

Effect of Organic Acids and Probiotics on *Salmonella enteritidis* Infection in Broiler Chickens

A.D. Wolfenden¹, J.L. Vicente^{1,3}, J.P. Higgins¹, R.L. Andreatti Filho²,
S.E. Higgins¹, B.M. Hargis¹ and G. Tellez¹

¹Department of Poultry Science, University of Arkansas, Fayetteville AR 72701, USA

²College of Veterinary Medicine and Animal Science (FMVZ),

Sao Paulo State University (UNESP), Botucatu, SP, Brazil, 18618-000

³Sigrah Zellet de Mexico S.A. de C.V., Mariano Escobedo No. 10, Col.
Tezontepec, Cuernavaca Morelos, Mexico 62250

Abstract: The effect of an organic acid mixture (OA) and a *Lactobacillus*-based probiotic culture on *Salmonella enteritidis* (SE) infection in broiler chicks was evaluated. In exp. 1, chicks were challenged by oral gavage with SE, held in chick boxes for 2 h and randomly assigned to either untreated control or continuous OA treatment in the drinking water. Crop and cecal tonsils were cultured at 48 h and 5 d post-challenge for recovery of SE. Recovery of SE in the crop and cecal tonsils at 48 h was significantly ($p < 0.05$) lower in the OA treated group as compared to control chickens but not different at 5d. In exps.2 and 3, chicks were SE challenged, held in chick boxes for 2 h and randomly assigned to either untreated control, probiotic, OA, or probiotic+OA. After 24 or 48 h, crop and cecal tonsils were cultured for the presence or absence of SE. After 24 h, probiotic or probiotic+OA significantly reduced SE recovery from the crop as compared to controls. All treatments reduced SE recovery from the cecal tonsils at 24 h. While no significant differences were observed in SE recovery from crop at 48 h, SE recovery from probiotic and or probiotic+OA groups was significantly lower than the controls in the cecal tonsils. These data suggest that combination treatment with the selected OA and *Lactobacillus*-based probiotic culture is more effective than individual treatment for *Salmonella* reduction in chicks.

Key words: *Salmonella*, organic acid, probiotic, *Lactobacillus*

Introduction

Increased pressure by consumers and regulatory agencies for reduced or even elimination of the use of antibiotics in food producing animals has created a need to find alternatives to maintain healthy and productive animals. The use of certain lactic acid bacteria as probiotics has been proposed for many years. These probiotic bacteria have been shown to prevent enteric disease, as well as, improve the overall health of poultry (Tellez *et al.*, 2006). Another alternative to antibiotics is the use of certain organic acids. Direct acidification of the water with organic acids could significantly reduce the amount of recoverable *Salmonella* on the carcasses or in the crops and cecal tonsils when used during the pre-slaughter feed withdrawal period (Byrd *et al.*, 2001; Jarquin *et al.*, 2007). In the present study a commercially available *Lactobacillus*-based probiotic culture and water acidifier were used in combination to reduce *Salmonella enteritidis* in the crop and cecal tonsils of broiler chicks.

Materials and Methods

Salmonella amplification: A primary poultry isolate of *Salmonella enteritidis* (SE), phage type 13A, was originally obtained from the National Veterinary Services Laboratory (Ames, Iowa). This isolate was selected for

resistance to nalidixic acid (NA)¹. For these experiments, *Salmonella* was grown in tryptic soy broth (TSB) for approximately 8 h. The cells were washed three times with 0.9% sterile saline by centrifugation (3,000 x g) and the approximate concentration of the stock solution was determined spectrophotometrically. The stock solution was serially diluted and confirmed by colony counts of three replicate samples (0.1 mL/replicate) that were spread plated on xylose lactose differential agar (XLD)² plates containing 25 µg/mL novobiocin (NO)³ and 20 µg/mL NA. The colony-forming units of *Salmonella* determined by spread plating were reported as the concentration of *Salmonella* (in cfu/mL) for *in vitro* experiments and total colony-forming units for *in vivo* challenge experiments.

Probiotic culture: Eleven lactic acid bacterial isolates, of poultry gastrointestinal origin, were previously selected and described (Higgins *et al.*, 2005). This commercial product (FM-B11™)⁴ was diluted in reconstituted powdered skim milk to an expected concentration of 4 x 10⁶ cfu/mL for oral gavage of chicks for these studies. Actual cfu administered per chick from each experiment were determined retrospectively from spread plating on Mann Rogosa sharp agar⁵.

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Table 1: *S. enteritidis* recovery from crop and cecal tonsils of broiler chicks at 24 h and 48 h post-treatment

Experiment	Treatment	24 hours		48 hours	
		Crop	Cecal tonsils	Crop	Cecal tonsils
1	Control	ND ¹	ND	100 % (20/20)	100 % (20/20)
	Organic Acids	ND	ND	75 % (15/20)*	55 % (11/20)*
2	Control	100 % (20/20)	60 % (12/20)	100 % (20/20)	70 % (14/20)
	Probiotic	75% (15/20)*	25 % (5/20)*	90 % (18/20)	20 % (4/20)*
	Organic Acids	100 % (11/11)	45 % (5/11)*	100 % (11/11)	91 % (10/11)
	Probiotic and Organic Acids	40 % (6/15)**	13% (2/15)*	87 % (13/15)	20 % (3/15)*
3	Control	ND	75 % (15/20)	100 % (20/20)	100 % (20/20)
	Probiotic	ND	5 % (1/20)*	100 % (20/20)	40 % (8/20)*
	Organic Acids	ND	65 % (13/20)	100 % (20/20)	95 % (19/20)
	Probiotic and Organic Acids	ND	15 % (3/20)*	42 % (8/19)*	21 % (4/19)*

¹Indicates significant (p<0.05) differences was observed between control and treated within a single experiment in each column, **Significantly (p<0.05) different than all groups within a single experiment in each column, ¹Not Determined

Table 2: Experiment 3, Log₁₀ *S. enteritidis* recovery from crop of broiler chicks at 24 h and 48 h post-treatment

	24 hours	48 hours
Control	4.83±0.37	3.25±0.22
Probiotic	2.83±0 ¹	2.78±0.88
Organic Acids	3.96±0.09 ¹	2.69±0
Probiotic and Organic Acids	2.69±0.29 ¹	1.26±0.32 ²

¹Significantly different than control (p<0.05), ²Significantly different than all groups (p<0.05)

Experimental design: In experiment 1, 80 day-of-hatch broiler chicks were obtained from a local hatchery. The chicks were randomized and challenged with 2.8 x 10⁴ cfu SE. The chicks were then held in chick boxes for 2 hours and then randomly assigned to either untreated control or continuous OA (Perform-Max Optimizer IITM) treatment in the drinking water at a dilution of 1:128. For each of the experiments the chicks were housed in brooder batteries with food and water *ad libitum*. At 48 hr or 5 days post-challenge, 20 chicks/treatment/time, were humanely killed by CO₂ inhalation and crop and cecal tonsils were aseptically harvested separately. *Salmonella* recovery procedures have been previously described by our laboratory and were followed with some modifications (Tellez *et al.*, 1993). Briefly, crop and cecal tonsils were enriched in 10 mL of tetrathionate broth overnight at 37°C. Following enrichment, each sample was streaked for isolation on XLD plates containing 25 µg/mL NO and 20 µg/mL NA. The plates were incubated at 37°C for 24 h and examined for the presence or absence of the antibiotic resistant SE.

In experiments 2 and 3, 160 day-of-hatch broiler chicks were obtained, randomized and challenged with 2.4 x 10⁴ cfu SE. All chicks were then held for one hour in chick boxes and then 80 chicks were garaged with 0.25 ml of probiotic (1.8 x 10⁷cfu). All chicks were held for an additional hour and placed in brooder batteries. Chicks were assigned to either untreated control, probiotic treatment only, OA treatment only, or probiotic+OA treatment groups. After 24 or 48 hours post-treatment, 20 chicks/group/time were humanely killed by CO₂ inhalation and isolation for *Salmonella* incidence was preformed as in experiment one. In experiment 3, crops were homogenized within sterile sample bags⁷ using a rubber mallet. Sterile saline (5 mL) was added to each

sample bag and hand stomached with the cecal contents. Dilutions were spread plated on XLD plates containing 25 µg/mL NO and 20 µg/mL NA. The plates were incubated at 37 C for 24 h and cfu of SE per crop were determined.

Statistical analysis: The incidence of *Salmonella* recovery within experiments was compared using the chi-square test of independence (Zar, 1984) testing all possible combinations to determine significant (p<0.05) differences between control and treated groups. Cecal cfu data were converted to log₁₀ cfu numbers then compared using the GLM procedure of SAS (SAS Institute, 2002) with significance reported at p<0.05.

Results and Discussion

Results of experiments 1-3 are summarized in Table 1. In experiment 1, treatment with OA in the drinking water caused a significant reduction (p<0.05) in SE recovery from the cecal tonsils when compared with the controls (OA treated = 55% vs. controls = 100%). Also treatment with OA caused significant less (p<0.05) SE recovery from the crop (75%) as compared to controls (100%). In experiment 2, at 24hr, treatment with probiotic or probiotic+OA significantly (p<0.05) reduced SE recovery from the crop as compared to controls (75, 40 and 100%, respectively). All treatments reduced (p<0.05) SE recovery from the cecal tonsils at 24h (control: 60%, probiotic: 25%, OA: 45%, probiotic+OA: 13%). While no significant differences were observed in SE recovery from crop at 48 h, SE recovery from probiotic and probiotic+OA groups was significantly lower than the controls in the cecal tonsils, 20%, 20% and 100%, respectively (Table 2).

In experiment 3, probiotic or probiotic+OA caused reduced cecal tonsil SE recovery as compared to controls at 24 h (5%, 15% and 75%, respectively) and at 48 h (40%, 21% and 100%, respectively) and probiotic+OA treatment again reduced SE recovery incidence in crops at 48 h as compared to controls, 42% vs. 100%, respectively (Table 2). When SE recovery was evaluated after 24 h, >2.5 log₁₀ reductions of SE recovery were observed from crop of probiotic or probiotic+OA

treated chicks and $>1 \log_{10}$ reductions of SE recovery were observed from crop of chicks treated with only OA (Table 2). When SE recovery was evaluated only from samples that were crop positive following enrichment after 48 h, $>1.5 \log_{10}$ reductions of SE recovery were observed from crop of probiotic + OA treated chicks (Table 2).

In these experiments, OA alone was not able to consistently reduce SE; however, the combination of the probiotic and OA consistently and significantly reduced SE in both the crop and cecal tonsils of broiler chicks. Probiotic alone consistently reduced SE recovery in the cecal tonsils. The OA most likely is killing SE in the crops because these organic acids are most likely being absorbed in upper gastrointestinal tract of the birds (Hume *et al.*, 1993; Avila *et al.*, 2003; Byrd *et al.*, 2001). The combination of the probiotic+OA may be most effective because the likely site of killing for the OA is the crop and the mode of action for the probiotic has been shown by previous research in our laboratory to be effective at reducing *Salmonella* colonization of the ceca and cecal tonsils (Higgins *et al.*, 2007). Probiotic has also been shown to improve body weights of turkeys experiencing idiopathic diarrhea under commercial conditions (Higgins *et al.*, 2005) and to increase performance of production turkeys under commercial conditions (Torres-Rodriguez *et al.*, 2007). This water acidifier has been shown to decrease *Salmonella* in market age broilers when administered during the pre-slaughter feed withdrawal period (Jarquin *et al.*, 2007). Previous research has suggested that administration of OA during the pre-slaughter feed withdrawal period could lead to carcass shrinkage (Byrd *et al.*, 2001). While this evidence was shown when using lactic acid alone, Perform-Max Optimizer II™ was developed as a combination of organic acids used in combination at low individual concentrations so that water consumption was not discouraged (Jarquin *et al.*, 2007). Organic acids are a readily available energy source for both the chicken and the bacteria; therefore, it is important that the organic acids be administered in high enough concentrations to be bactericidal and low enough concentrations to be voluntarily consumed by the birds. Perform-Max Optimizer II™ did not cause a significant decrease in water consumption in broiler chicks in these experiments (data not shown).

These results suggest that the combination of these lactic acid bacteria based probiotic and water acidifier are more effective than individual administration for SE reduction in chicks.

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¹Catalog No. N-4382, Sigma, St. Louis, MO 63178

²Catalog No. 278820, Becton Dickinson, Sparks, MD 21152

³Catalog No. N-1628, Sigma, St. Louis, MO 63178

⁴Catalog No. 41069, IVS-Wynco LLC, Springdale, AR 72762

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⁶Catalog No. R1148, Sigma, St. Louis, MO 63178

⁷Catalog No. B00679WA, Nasco, Fort Atkinson, WI 53538-0901