



Heterophil Phagocytic Activity Stimulated by *Lactobacillus salivarius* L61 and L55 Supplementation in Broilers with *Salmonella* Infection

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ABSTRACT: Newborn chicks are susceptible to *Salmonella enterica* serovar Enteritidis (SE). The objective of this study was to evaluate the effect of *Lactobacillus* probiotic isolated from chicken feces on heterophil phagocytosis in broiler chicks. A total of 150 newborn broiler chicks were divided into 5 groups (30 chicks per group) as follows: group 1 (normal control), given feed and water only, group 2 (positive control) given feed, water and SE infection, group 3 (L61 treated) given feed, water, SE infection followed by *Lactobacillus salivarius* L61 treatment, group 4 (L55 treated) given feed, water, SE infection followed by *L. salivarius* L55 treatment, and group 5 given feed, water, SE infection followed by *L. salivarius* L61 + L55 combination treatment. After SE infection, *L. salivarius* treatment lasted for 7 days. The results showed that *L. salivarius* L61 and *L. salivarius* L55 treatment, either alone or combination of both, increased the survival rate after SE infection, and upregulated heterophil phagocytosis and phagocytic index (PI). Conversely, chick groups treated with *Lactobacillus* showed lower SE recovery rate from cecal tonsils than that of the positive control group. The PI values of the chicken group with SE infection, followed by the combination of *L. salivarius* L61 and *L. salivarius* L55 were the highest as compared to either positive control or normal control group. Two *Lactobacillus* strains supplementation group showed significantly ($p < 0.05$) higher PI value at 48 h than 24 h after treatment. (**Key Words:** Heterophil, *Lactobacillus*, *Salmonella* Enteritidis, Broiler Chicks)

INTRODUCTION

Foodborne illness is usually caused by pathogenic bacteria contamination in food of animal origin such as *Salmonella*. Salmonellosis in poultry is mainly caused by *Salmonella enterica* serovar Enteritidis (SE) that can survive in the digestive tract of poultry for several months and contaminate both meat and eggs. Reduction of *Salmonella* infection in poultry is very important to minimize the loss of chicks and to prevent contamination of meat. Due to an overuse of antibiotic to treat diseases in animals, pathogens have developed a high resistance to antibiotics. *Salmonella* isolated from chicken has been reported resistant to many antibiotics such as tetracycline, erythromycin, and colistin (Cardoso et al., 2006). To avoid

antibiotic residue in meat for consumption, animal rearing has turned to the use of alternatives to antibiotics, such as herbs and probiotics. The largest group of microorganisms used for probiotics is lactic acid-producing bacteria, such as *Lactobacillus* which is a normal flora found in the gastrointestinal tract of animals including humans (Gilliland et al., 1975). It has been recognized as a safe alternative. To qualify *Lactobacillus* strains as probiotics, *in vivo* studies are necessary to clarify their basic properties including survival in the gut environment, antagonistic property to other intestinal pathogens and the ability to adhere to the intestinal epithelial cells (Nousiainen and Setälä, 1998). However, some studies have reported that probiotic supplementation in chicken feed did not affect their growth (O'Dea et al., 2006). Limitation of probiotics used in animals may be due to uncertainty of their source and whether they specific to a particular animal species. Adhesion to animal epithelial cells of the gastrointestinal tract is supposed to determine host-specificity (Piątek et al., 2012). Immune response of broilers to SE infection has

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been reported in chicks aged 1 to 7 days after hatching; it is sensitive to infection and the broilers still may have a high mortality rate (Barrow et al., 1987). However, many studies reported a probiotic effect that reduces SE infection but there still is a lack information regarding some immune responses such as phagocytic activity in broiler chicks (Bai et al., 2013). Therefore, this study investigated the effect of a *Lactobacillus* strain isolated from chicken feces that had basic probiotic properties and anti-*Salmonella* activities *in vitro* (Sornplang and Leelavatcharamas, 2010), on treating of SE infection and also the effect on phagocytic activity of heterophils in broiler chicks.

MATERIALS AND METHODS

Bacterial strains

SE was isolated from a broiler chicken farm in Khon Kaen province, Thailand. *Lactobacillus salivarius* L61 and *L. salivarius* L55 were isolated from chickens feces, identified by nucleotide sequence analysis of 16S rRNA genes compared to GenBank with 99% to 100% similarity (accession number: CP0003233.1) and these strains can inhibit *Salmonella* growth *in vitro*, reported in our previous study (Sornplang and Leelavatcharamas, 2010). Both SE and *Lactobacillus* isolates were kept as freeze dried specimens at -70°C in a freezer in the Department of Veterinary Public Health, Faculty of Veterinary Medicine, Khon Kaen University, Thailand.

Experimental design

A total of 150 newborn Arbor Acres chicks of mixed sex were randomly divided into 5 groups (6 pens per group and 5 chicks/1.4×1.5 m/pen). Each group was raised in the open house system under natural conditions. Feed and water were provided *ad libitum* throughout the experimental period. The feeding trial lasted for 7 days. Nutrition provided by feed was as recommended by the National Research Council (1994) with no antibiotics and *Salmonella* free. Chicks were challenged with SE by oral gavage of 50% lethal dose (10^6 colony forming unit, CFU in 0.1 mL) (Pascual et al., 1999). The experimental groups were as follows: Group 1 provided feed and water only, group 2 provided water, feed and SE challenged (positive control group), group 3 provided water, feed, SE challenged and one hour later, *L. salivarius* L61 was supplemented by oral gavage at 10^9 CFU (Chen et al., 2012), group 4 provided water, feed, SE challenged and one hour later, *L. salivarius* L55 was supplemented by oral gavage at 10^9 CFU and group 5 provided water, feed, SE challenged and one hour later *Lactobacillus* L61 combination with L55 strains was supplemented by oral gavage at 10^7 CFU. Twenty four and 48 hours after *Lactobacillus* gavage, one chick was randomly selected from each of the 6 pens per treatment (6

chicks/treatment). Each chick was slaughtered by cervical dislocation and examined for the presence of SE. The experimental protocol was approved by Khon Kaen University Animal Ethics Committee.

SE detection and isolation

Cecal tonsil samples were dissolved in the tetrathionate enrichment broth (catalog no. 210420, Becton Dickinson, Sparks, Maryland, USA) and streaked on xylose lactose differential agar plate containing novobiocin 25 $\mu\text{g}/\text{mL}$ and nalidixic acid 20 $\mu\text{g}/\text{mL}$. These plates were incubated at 37°C for 24 hours and then checked for SE (Higgins et al., 2007).

Heterophil isolation

Blood samples were randomly collected from ulnar vein for one chick from each of the 6 pens per treatment (6 chicks/treatment) at 24 and 48 hours after the treatment. Heterophils were isolated by the method of Lowry et al. (2005). In brief, 1 mL of blood sample was collected in a test tube containing ethylene diamine tetra-acetic acid, and red blood cells were sedimented with 3% Dextran 500 (Amersham Biosciences Co., Ltd., Uppsala, Sweden). The heterophil rich suspension was mixed with a discontinuous Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO, USA) and centrifuged at $250\times g$ for 30 min. The purity of the heterophil suspension consisted of 90% heterophils and 10% macrophages and >95% viable as determined by microscopic examination of Wright-stained smears.

Heterophil phagocytosis

Heterophils were isolated from peripheral blood and adjusted to 5×10^6 cells/mL, combination of live SE of 5×10^7 cells/mL (ratio 1:10). The protocol was as described by Lowry et al. (2005). Briefly, the mixture was centrifuged and incubated at 39°C in 5% CO_2 for 1 h. Samples were submerged in an ice bath for 15 min to stop phagocytosis. Samples were pelleted by centrifugation, supernatants were decanted, and cell/bacteria pellets were resuspended in ice-cold clear RPMI 1640 (Carlsbad, CA, USA) and washed three times by repeating this process. Gentamicin solution was added to remove residual extracellular SE at the second wash. Sediment cells of the blood were smeared on a slide, stained with Wright's stain and examined microscopically. The results were expressed as percentage of heterophils with bacteria and phagocytic index (PI), where $\text{PI} = (\text{the percentage of heterophils containing bacteria}) \times (\text{the average number of bacteria per ingesting heterophil})$. Phagocytosis and PI values were counted from 200 heterophil cells in different microscope fields.

Bacterial preparation

Stock freeze-dried culture of *Lactobacillus* was

Table 1. Number of surviving chicks after *Salmonella* Enteritidis (SE) challenge and fed *Lactobacillus* in posthatch chicks

Treatments	Numbers of survival chick (%)		
	24 h	48 h*	7 d**
1. Feed and water only (basal diet, B)	29/30 (96.67)	22/23 (95.65)	14/16 (87.50)
2. B+SE challenged (positive control group)	18/30 (60.00)	7/12 (58.33)	0/1 (0.00)
3. B+SE+ <i>L. salivarius</i> L61	29/30 (96.67)	23/23 (100)	16/17 (94.12)
4. B+SE+ <i>L. salivarius</i> L55	29/30 (96.67)	22/23 (95.65)	15/16 (93.75)
5. B+SE+ <i>L. salivarius</i> L61+ <i>L. salivarius</i> L55	30/30 (100)	24/24 (100)	18/18 (100)

* Total chicks per group were counted at 24 h survival and excluded from randomly slaughtered for each group of 6 chicks.

** Total chicks per group were counted at 48 h survival and excluded from randomly slaughtered for each group of 6 chicks.

dissolved into 0.4 mL de Man, Rogosa, and Sharpe (MRS) broth. It was cultured in MRS agar, and then incubated at 37°C overnight or for 18 hours and bacterial growth (adjusted to 10⁹ CFU/mL) was measured by the Optical Density (OD₆₀₀). Stock culture of SE was inoculated into tryptic soy agar (Difco, Sparks, Maryland, USA) containing nalidixic acid, incubated at 37°C for 18 hours and dissolved in 0.1% peptone water to measure the OD (adjusted to 10⁶ CFU/mL).

Statistical analysis

Observation values of the mortality, SE recovery, phagocytosis and PI of chicks in each group was analyzed by analysis of variance. The means from these values among groups were further analyzed by Tukey HSD test at the 0.05 level of significance in a completely randomized design.

RESULTS AND DISCUSSION

This study showed that in the chicks group SE challenged without *Lactobacillus* treating the mortality was 100% by 7-day-old. Chick groups with SE challenged and treated by *Lactobacillus* exhibited significantly higher survival rate at 1, 2, and 7 days post-treatment compared to the SE challenge group and normal control group (Table 1). Chicks with SE challenged and supplemented with two *Lactobacillus* strains (L61+L55) showed 100% survival up to 7 days. Kramer et al. (2001) reported that chicks challenged with SE at the age of 1 day survived for 28 days; the mortality rate was higher than the chicks challenged with SE at the age of 7 days. This may be due to the

immune system of 1-day-old chicks that are only partially developed as a similar study of Chen et al. (2012) who reported a cytokine stimulating neutrophils function was lower expression in neonate chicks than the older chicks. Heterophils of broiler chicks have been reported to move into the abdomen in significantly increased quantities after SE challenge (Swaggerty et al., 2005). In this study, survival rate of chicks depended on SE recovery in cecal tonsils. Chicks with SE challenge and treated by *Lactobacillus* groups showed significantly lower SE recovery in cecal tonsils compared to positive control group and group 1 (Table 2). This study showed one chick infected with SE in the non-infected control group (Table 2). This result can be explained by some broiler chicks being naturally contaminated with *Salmonella* from their environment, such as bedding materials (Gustafson and Kobland, 1984).

Day-of-hatch chicks inoculated with SE had no SE detected in their cecal tonsils when supplemented with probiotics for 24 h (Higgins et al., 2010) or for 3 d (Chen et al., 2012) before the SE inoculation. Our study showed a success of the probiotic treating SE infection in chicks that reduced SE in cecal tonsil when treated by probiotic within 1 h after the infection. Supplementation of *Lactobacillus* L61 and *Lactobacillus* L61 together with *Lactobacillus* L55 can eliminate SE infection at 48 and 24 h post-treatment, respectively. Phagocytosis is an innate immunity, first response to body inflammation or infection. In present study, chick heterophils were activated which challenged with SE. Although, some infected chicks may show a heterophil engulfed SE before treating with the *Lactobacillus* but all phagocytose heterophils engulfed only one SE per one

Table 2. The *Salmonella* Enteritidis (SE) recovery from cecal tonsils of experimental chicks (6 chicks/group) at 24 h and 48 h after treatment

Treatments	Number of chicks with SE found per chicks examined (%)	
	24 h	48 h
1. Feed and water only (basal diet, B)	0/6 (0)	1/6 (16.67)
2. B+SE challenged (positive control group)	6/6 (100)	6/6 (100)
3. B+SE+ <i>Lactobacillus salivarius</i> L61	1/6 (16.67)	0/6 (0)
4. B+SE+ <i>L. salivarius</i> L55	2/6 (33.33)	1/6 (16.67)
5. B+SE+ <i>L. salivarius</i> L61+ <i>L. salivarius</i> L55	0/6 (0)	0/6 (0)

Table 3. Effects of *Lactobacillus* treatment on chicks' heterophil phagocytosis at 24 h and 48 h after *Salmonella* Enteritidis (SE) infection

Treatments	Percentage of heterophils+SE		Average SE: heterophil		Phagocytic index	
	24 h	48 h	24 h	48 h	24 h	48 h
1. Feed and water only (basal diet, B)	60	62	1.3	1.27	78.00	78.74
2. B+SE challenged (positive control group)	50	51	1.12	1.12	56	57.12
3. B+SE + <i>Lactobacillus salivarius</i> L61	74	75	2.30	2.29	170.20*	171.75*
4. B+SE + <i>L.salivarius</i> L55	72	73	2.19	2.19	157.68*	159.87*
5. B+SE + <i>L.salivarius</i> L61+ L55	91	96	4.78	5.20	434.98*	499.20*/**

* Indicates significant ($p<0.05$) differences were observed between normal control, or positive control and treated in each column.

** Indicates significant ($p<0.05$) differences were observed between 24 and 48 h treated in each row.

heterophil (data not shown). Chick groups with SE challenged and treated by *Lactobacillus* supplementation can significantly improve ($p<0.05$) phagocytosis and PI value at 24 and 48 h after treatment compared to positive control group and normal control group (Table 3). In this study, supplementation of *Lactobacillus* probiotic at 1 h post SE challenged can eradicate SE infection in broiler chicks by 24 h after treatment. This result was different from the study of Higgins et al. (2010) who reported *Lactobacillus* treatment after 24 h of *Salmonella* infection cannot reduce SE in chicks. The results of *Lactobacillus* supplementation within 1 h post SE infection of chicks in our study indicated that this was an advantageous time to eliminate the SE infection. Multi-strains or high dose ($\geq 10^6$ CFU/mL) of probiotic supplementation showed a positive effect on eradication of intestinal infection as a similar report of Khodadad et al. (2013). In this study, chick heterophils were stimulated efficiently during 24 to 48 h post-treatment. Chick group with SE challenge and treated by combination of *Lactobacillus* L61 and *Lactobacillus* L55 exhibited a significantly higher PI value at 48 h than that the PI value at 24 h post-treatment ($p<0.05$). In addition, supplementation with two strains of *Lactobacillus* in this study eradicated SE infection in chicks by 48 h after treatment. This result is due to the greater innate immune response to SE in probiotic-fed chickens. In conclusion, supplementation of *Lactobacillus* probiotic (*Lactobacillus* L61 or L55) can treat *Salmonella* infection in young chicks when supplemented within 1 h after infection. This result indicates that *Lactobacillus* probiotic may be used to prevent *Salmonella* infection in young chicks when supplemented at an optimal time of posthatch to 2-d-old chicks because their heterophils were more stimulated at this time.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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