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Screening of Lactic Acid Bacteria Isolated from Chicken Ceca for *In vitro* Growth Inhibition of *Salmonella enteritica* Serovar Enteritidis

[1]Preeyaporn Surachon, [2]Peerapol Sukon, [1]Prapansak Chaveerach,
[1]Panya Waewdee and [1]Chaiyaporn Soikum
[1]Department of Veterinary Public Health, [2]Department of Anatomy,
Faculty of Veterinary Medicine, Khon Kaen University, 40002 Khon Kaen, Thailand

Abstract: Salmonellosis in humans caused by consumption of contaminated poultry products with *Salmonella enteritica* serovar enteritidis (*S. enteritidis*) is still a public health problem. Many efforts have been developed to eradicate or reduce Salmonella loads in poultry industry. Use of normal microbiota (e.g., Lactic Acid Bacteria (LAB)) against the pathogen is an alternative of antibiotics used and is under extensive investigations. Therefore, the objective of this study was to screen and identify the LAB strain showing the greatest growth inhibition against *S. enteritidis*. LAB were isolated from chicken ceca of five clinically healthy broilers (age, 42-50 days). The bacteria were grown in MRS broth and on the plate with selective media Rogosa agar. For screening of the inhibitory effects of the isolated LAB against *S. enteritidis*, we used disc diffusion and agar well diffusion methods. In this study, 56 isolates exhibited inhibitory effect against *S. enteritidis* but only thirteen isolates producing a clear zone as large as 19 mm or greater were selected for acid tolerance test. In this test, three isolates did survive at pH 2.5 for 18 h but only 1 isolate was subjected for evaluation by coculture with *S. enteritidis* and for 16S rDNA sequencing. This isolate was able to grow in the coculture medium and at the same time, inhibited the growth *S. enteritidis*. This isolate was identified as *Lactobacillus salivarius* TP4.2-2.

Key words: Chicken, lactic acid bacteria, *Lactobacillus salivarius*, *Salmonella enteritidis*, growth inhibition, Thailand

INTRODUCTION

S. enteritidis is a causative agent of gastroenteritis in humans worldwide. The infected patient often receives the pathogen through, consumption of contaminated (usually raw or undercook) poultry products (European Food Safety Authority, 2009). However in chickens, *S. enteritidis* does not cause severe symptoms but can efficiently invade various internal organs including ovaries causing the infection in the eggs thus asymptomatic infected chickens play an important role in Salmonella propagation. Many strategies (e.g., biosecurity, vaccination, acidification of feed and drinking water, modification of the diet and use of feed additives such as probiotics, prebiotics and synbiotics) have been developed to eradicate or reduce Salmonella in chickens at farm level (Vandeplas *et al*., 2010). Although, these strategies are associated with significant reduction in the incidence of Salmonella outbreaks in humans, *S. enteritidis* remains a problem in poultry industry and no strategies guarantee the complete eradication of the pathogen from the farm. According to the World Health Organization and the Food and Agriculture Organization of the United Nation, probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host. Among bacterial probiotics, LAB especially *Lactobacillus* sp. play an important role in health and function of human and animal Gastrointestinal (GI) tracts. LAB have ability to ferment, produce lactic acid and antimicrobial substances and colonize GI tract of the host (De Keersmaecker *et al*., 2006; Jin *et al*., 1996; Lima *et al*., 2007; Stern *et al*., 2006). In poultry industry, LAB have been used as probiotics for growth promotion and control of intestinal pathogens (e.g., *Salmonella* sp.) for several decades (Mead, 2000) and they are still under currently extensive investigations (Angelakis and Raoult, 2010; Musikasang *et al*., 2009; Taheri *et al*., 2009). However, such use is still not universal acceptance because, unlike the use of antibiotics, the results still vary. These variations may arise from many factors such as probiotic itself, dose and route of administration and host factors (e.g., age, breed, health status and husbandry) (Chichlowski *et al*., 2007). Screening or selecting LAB as

Corresponding Author: Peerapol Sukon, Department of Anatomy, Faculty of Veterinary Medicine, Khon Kaen University, 40002 Khon Kaen, Thailand

a source of chicken probiotics is a primarily crucial step for the success in poultry industry. Several criteria have been used for example, colonization ability, antibacterial effects against enteric pathogens, resistance to bile salts and acidic pH and aggregation (Taheri *et al*., 2009).

In chickens, cecum is a fermentation vat resulting in the highest number of microbial population in the gastrointestinal tract and is the most common site infected with enteric pathogens such as *Salmonella* sp. (Jozefiak *et al*., 2004). These pathogens are secreted with the manure resulting in environment contamination.

Therefore, the objective of this study was to screen LAB isolated from the cecum of clinically healthy mature chickens with the screening criteria focused primarily on anti *S. enteritidis* and ability to survive in acidic pH.

MATERIALS AND METHODS

Isolation of LAB: In this study, 5 clinically healthy broilers (age, 42-50 days) were used for LAB isolation. The broilers were free of *S. enteritidis* infection. Use of animals was approved by Animal Ethic Committee of Khon Kaen University. The broilers were killed by cervical dislocation then the abdomen was exposed and each cecum in both sides was removed aseptically. The cecal content was used for bacterial isolation. The content (1 g) was transferred into 9 mL of MRS broth (Oxoid, Hampshire, England) and incubated in microaerophilic environment with BD GasPack (Becton, Dickinson and Company, Sparks, MD, USA) at 37°C for 48 h. The growing bacteria were then spread-plated onto selective media Rogosa agar (Oxoid, Hampshire, England). The plate was incubated microaerophilically at 37°C for 48 h. Colonies were randomly selected from each agar plate and then were purified by subculturing in MRS broth and re-streaking onto MRS agar 3 times.

Characterization of LAB

Gram staining and bacterial morphology: A loopful of each pure culture was placed on a clean glass slide. Then, it was allowed to air-dry and fixed over Bunsen-burner flame. The slide was flooded with crystal violet for 1 min, washed off and flooded with gram's iodine for 1 min, washed off and flooded with acetone alcohol for 15 sec and counter stained with safranin solution for 30 sec. The slide was air-dry and observed under the microscope with oil-immersion lens (x1000 magnification). Only gram-positive isolates were selected for Catalase test.

Catalase test: A loopful of each selected gram-positive isolate was aseptically transferred into 3% hydrogen peroxide on a glass slide. It was observed for air bubbles (positive result) or no air bubbles (negative result). At this stage, only gram-positive and catalase-negative isolates were selected and stored at -70°C in MRS broth containing 20% (v/v) glycerol for further use. The isolates were subcultured twice before all of the following tests.

Inhibitory effects of the isolated LAB against *S. enteritidis*

Preparation and pH measurement of culture supernatant: The supernatants of all 56 isolates of LAB were prepared by modification of the methods described previously (Chaveerach *et al*., 2004). Each isolate from the stock was grown in the tube containing MRS broth, incubated at 37°C under microaerophilic condition for 48 h and then the tube was centrifuged at 4500 rpm at 4°C for 15 min. The supernatant was filtered through filter paper (pore size, 0.2 micron) and divided into 2 portions: the first for pH measurement by using pH meter, the second for agar disc diffusion and agar well diffusion tests.

Preparation of *S. enteritidis*: In this study, researchers used *S. enteritidis* isolated from 3 days old broiler chicks (the bacterial serotype was confirmed by Thailand National Institute of Animal Health). *S. enteritidis* was grown in tryptic soy broth (Hardy Diagnostics, Santa Maria, CA, USA) at 37°C for 24 h until reached concentration of 1×10^{8} cfu mL^{-1} (equivalent to MacFarland standard No. 0.5) and then diluted to 10^{6} cfu mL^{-1} by using 0.1% peptone water for further use.

Agar disc diffusion test: The surface of a plate containing MH agar (Hardy Diagnostics, Santa Maria, CA, USA) was swabbed with tryptic soy broth containing *S. enteritidis* 10^{6} cfu mL^{-1}. A paper disc (diameter, 6 mm) was soaked with 20 μL of each supernatant as described previously. About 7 soaked paper discs were placed on the surface of each plate (6 at the periphery and 1 at the center). The plates were incubated at 37°C for 24 h. Each plate was examined for clear inhibition zones around the wells. Diameter of the clear zone was measured by using a vernier caliper.

Agar well diffusion test: Methods for preparing this test were similar with those of the agar disc diffusion test except using a paper disc. In agar well diffusion test, 7 wells (6 at the periphery and 1 at the center each 6 mm in diameter) were made in the agar plate and 80 μL of the culture supernatant of LAB was transferred into each well. The plates were incubated at 37°C for 24 h. Each plate was examined for clear inhibition zones around the wells. Diameter of the clear zone was measured by using a vernier caliper.

Acid tolerance test: The samples of isolated LAB for acid tolerance test were selected from those that showed anti *S. enteritidis* by agar well diffusion method with diameter of clear zone, 19 mm or greater. The acid tolerance test was modified from the previous study (Ehrmann *et al*., 2002). The selected isolates were grown in MRS broth and then were centrifuged at 4500 rpm for 15 min at 4°C. The pellet was collected in a sterile tube and was washed twice with PBS, pH 7.0 before inoculation in MRS broth adjusted to pH 2.5 (by addition of 1 M HCl). The surviving cells were counted by plating on MRS. Only 1 strain with the greatest survival from acid tolerance test was selected for the next steps.

Bacterial identification: The selected strain was identified by 16S rDNA sequencing which was done by BIOTECH culture collection (Thailand Science Park, Thailand). This method included: PCR amplification of 16S rDNA; direct sequencing of 16S rDNA and sequence analyses.

Coculture growth curves: The interference of the selected strain (*L. salivarius*) with the growth of *S. enteritidis* was done by modification of the previous study (Drago *et al*., 1997). A bottle containing 5 mL of MRS broth and 5 mL of MH broth was inoculated with 10^8 cfu mL^{-1} of both the *L. salivarius* and *S. enteritidis*. The tubes were incubated at 37°C under continuous agitation and microaerophilic conditions for 24 h and 48 h. Then, the serial 10-fold dilutions were plated on MRS agar to evaluate the *L. salivarius* growth or on XLD agar (Difco, Becton Dickinson, Sparks, MD, USA) to evaluate *S. enteritidis* growth. The MRS agar plates were incubated at 37°C in under microaerophilic environment for 48 h but the XLD agar plates were incubated at 37°C for 24 h. Pure cultures of each strain were also subjected to the same conditions and used as controls. Additionally, pH of the culture solution was measured at 24 and 48 h after coincubation.

Statistical analysis: Fisher's exact test was used to compare number of isolates with ability to produce a clear zone (positive result) between agar disc diffusion and agar well diffusion tests. For assessing the association between diameter of growth inhibition of *S. enteritidis* and pH of supernatant of LAB strains, data of both parameters are not normally distributed. Therefore, Speaman's rho correlation coefficient was used for determining the association. All statistical analyses were done by using SPSS Version 17 (SPSS Inc, Chicago).

RESULTS

Isolation and characterization of LAB: After the colonies were randomly selected from each agar plate and then were purified by subculturing in MRS broth and re-streaking onto MRS agar 3 times, 56 strains of LAB were isolated. All isolated strains had the characteristics of turbid-white round colonies (diameters, 0.1-0.4 mm) when examined with naked eye, rods and non-motile when examined under the light microscope, gram-positive and catalase-negative.

Inhibitory effects of isolated LAB against *S. enteritidis*: After 56 isolates of LAB were tested by using disc diffusion and agar well diffusion methods against *S. enteritidis*, results of the test were showed in Table 1. It was found that number of isolates producing inhibition zones were observed significantly more in agar well diffusion method (54/56 isolates) but less in disc diffusion method (37/56) ($p<0.001$). In addition, decrease in pH of supernatant of LAB was associated with increase in diameter of growth inhibition of *S. enteritidis* (Fig. 1). At

Table 1: Disc diffusion and agar well diffusion methods for LAB test against *S. enteritidis*

	Methods	
Inhibition zone	Disc diffusion	Agar well diffusion
No	19	2
Yes	37	54

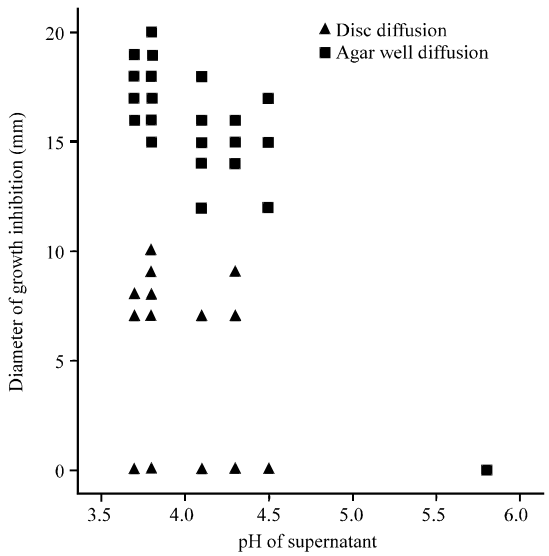

Fig. 1: Correlation between diameter of growth inhibition and pH of supernatant. In both agar disc diffusion and agar well diffusion tests, decrease in pH of supernatant of LAB is significantly associated with increase in diameter of growth inhibition of *S. enteritidis* (Speaman's rho correlation coefficient = – 0.548 ($p<0.001$) for agar disc diffusion test and = – 0.628 ($p<0.001$) for agar well diffusion test)

Table 2: Results of coculture test between *L. salivarius* and *S. enteritidis*

	Control				Coculture of *L. salivarius* and *S. enteritidis*		
Time (h)	*L. salivarius* (log cfu mL^{-1})	pH	*S. enteritidis* (log cfu mL^{-1})	pH	*L. salivarius* (log cfu mL^{-1})	*S. enteritidis* (log cfu mL^{-1})	pH
0	7.38	ND	8.14	ND	7.38	8.14	ND
24	7.27	3.92	8.74	5.44	7.24	4.05	4.01
48	6.32	3.81	5.48	4.96	6.05	2.53	3.87

Values of log cfu mL^{-1} and pH are the average values from two replicates, ND = Not Determined

this step, thirteen isolates with the greatest inhibition zone 19-20 mm from an agar well diffusion test were selected.

Acid tolerance test: Of thirteen selected isolates, three isolates survived at pH 2.5 for 18 h but only 1 strain with the greatest survival was selected.

Coculture growth curve: *S. enteritidis* was significantly reduced a ter co-incubation with the selected LAB strain for 24 and 48 h. However, pH in both coculture and in pure culture slightly decreased at 24 and 48 h after incubation. Data from coculture growth curve study were showed in Table 2.

Bacterial identification: Only 1isolate surviving at pH 2.5 for 18 h was subjected for 16 rDNA sequence. This isolate was 99.9% identity with *Lactobacillus salivarius*. With the assigned strain, it was called as *Lactobacillus salivarius* TP4.2-2.

DISCUSSION

In this study, we found that *L. salivarius* isolated from chicken ceca not only strongly inhibited *S. enteritidis* in various *in vitro* tests but also capably survived at pH 2.5 for at least 18 h. The results confirmed and extended the previous *in vitro* studies (Ehrmann *et al*., 2002; Lima *et al*., 2007; Miyamoto *et al*., 2000; Nouri *et al*., 2010).

Although among the, potential probiotics, LAB are reported to have important effects in poultry (Ehrmann *et al*., 2002; Taheri *et al*., 2009), there are several factors associated with the success of probiotics used in animals (Chichlowski *et al*., 2007).

These factors establish criteria that should be used to select appropriated LAB. *In vitro* tests as selection criteria are necessary to reduce the number of strains and find the most effective organisms because *in vivo* tests are time-consuming and often expensive. In *in vitro* tests, there are several criteria used for the selection such as acid and bile tolerance tests, anti-pathogenic tests, aggregration tests, adhesion tests, colonization tests (Taheri *et al*., 2009) however in this study, we focused specifically on the selection of LAB for anti *S. enteritidis* which is an importantly zoonotic pathogen causing salmonellosis in humans.

In this study although, we did not know the exact mechanisms how LAB inhibited *S. enteritidis in vitro*, decrease pH of the supernatant of LAB was associated with increase diameter of inhibition zone. Thus, this result indicated that lowering pH of the supernatant (probably due to lactic acid) might play a role in inhibiting *S. enteritidis*.

This result was similar with that of the previous report (Taheri *et al*., 2009). However, we cannot rule out other mechanisms; for example, *L. salivarius* can produce antimicrobial compounds called bacteriocin (Stern *et al*., 2006).

In this study, we also found that *L. salivarius* did survive and was able to inhibit the growth of *S. enteritidis* in coculture medium. *L. salivarius* can be found as a normal flora in many parts of chicken's gastrointestinal tracts (Abbas Hilmi *et al*., 2007; Miyamoto *et al*., 2000). In addition, *L. salivarius* can prevent *S. enteritidis* colonization in chickens (Pascual *et al*., 1999). Due to the different amounts of the supernatant used in each method, it is not surprising that a diameter of an inhibition zone in agar disc diffusion method is narrower than that in agar well diffusion method.

The diameter or radius usually varies from studies to studies (Cadirci and Citak, 2005). For acidic pH tolerance tests, the previous studies normally incubated the bacterial dilutions with acid solution for a few hours (Ashraf *et al*., 2009; Jacobsen *et al*., 1999; Taheri *et al*., 2009) but in this study we allowed the incubation time lasting for 18 h in order to keep the number of the selected LAB strains at minimum.

There were several tests (e.g., acid and bile tolerance tests, anti-pathogenic tests, aggregration tests, adhesion tests and colonization tests) suggested for *in vitro* screening of LAB toward their selection as a source of chicken probiotics (Taheri *et al*., 2009) but the selection criteria of LAB in this study were focused primarily on

how to select the best strain capable for inhibiting *S. enteritidis* therefore, this study had some limitations.

CONCLUSION

This study showed that *L. salivarius*, one of the LAB isolated from chicken ceca, strongly inhibited *S. enteritidis* in various *in vitro* tests indicating its potentials for further investigations toward its selection as a source of chicken probiotics.

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