-range chicken;* Salmonella

ABSTRAK

Sebanyak lapan strain Lactobacillus *dan dua strain* Salmonella *dipencilkan daripada usus ayam kampung Malaysia. Penilaian berdasarkan kajian* in vitro *seperti ujian agregasi, koagregasi, kerintangan terhadap garam hempedu, kerintangan terhadap pH asid, dan ujian aktiviti perencatan telah dilakukan. Pencilan* Lactobacillus *tersebut ialah* Lactobacillus fermentum *IA,* Lactobacillus fermentum *IB,* Lactobacillus fermentum *IC,* Lactobacillus fermentum *ID,* Lactobacillus salivarius *subsp.* salicinus *IE,* Lactobacillus salivarius *subsp.* salicinus *IF,* Lactobacillus salivarius *subsp.* salivarius *IG, dan* Lactobacillus *spp. IH. Sedangkan pencilan salmonella yang didapatkan ialah* Salmonella *spp. 3B21 and* Salmonella *spp. 1A12. Kemampuan agregasi dan juga ketahanan terhadap pH 2.5 dijumpai pada* Lactobacillus fermentum *ID,* Lactobacillus salivarius *subsp.* salicinus *IF,* Lactobacillus salivarius *subsp.* salivarius *IG, dan* Lactobacillus *spp. IH. Pencilan yang paling tahan terhadap garam hempedu 1% ialah* Lactobacillus fermentum *ID, tetapi* Lactobacillus *tersebut menunjukkan aktiviti perencatan yang lemah terhadap* Salmonella *spp. Koagregasi terbaik dan aktiviti perencatan yang paling kuat terhadap* Salmonella *spp. dijumpai pada* Lactobacillus salivarius *subsp. salivarius IG. Meskipun tidak begitu tahan di dalam kehadiran garam hempedu 0.5 dan 1% (w/v), masa lag* Lactobacillus salivarius *subsp.* salivarius *IG dan juga* Lactobacillus *spp. IH di dalam kehadiran garam hempedu 0.3% (w/v) adalah yang paling singkat. Berdasarkan ciri-ciri agregasi yang baik, koagregasi yang terbaik, kerintangan terhadap pH 2.5 dan garam hempedu 0.3% (w/v), serta aktiviti perencatan yang paling kuat terhadap* Salmonella *spp.,* Lactobacillus salivarius *subsp.* salivarius *IG keluar sebagai calon terbaik probiotik ayam.*

Kata kunci: Aktiviti perencatan; ayam kampung Malaysia; Lactobacillus*;* Salmonella

INTRODUCTION

The reason for the isolation and identification of *Lactobacillus* strains from free-range Malaysian chicken were to screen for potential probiotic strains that have the specific association with *Salmonella* species prevalent in the Malaysian poultry industry. We believe that *Lactobacillus* isolated from free range chicken has more potential then that isolated from broiler chicken. Problems with *Salmonella* have occurred over the past few decades, and these problems have been addressed using several

means. For the past four decades, antibiotics have been supplemented to animal and poultry feed to improve growth performance and efficiency and protect animals from adverse effects of pathogenic and non-pathogenic enteric microorganisms (Ferket et al. 2002). There are also reports that antibiotics could increase the colonization of the chicken gut by salmonellae, creating a potential public health problem (Fuller 1999). The feeding of antibiotics also resulted in the retention of antibiotics in animal tissue, imbalances in normal intestinal flora, reduced beneficial intestinal microbial populations, and the generation of antibiotic-resistant bacteria (Reid & Friendship 2002; Schneitman 2002). To overcome these problems, efforts have been directed towards the development and use of probiotics in food animals (Reid & Friendship 2002).

A probiotic is a "live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller 1989). Probiotics reacts to intestinal pathogens by the production of antibacterial compounds, including lactic and acetic acid antibiotic-like substances, competition for nutrients, and adhesion sites, increased and decreased enzyme activity, increased antibody levels and increased macrophage activity (Hose and Sozzi 1991). Probiotic supplementation of the intestinal microflora in poultry, especially with *Lactobacillus* species, showed beneficial effects on resistance to infectious agents such as *Escherichia coli* (Jin et al. 1996), *Salmonella* sp. (Pascual et al. 1999), *Campylobacter* sp. (Stern et al. 2001) & *Eimeria acervulina* (Dalloul et al. 2005).

Lan et al. 2003 have demonstrated that there is a difference in probiotic characteristics of *Lactobacillus* strains within the same species but from different origin. As such we believe that probiotic strains for chicken should originated from chicken in the same environment.

Screening and application of probiotic *Lactobacillus* isolated from chicken have been widely studied, but the study using free-range Malaysian chicken has never been fully investigated. This paper reports the potential of a local *Lactobacillus* isolate as probiotic for chicken based on aggregation, co-aggregation, bile resistance, and tolerance to acidic pH were tested. Inhibitory activity of *Lactobacillus* strains against two strains of *Salmonella* spp. based on an agar spot test, well diffusion assay, and blank disc method were also investigated.

Materials and Methods

Isolation and Identification of *Lactobacillus*

The entire intestine of a Malaysian chicken was removed from the body cavity, aseptically minced, diluted 1/10 (peptone 0.1% w/v, NaCl 0.85% w/v) and homogenized using a blender for 2 min. Serial 10-fold dilution from the homogenate were made and plated on deMan, Rogosa and Sharpe (MRS) agar (Merck). The incubation was carried out anaerobically for 2 d at 37°C. After incubation, bacterial isolates were randomly sampled and subcultured on MRS broth (Merck) at 37°C in anaerobic conditions. Bacterial isolates that were gram-positive and catalase-negative rods were selected for further identification by the API 50CHL kit system (BioMerieux, France). All isolates were identified by using the API WEB software version 5.0 from BioMerieux and *Bergey's Manual of Determinative Bacteriology* (Buchanan & Gibson 1974) for comparison of assimilation and/or fermentation pattern. All identified strains were kept at -70°C in MRS broth with glycerol (30% v/v). Lactobacilli were activated and grown in a MRS medium.

Isolation and Identification of *Salmonella*

The homogenate were incubated for 24 h at 37°C. One portion of the homogenate was diluted 1/10 into 10 mL of tetrathionate broth and incubated at 37°C for 24 h, and a second portion was diluted 1/10 into 10 mL Rappaport-Vassiliadis broth and incubated at 37°C. After 24 h, 10 µL of each culture were streaked onto one plate each of brillian green agar (Oxoid) and xylose lysine desoxycholate agar (Oxoid). Plates were incubated for 18 to 24 h at 37°C before assessment for the presence of characteristic presumptive *Salmonella* colonies. At least one presumptive *Salmonella* colony was chosen from every plate containing presumptives. These colonies were grown overnight on brain heart infusion (BHI) agar (Oxoid) at 37°C prior to confirmation. Isolates were screened using the urease and oxidase test, followed by API 20E analysis (BioMerieux, France) for all urease- and oxidase-negative isolates (Plummer et al. 1995). All identified strains were kept at -70°C in BHI broth with glycerol (30% v/v). Subculture in BHI medium (24 h, 37°C) were made before use in the experiment.

Aggregation Test

Aggregation test was performed as described by Jankovic et al. (2003). The aggregation phenotype was scored positive if the overnight cultures were clear with cells clumped at the bottom of the tube (Figure 2). The strains were considered nonaggregating if the overnight cultures were turbid.

Co-aggregation Test

The co-aggregation test was performed as described by Handley et al. (1987). Suspensions of *Lactobacillus* spp. and *Salmonella* spp. were adjusted to an optical density (OD) of 0.5 ± 0.02 measured at 600 nm. A suspension (0.5 mL) of the *Salmonella*, and a similar suspension (0.5 mL) of the test *Lactobacillus* sp., were mixed in a glass test tube, mixed thoroughly using a vortex. Control tubes contained 1.0 mL of a suspension of each bacterial species. The OD of the bacterial mixture and for the bacterial suspension alone were measured at 600 nm after incubation at 37°C for 4 h. The percentage of co-aggregation was calculated using the equation:

$$\frac{(A+B)/2 - C}{(A+B)/2} \times 100.$$

where A and B represent the OD (600 nm) in control tubes of containing only *Lactobacillus* spp. or *Salmonella* spp., respectively, after 4 h incubation; C represents the OD (600 nm) of the mixed culture after the same period of incubation. The experiment was repeated three times with duplicates each time.

TOLERANCE TO LOW PH

Tolerance of the isolates to low pH was tested as follows: overnight cultures of the isolated strains were centrifuged at 4000 rpm for 15 min at 4°C. After resuspending the pellet in the same buffer of saline solution, it was diluted 1/10 in sterile phosphate-buffered saline (PBS) at pH2.0, 2.5, 3.0 and 3.5. After 3 h at 37°C, the appropriate dilutions were plated on MRS agar and incubated at 37°C for 48 h. This method was a modification based on Gusils et al. (2002) and Ehrmann et al. (2002). All tests were carried out in triplicate.

BILE SALTS RESISTANCE

The *Lactobacillus* strains were grown overnight in MRS broth and 10 mL of the culture suspensions adjusted the optical density to 0.5 ± 0.02 at 600 nm were inoculated into 250 mL Erlenmeyer flasks containing 100 mL of MRS broth with 0.1, 0.3, 0.5 and 1% (w/v) bile salts (Oxoid) or without bile salts (which acted as controls). The absorbance at 600 nm was adjusted to 0.5 ± 0.02 in order to standardise the number of bacteria (10^7-10^8 CFU mL^{-1}). The cultures were incubated in shaker incubator (Infors HT, Multitron) at 37°C. Absorbance was measured at 600 nm at 2, 4, 6, 8, 10, 12, 18 and 24 h against the corresponding non inoculated blanks. The experiment was repeated twice and each reading represents the mean of three observations.

INHIBITORY ACTIVITY

For detection of inhibitory activity, the agar spot test, the well diffusion assay, and the paper blank disc method were used. These methods were modification based on those described by Schillinger & Lucke (1989), Jin et al. (1996), and Nowroozi et al. (2004). For the agar spot test, overnight culture of *Lactobacillus* spp. were spotted (3 mm) onto the surface of MRS agar (Merck) plates and incubated anaerobically in anaerobic jar for 24 h at 37°C to allow colonies to develop. Approximately 5×10^9 CFUs of *Salmonella* spp. in 15 mL of BHI agar (Merck) were poured on the plates in which *Lactobacillus* was grown. After incubation for 24 h at 37°C, the radius of the clear inhibition zone around *Lactobacillus* was recorded. The test for each *Lactobacillus* strain against *Salmonella* spp. was carried out three times with duplicates each time.

For the well diffusion assay, plates containing solidified Nutrient Agar (20 mL) overlaid with 12 mL of Nutrient agar (1% agar in Nutrient Broth, Merck) were inoculated with 75 µL of an overnight culture of *Salmonella* spp. Five wells, four at periphery and one at the centre, each 5 mm in diameter, were made in the agar using a cork borer and 100 µL of cell-free culture supernatant (CFCS) of a *Lactobacillus* strain were transferred into each periphery well. At the centre, 100 µL MRS broth was transferred into the well as a control. The plates were incubated aerobically for 24 h at 37°C, and then observed for clear inhibition zones around the well. The test for each cell-free culture supernatant (CFCS) of *Lactobacillus* strain against *Salmonella* spp. was carried out three times with duplicates each time.

For the blank disc method, five sterile paper blank discs, four at periphery and one at the centre, were placed on the Nutrient agar (Merck) in which 50 µL of an overnight culture of *Salmonella* spp. was inoculated. Fifty microlitres of cell-free culture supernatant (CFCS) of a *Lactobacillus* strain were dropped into each periphery paper blank disc. At the centre, 50 µL MRS broth was transferred into the paper blank disc as a control. The plates were incubated aerobically for 24 h at 37°C, and then observed for clear inhibition zones around the paper blank discs. The test for each cell-free culture supernatant (CFCS) of *Lactobacillus* strain against *Salmonella* spp. was carried out three times with duplicates each time.

PREPARATION OF CELL-FREE CULTURE SUPERNATANT (CFCS)

The *Lactobacillus* strains were grown anaerobically in MRS broth (Merck) for 24 h at 37°C. Bacterial cells were removed by centrifuging the culture at 4000 rpm for 15 min at 4°C. The supernatant was sterilized by filtration (0.22 µm-pore size, cellulose acetate filter, Millipore) and used immediately or stored at – 70°C until use within 24 h.

RESULTS AND DISCUSSION

LACTOBACILLUS AND *SALMONELLA* ISOLATION

Twenty-eight isolates of *Lactobacillus* were isolated on MRS medium from chicken intestine. Eight of them were selected for further assays because of their ability to inhibit indicator strains (*Salmonella* spp.). They were four strains of *Lactobacillus fermentum*, two of *Lactobacillus salivarius* subsp. *salicinus*, one of *Lactobacillus salivarius* subsp. *salivarius*, and one of *Lactobacillus* spp. (Table 1). For isolation of *Salmonella* from the same source, a total two *Salmonella* spp. isolates (Table 1) were isolated.

According to (Hammes & Hertel 2006; Walter 2005), *Lactobacillus* species commonly detected in the gastrointestinal tract of chicken were *Lactobacillus acidophilus*, *Lactobacillus crispatus*, *Lactobacillus gallinarum*, *Lactobacillus johnsonii*, *Lactobacillus animalis*, *Lactobacillus salivarius*, *Lactobacillus agilis*, *Lactobacillus aviarius*, *Lactobacillus reuteri*, *Lactobacillus fermentum*, and *Lactobacillus brevis*.

TABLE 1. Isolated *Lactobacillus* and *Salmonella* strains

Code	Biochemical identification
IA	Lactobacillus fermentum
IB	*Lactobacillus fermentum*
IC	*Lactobacillus fermentum*
ID	*Lactobacillus fermentum*
IE	*Lactobacillus salivarius* subsp. *salicinus*
IF	*Lactobacillus salivarius* subsp. *salicinus*
IG	*Lactobacillus salivarius* subsp. *salivarius*
IH	*Lactobacillus* spp.
3B21	*Salmonella* spp.
1A12	*Salmonella* spp.

Therefore, our findings are in agreement with the above references.

AGGREGATION TEST

Of the 8 lactobacilli isolated from gastrointestinal (GI) tracts of free-range Malaysian chicken, aggregation activity was found in 6 isolates (Table 2). The results suggest that these *Lactobacillus* strains have the ability to aggregate in the GI tract. Huis in't Veld et al. (1994) suggested that one of the proposed mechanisms that could increase the potential of bacteria to survive and persist in the GI tract is their ability to aggregate. Therefore, the six isolates have potential to survive. These capabilities may be due to a secreted protein of 32 kDa as reported by Reniero et al. (1992). This protein is found in supernatant and acts as aggregation-promoting factor (APF).

CO- AGGREGATION TEST

Co-aggregation between *Lactobacillus* and *Salmonella* shows low percentage (Table 2). The co-aggregation percentage of *Lactobacillus salivarius* subsp. *salivarius* IG with the two *Salmonella* strains showed the highest percentage (12.4 and 13.8%).

Co-aggregation tests represent simple and reliable methods applicable to a large number of the test strains for screening *lactobacillus* as reported by Gusils et al. (1999). These properties are thought to be linked to the ability to interact closely with undesirable bacteria. Because of co-aggregation percentage is high, *Lactobacillus salivarius* subsp. *salivarius* IG is thought to be very potential as probiotic for chicken comparing to the others. Co-aggregation data may explain that *Lactobacillus salivarius* subsp. *salivarius* IG have the ability to trap *Salmonella* in the GI tract.

TOLERANCE TO LOW PH (2–3.5)

Table 2 shows that all the tested strains survived 3h of exposure at pH3 and 3.5. However, none of the strains tested survived at pH2. On the other hand, there were four strains that survived at pH2.5, i.e. *Lactobacillus fermentum* ID, *Lactobacillus salivarius* subsp. *salicinus* IF, *Lactobacillus salivarius* subsp. *salivarius* IG, and *Lactobacillus* spp. IH. Jacobsen et al. (1999) suggested that the pH of 2.5 seemed to be damaging to the bacteria. Thus, the four strains exhibited good pH tolerance for probiotic use.

BILE SALTS RESISTANCE

The results show that bile salts exerted a slight inhibitory effect on the growth of the 8 *Lactobacillus* strains. *Lactobacillus fermentum* ID is the isolate most tolerant to bile salts 0.5 and 1% because its lag time is the shortest, whilst the lag time for *Lactobacillus salivarius* subsp. *salicinus* IE is the longest. In the presence of 0.3% bile salts, the growth of *Lactobacillus salivarus* subsp. *salivarius* IG and *Lactobacillus* spp. IH showed shorter lag time than the others (Figure 1-3). This result further support our previous data, thus *Lactobacillus salivarius* subsp. *salivarius* IG has good probiotic characteristic. This is because resistance

TABLE 2. Aggregation, co-aggregation and tolerance to low pH of *Lactobacillus* strains isolated from free-range Malaysian chicken intestine

	Lactobacillus strains							
	IA	IB	IC	ID	IE	IF	IG	IH
Aggregation	+ [a]	- [b]	-	+	+	+	+	+
Co-aggregation with:								
Salmonella 3B21	2.9±2.3	1.9±1.4 [c]	2.3±1.1	3.4±1.2	3.4±2.2	6.9±1.6	12.4±1.3	6.4±1.9
Salmonella 1A12	1.0±2.0	1.2±1.7	2.8±2.5	3.6±3.7	5.9±4.6	4.5±29	13.8±2.1	5.1±2.8
Tolerance to low pH	-	-	-	-	-	-	-	-
2	-	- [e]	-	+	-	+	+	+
2.5	+	+	+	+	+	+	+	+
3	+ [d]	+	+	+	+	+	+	+
3.5								

a. aggregating; b. nonaggregating; c. mean of co-aggregation percentage (%) ± standard deviation
d. indicates growth; e. indicates no growth

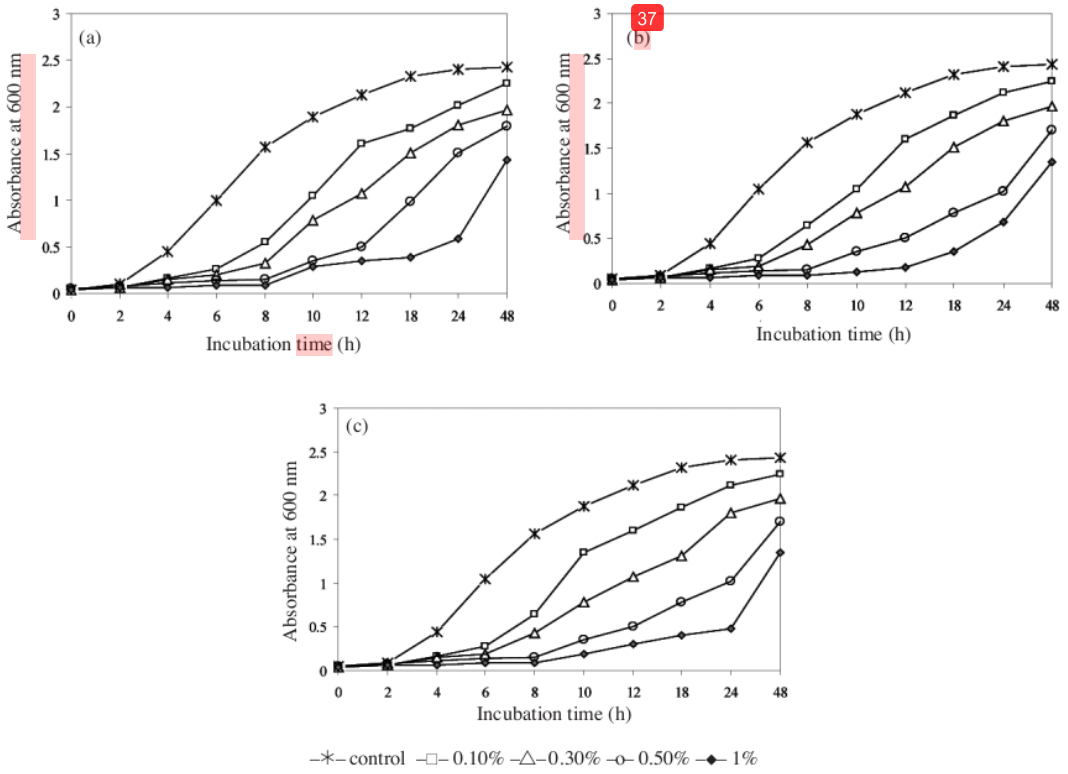


FIGURE 1. The growth profiles of *Lactobacillus* strains (a-c) on MRS broth as measured by the absorbance at 600 nm in the presence of 0.1-1.0% (w/v) bile salts. Control cultures without bile salts. (a) *Lactobacillus fermentum* IA, (b) *Lactobacillus fermentum* IB and (c) *Lactobacillus fermentum* IC

to pH and bile salts is of great importance in survival and growth of probiotic in the intestinal tract (Havenaar et al. 1992). As it is related to specific enzyme activity-bile salt hydrolase (BSH) which helps hydrolyze conjugated bile, thus reducing its toxic effect (Du Toit et al. 1998).

INHIBITORY ACTIVITY

The inhibitory activity of *Lactobacillus* isolates against *Salmonella* spp. is presented in Table 3 and Figure 4. A total of eight *Lactobacillus* strains isolated were found to produce inhibition zones against the two strains of *Salmonella* spp. Based on an agar spot test, the radii of their inhibition zones ranged from 4.8 to 12.4 mm. *Lactobacillus salivarius* subsp. *salivarius* IG was found to be the most effective in inhibiting the growth of *Salmonella* spp. with 10.6 and 12.4 mm of clear inhibition zone against the two *Salmonella* strains, whereas *Lactobacillus fermentum* IC was the least effective.

The growth of the two *Salmonella* strains was also inhibited by the cell-free culture supernatant (CFCS) of the eight *Lactobacillus* strains. Based on well diffusion assay and blank disc method data in Table 3, the result also showed that the CFCS of *Lactobacillus salivarius* subsp. *salivarius* IG exhibited the highest inhibition to growth of the two *Salmonella* strains based on the size of inhibition zones. This indicates that inhibitory factor(s) secreted into environment.

Our results are in agreement with the report of Gariga et al. (1998) and Walter (2005) that *Lactobacillus salivarius* as the predominant species among gastrointestinal microbiota of young chickens. Pascual et al. (1999) too reported good potential of *Lactobacillus salivarius* to reduce *Salmonella enteritidis* colonization *in vivo*. Together with its ability to colonize the gastrointestinal tract of chicken after a single inclusion in the feed mixture, highlights it as a suitable strain for widespread use in the avian industry in order to minimize *Salmonella* colonization.

The antibacterial properties of lactobacilli have been related to their metabolic products such as organic acids, bacteriocins, and hydrogen peroxide (Ehrmann et al. 2002). The ability of the lactobacilli to produce toxic metabolites such as lactic acid, hydrogen peroxide, and bacteriocin has been suggested as be responsible for their ability to inhibit other bacteria (Juven et al. 1992). Langhendries et al. (1995) reported that lactobacilli exert their protective or therapeutic effect through reduction of gut pH by stimulating the lactic acid producing microflora.

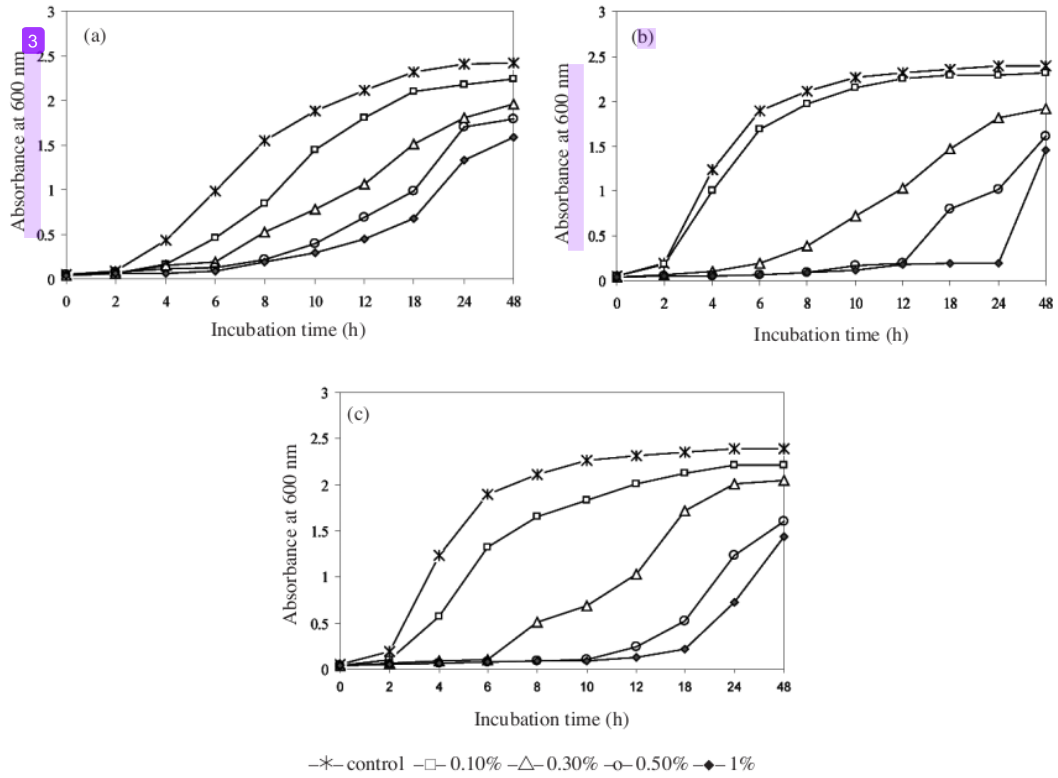


FIGURE 2. The growth profiles of *Lactobacillus* strains (a-c) on MRS broth as measured by the absorbance at 600 nm in the presence of 0.1-1.0% (w/v) bile salts. Control cultures without bile salts. (a) *Lactobacillus fermentum* ID; (b) *Lactobacillus salivarius* subsp. *salicinus* IE and (c) *Lactobacillus salivarius* subsp. *salicinus* IF

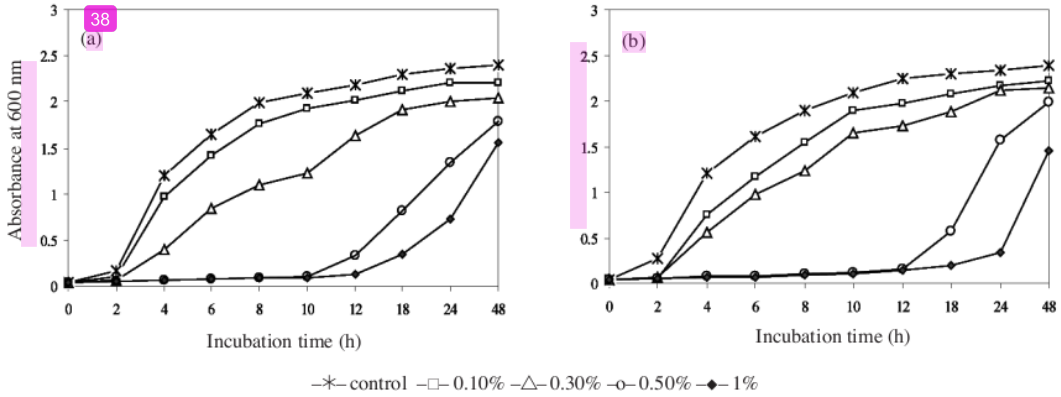
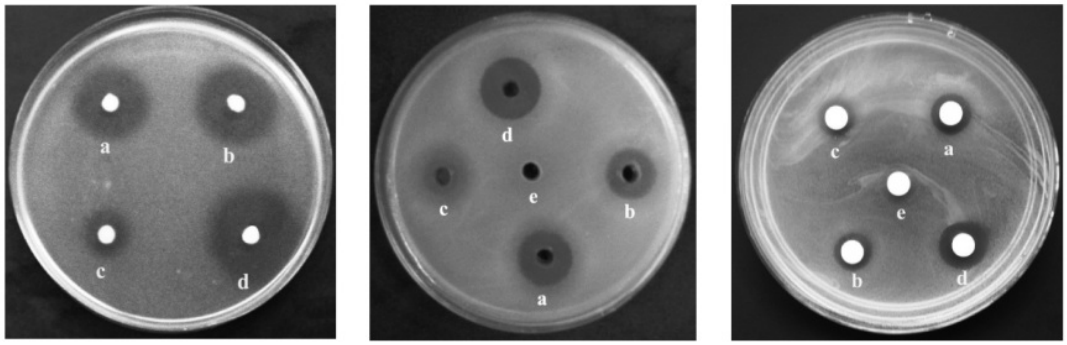


FIGURE 3. The growth profiles of *Lactobacillus* strains (a-b) on MRS broth as measured by the absorbance at 600 nm in the presence of 0.1-1.0% (w/v) bile salts. Control cultures without bile salts.
(a) *Lactobacillus salivarius* subsp. *salivarius* IE and (b) *Lactobacillus* spp. IH

TABLE 3. Inhibitory activity of intestinal *Lactobacillus* spp. against *Salmonella* spp. of Malaysian free range chicken intestine

Lactobacillus isolates	Radius of clear inhibition zones (mm) of *Salmonella* spp.					
	Agar spot test		Well diffusion assay		Blank disc method	
	Salmonella 3B21	*Salmonella* 1A12	*Salmonella* 3B21	*Salmonella* 1A12	*Salmonella* 3B21	*Salmonella* 1A12
Lact. fermentum IA	5.2	6.3	5.7	5.3	5.3	5.7
Lact. fermentum IB	5.3	6.1	6.0	6.4	5.0	5.6
Lact. fermentum IC	4.8	5.5	4.9	5.8	4.2	5.1
Lact. fermentum ID	5.7	5.6	5.5	6.0	5.2	5.9
Lact. salivarius ss. *salicinus* IE	10.3	10.9	7.8	8.3	6.5	7.0
Lact. salivarius ss. *salicinus* IF	9.9	10.1	8.0	8.8	6.3	6.5
Lact. salivarius ss. *salivarius* IG	10.6	12.4	8.4	8.9	7.3	8.7
Lact. spp. IH	10.0	11.5	6.9	7.8	6.2	7.2

Each value was the mean of three repeat experiments with a duplicate each



(a) (b) (c)

FIGURE 4. Inhibition zone of *Salmonella* spp. 3B21 against *Lactobacillus* strains by agar spot test (a) well diffusion assay, (b) blank disc method (c). a. *Lactobacillus salivarius* subsp. *salicinus* IF; b. *Lactobacillus* spp. IH; c. *Lactobacillus fermentum* ID; d. *Lactobacillus salivarius* subsp. *salivarius* IG; e. MRS broth.

However, characterization of the inhibitory substance(s) was not carried out in this study. Makras et al. (2006) reported that the antibacterial activity of *L. acidophilus* IBB 801, *L. amylovorus* DCE 471, *L. casei* Shirota and *L. rhamnosus* GG was solely due to the production of lactic acid. The same result has been reported by Jin et al. (1996), that inhibitory activities of *Lactobacillus* culture supernatant against pathogen bacteria were not due to the production of hydrogen peroxide or bacteriocin but probably due to the production of organic acids. Makras et al. (2006) suggested that lactic acid produced was responsible for significant inhibitory activity upon invasion of *Salmonella* into Caco-2/TC7 cells.

CONCLUSION

Eight of twenty eight isolates of *Lactobacillus* isolated from free-range Malaysian chickens intestine were identified and evaluated based on *in vitro* studies that include aggregation, co-aggregation, growth with bile salts, tolerance to acidic pH, and inhibitory activity. *Lactobacillus salivarius* subsp. *salivarius* IG were found as the best candidate as probiotic for chicken based on good aggregation properties, strongest co-aggregation, tolerance to acidic pH (2.5) and bile salts (0.3%) and strongest inhibitory activity against *Salmonella* spp.

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