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Effect of Organic Acids on *Salmonella* Typhimurium Infection in Broiler Chickens

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Abstract: An alternative to antibiotics is the use of certain organic acids for routinely encountered pathogens in the poultry industry. Direct acidification of drinking water with organic acids could significantly reduce the amount of recoverable *Salmonella* Typhimurium (ST) from the crop and cecal tonsils when used during the pre-slaughter feed withdrawal period. In the present study, *in vitro* and *in vivo* evaluations were conducted to compare a commercially available water acidifier (Optimizer®), *versus* two formulations of organic acid mix (OAM), made up of of acetic, citric and propionic acids at a final concentration of either 0.031% or 0.062%, to reduce *Salmonella* Typhimurium in the crop and cecal tonsils of broiler chicks during a 24 h period. The two OAM showed better *in vitro* activity to reduce *Salmonella* when compared to control. *In* vivo, the OAM (0.062%) had a similar effect as Optimizer® showing a significant reduction in total number of ST positive cecal tonsils, and reducing the number of ST in the crop when compared with controls ($P < 0.05$). All treatments reduced the number of ST recovered from crop contents at 24 h. This new formulation of OAM has great potential as a crop sanitizer and will be further evaluated under conditions similar to commercial chickens.

Key words: *Salmonella*, organic acid, chickens

INTRODUCTION

Salmonella enterica causes an estimated 1.4 million cases of foodborne illnesses annually in the United States, resulting in over 15,000 hospitalizations (Voetsch *et al*., 2004a,b). Poultry and poultry products have been identified by some researchers as the most important source of transmission of *Salmonella* to the human population (Lynch *et al*., 2006). 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. These pressures are a challenge for the poultry industry for controlling *Salmonella* not only at the farm level, but also within processing and manufacturing plants (Hargis *et al*., 1995; Corrier *et al*., 1999a; Hinton *et al*., 2000; Mikolajczyk and Radkowski, 2002). An 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 (Van Immerseel *et al*., 2006; Alali *et al*., 2010; Vandeplas *et al*., 2010); however, 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, Optimizer® 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; Wolfenden *et al*., 2007; Vicente *et al*.,2007a,b,c). 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 in the presence of organic matter, and low enough to be voluntarily consumed by the birds. In the present study, we compared a commercially available water acidifier (Optimizer®, Pacific Vet Group, Fayetteville, AR 72703), *versus* a new formulation of organic acid mix (OAM) to reduce *Salmonella* Typhimurium in the crop and cecal tonsils of broiler chicks.

MATERIALS AND METHODS

***Salmonella* amplification:** A primary poultry isolate of *Salmonella* Typhimurium (ST) was used in these experiments. This isolate was selected for resistance to nalidixic acid (NA)[1]. For these experiments, ST was grown in tryptic soy broth (TSB)[2] 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 at 625 nm. The stock solution was serially diluted and confirmed by colony counts of three replicate samples (0.1 mL/replicate) that were spread plated on brilliant green agar (BGA)[3] plates containing 25 µg/mL novobiocin (NO)[4] and 20 µg/Ml nalidixic acid (NA). The colony-forming units of

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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.

Experimental Design - *in vitro* crop assay: An assay previously described (Barnhart *et al*., 1999) was used with modifications. Briefly, 1.25g of unmedicated chick starter feed was measured into 13×100 mm borosilicate tubes and autoclaved. The feed was suspended in 4.5 mL sterile saline and inoculated with 0.5 mL of a *Salmonella* Typhimurium culture containing approximately 10^4 cfu/mL. The tubes were treated with either: 1) saline as a control; 2) OAM, having a final concentration of acetic, citric and propionic acids at 0.031 % or; 3) OAM, having a final concentration of acetic, citric and propionic acids at 0.062 %. Each sample was run as triplicate, each treatment had 5 replicates, and the entire assay was repeated in 2 additional trials. After administering the treatment, the tubes were vortexed and incubated at 37°C for 30 minutes and an additional 6 h. The tubes were then agitated and 20 µL of the content was serially diluted and plated as triplicates on BGA containing novobiocin and nalidixic acid. Typical ST colonies were counted after 24 h of incubation.

Experimental design with chickens: In experiment 1, 64 day-of-hatch broiler chicks were obtained from a local hatchery. Chicks were randomized and challenged with 2 x 10^5 cfu/mL of ST. The chicks were then held in chick boxes for 1 h and then randomly assigned to 1) untreated control or continuous treatment in the drinking water with: 2) Optimizer® at commercial recommended doses; 3) OAM, having a final concentration of acetic, citric and propionic acids at 0.031 % or; 4) OAM, having a final concentration of acetic, citric and propionic acids at 0.062%. Chicks were housed in brooder batteries with food and water *ad libitum*. At 24 hr post-challenge, chicks were humanely killed by CO_2 inhalation and crop, both ceca 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 BGA 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 ST. Ceca were weighed and then homogenized within sterile sample bags[5] using a rubber mallet. Sterile saline (4X weight to volume) was added to each sample bag and hand stomached with the cecal contents. Dilutions were spread plated on BGA plates containing 25 g/mL NO and 20 µg/mL NA. The plates were incubated at 37°C for 24 h and cfu of ST per ceca were determined.

In experiment 2, 80 day-of-hatch broiler chicks were obtained from a local hatchery. Chicks were randomized and challenged with 2 x 10^5 cfu/mL of ST. The chicks were then held in chick boxes for 1 h and then randomly assigned to 1) untreated control or continuous treatment in the drinking water with: 2) Optimizer® at commercial recommended doses; 3) OAM, having a final concentration of acetic, citric and propionic acids at 0.031 % or; 4) OAM, having a final concentration of acetic, citric and propionic acids at 0.062 %. Chicks were housed in brooder batteries with food and water *ad libitum*. At 24 hr post-challenge, chicks were humanely killed by CO_2 inhalation and crops were aseptically harvested, weighed and were homogenized within sterile sample bags using a rubber mallet. Sterile saline (4X weight to volume) was added to each sample bag and hand stomached with the crop contents. Dilutions were spread plated on BGA plates containing 25 µg/mL NO and 20 µg/mL NA. The plates were incubated at 37°C for 24 h and cfu of ST per crop were determined. Following this, crops were enriched with a 2X solution of tetrathionate broth overnight at 37°C. Following enrichment, each sample was streaked for isolation on BGA 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 ST.

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 and then compared using the GLM procedure of SAS (SAS Institute, 2002) with significance reported at $P < 0.05$.

RESULTS AND DISCUSSION

Salmonella colonization of poultry flocks can occur via horizontal transmission (Bailey *et al*., 2002; Kim *et al*., 2007; Alali *et al*., 2010; Vandeplas *et al*., 2010). Once cecal tonsil colonization is established, the bacterium is consistently shed in the feces (Bailey *et a*l., 2002; Foley *et al*., 2008). Feed Withdrawal induces pecking of the contaminated litter which may contaminate the crop (Corrier *et al*., 1999c) and if the crop is ruptured during processing, *Salmonella* may contaminate raw poultry products (Corrier *et al*., 1999b). Because the crop is more likely to rupture than the ceca, the crop represents an important source of *Salmonella* contamination to carcasses (Hargis *et al*., 1995; Corrier *et al*., 1999a). Table 1 summarizes the results of effect of OAM on ST in an *in vitro* crop assay. In 3 independent trials, the 0.031% OAM reduced ST by 6 h and the 0.062 % OAM was also efficacious. However, when 0.062 % OAM was tested in chickens, it had a similar effect as Optimizer® showing a significant reduction in total number of ST

Table 1: Effect of organic acid mix (OAM) on *Salmonella* Typhimurium (ST) in an *in vitro* crop assay

	Trial 1		Trial 2		Trial 3	
	30 minutes	6 hours	30 minutes	6 hours	30 minutes	6 hours
Control (ST)	6.25±0.13[a]	7.09±0.09[a]	7.42±0.03[a]	7.07±0.04[a]	4.95±0.13[a]	5.99±0.22[a]
0.031% OAM	6.08±0.8[a]	5.98±0.01[b]	7.43±0.03[a]	5.86±0.03[b]	4.88±0.24[a]	4.56±0.07[b]
0.062% OAM	ND	ND	7.39±0.04[a]	6.24±0.12 [b]	4.70±0.22[b]	4.56±0.07[b]

Organic acids mix= acetic, citric, and propionic acid. ND= Not determined. Data are expressed as log_{10} mean ± standard error. Values within columns with different lowercase superscripts differ significantly (P < 0.05).

Table 2: Experiment 1, effect of Optimizer® or organic acids mix (OAM) on *Salmonella* Typhimurium (ST) infection in broiler chicks during 24 hours period

Treatment	Crop Enrichment culture	Cecal tonsils Enrichment culture	Log_{10} ST/gram of ceca content
Control ST	15/16 (94%)	14/16 (87%)	2.43±0.35[a]
Optimizer ®	13/16 (81%)	3/16 (19%) **	0.22±0.22[b]
0.031% OAM	16/16 (100%)	12/16 (75%)	2.02±0.35[a]
0.062% OAM	13/16 (81%)	8/16 (50%) *	1.34±0.40[a]

Organic acids mix= acetic, citric, and propionic acid. Data of enrichment culture is expressed as positive/total chickens for each tissue sampled (%). * Indicates significant difference at P < 0.05. ** Indicates significant difference at P < 0.001.
Log_{10} ST/gram of ceca content data is expressed as mean ± standard error. Values within columns with different lowercase superscripts differ significantly (P < 0.05).

Table 3: Experiment 2, effect of Optimizer® or organic acids mix (OAM) on *Salmonella* Typhimurium (ST) infection in broiler chicks during 24 hours period

Treatment	Crop enrichment culture	Log_{10} ST/gram of crop content
Control ST	20/20 (100%)	5.21 ± 0.31[a]
Optimizer ®	18/20 (90%)	3.73 ± 0.25[b]
0.031% OAM	20/20 (100%)	3.96 ± 0.37[b]
0.062% OAM	18/20 (90%)	3.89 ± 0.22[b]

Organic acids mix= acetic, citric, and propionic acid
Data of enrichment culture is expressed as positive/total chickens for each tissue sampled (%).
Log_{10} ST / gram of crop content is expressed as mean ± standard error. Values within columns with different lowercase superscripts differ significantly (P < 0.05).

positive chickens in cecal tonsils (Table 2), and reducing the number of ST in the crop (Table 3) when compared with controls.

In the present study, Optimizer® reduced ST colonization in both crop and ceca (Tables 2 and 3) as has been previously reported (Jarquin *et al*., 2007; Wolfenden *et al*., 2007). In experiment 1, treatment with OAM in the drinking water caused a significant reduction (P<0.05) in ST recovery from cecal tonsils when compared with the controls (OA treated = 19% vs. controls = 87%). Also, treatment with OAM reduced 2.21 logs of ST when compared with controls (Table 2). While any of the treatments reduced recovery of ST from the crop by enrichment, all treatments reduced the number of ST recovered from crop content at 24 h (Table 3). The organic acids used in this study (citric, acetic and propionic) as well as others have been shown to be individually effective in reducing *Salmonella in vitro* (Van Immerseel *et al*., 2006). The biocidal efficacy and the effect on virulence of *Salmonella* differ with each organic acid treatment and each organic acid has a unique effect on bacteria normally present in the crop and gastrointestinal tract (Furuse *et al*., 1991; Byrd *et al*., 2001; Castro Gonzalez *et al*., 2001; Kubena *et al*., 2001). Characteristics of organic acids such as chain length, side chain composition, pkA values and hydrophobicity could be factors that effect biocidal activity (Van Immerseel *et al*., 2006). For these reasons, a mixture of organic acids was tested to reduce ST crop contamination. Further studies are being conducted to evaluate these new formulations of OAM during the pre-slaughter feed withdrawal period in commercial chickens to evaluate water consumption and bactericidal activity against *Salmonella* in the crop.

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