International Journal of Poultry Science 6 (3): 182-186, 2007
ISSN 1682-8356

The Evaluation of Organic Acids and Probiotic Cultures to Reduce *Salmonella enteriditis* Horizontal Transmission and Crop Infection in Broiler Chickens

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Abstract: An Organic Acid Mixture (OAM) was evaluated for efficacy against *Salmonella enteritidis* (SE) horizontal transmission and crop colonization in broiler chickens. The biocidal efficacy of the OAM (0.024% tannic, 0.042% lactic, 0.048% butyric and 0.048% acetic), was initially determined *in vitro* by treating a feed suspension inoculated with SE. The OAM was effective at reducing SE by at least 1 log unit in the feed suspension (P = 0.05). Treatment with the OAM was also assessed in combination with a probiotic treatment to evaluate effectiveness against horizontal transmission. One hundred newly hatched chicks were administered 3 treatments of OAM or probiotic, or 3 treatments of both OAM and probiotic over a 10 d period. Chicks (n = 20/group) were orally challenged with a 10^5 cfu mL-L culture of SE prior to treatment to act as sources for horizontal transmission. Ceca were collected and cultured for SE after 10 or 20 d. Horizontal transmission was reduced with OAM treatment solely (P = 0.05) or in combination with probiotic (P = 0.05). However, no apparent advantage to using both treatments was observed. The ability of the OAM to reduce SE crop contamination was determined by challenging market aged broilers with a 10^9 cfu mL-L culture of SE and administering the OAM in drinking water. Broilers were challenged with SE and immediately given the OAM or given the OAM 2 d prior to SE challenge. OAM was valuable in preventing horizontal transmission but did not eliminate crop colonization. The emergence of SE strains resistant to multiple antibiotics presents the need for alternative treatments and the results of these experiments show that an OAM may be a cost efficient and effective tool however the method of application of the OAM may alter effectiveness.

Key words: *Salmonella*, probiotic, organic acid, crop, ceca

Introduction

Salmonella colonization of poultry flocks can occur via horizontal transmission (Gast and Holt, 1999). Once cecal tonsil colonization is established, the bacterium is consistently shed in the feces (Baker *et al*., 1980). Feed Withdrawl (FW) induces pecking at the contaminated litter which may contaminate the crop (Corrier *et al*., 1999a) and if the crop is ruptured during processing, *Salmonella* may contaminate raw poultry products (Byrd *et al*., 2002). Because the crop is more likely to rupture than the ceca (Hargis *et al*., 1995) the crop represents an important source of *Salmonella* contamination to carcasses.

Poultry feed is a major source of *Salmonella* introduction to farms (Williams, 1981). Treating feed with acids has been found to reduce *Salmonella* contamination (Hinton and Linton, 1988). Thompson and Hinton (1997) found feeding chickens with acidified feed reduced the pH in the crop which resulted in a reduction in horizontal transmission of *Salmonella*. The mechanism of biocidal activity of acids is still unknown, but it was suggested that *Salmonella* does not have the ability to reduce intracellular pH, which causes a lethal accumulation of acid anions within the cell (Van Immerseel *et al*., 2006). Directly acidifying the drinking water was later evaluated and found to reduce the recovery of *Salmonella* from the crop (Byrd *et al*., 2001). However, a reduction in water consumption during the FW period was observed, which resulted in reduced carcass weight.

Probiotics have also been shown to be effective at reducing *Salmonella* colonization in chickens (Hume *et al*., 1998). Probiotic cultures may act in the gut by producing antibacterial substances (e.g., bacteriocins or colicins), altering immune responses or competing with pathogens for specific adhesion receptors on intestinal epithelia (Nava *et al*., 2005). In addition, increased numbers of some probiotic bacteria are associated with increases in organic acid concentrations (Humblot *et al*., 2005,) which correlates to a decrease in *Salmonella* colonization (Van Immerseel *et al*., 2005).

The objectives of this study were to determine if an Organic Acid Mixture (OAM) would reduce *Salmonella enteritidis* (SE) crop contamination and horizontal transmission. As opposed to one organic acid, a mixture of four acids at low concentration was tested in order to avoid problems of reduced water consumption. Administration of the OAM was also hypothesized to create a favorable environment that could possibly facilitate colonization by a probiotic culture and further reduce SE horizontal transmission. Therefore, the synergistic effect of a probiotic culture and OAM treatment was also assessed.

Materials and Methods

Bacterial strain and defined experimental probiotic culture: A primary poultry isolate of SE (PT 13A) was used for all experiments. The amplification, enumeration and culture protocols for the isolate have been described (Tellez *et al*., 1993). A defined experimental probiotic culture was selected *in vitro*, amplified and enumerated as previously described (Bielke *et al*., 2003). Briefly, the nine species comprising the culture, (5 *Escherichia*, 1 *Klebsiella*, 1 *Kluyvera* and 2 *Lactobacilli*) were grown overnight in tryptic soy broth (BD Bacto™ Laboratories, Sparks, MD) or MRS (EM Science, Gibbstown NJ) broth and concentrations were spectrophotometrically estimated. The volume of each culture was adjusted so that the final mixture contained roughly the same number of each isolate.

***In vitro* assay:** An assay previously described (Barnhart *et al*., 1999) was used with modifications. Briefly, 1.25 g 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 the SE culture containing approximately 10^5 cfu mL-1L. The tubes were treated with either the OAM, having a final concentration of tannic 0.024%, lactic 0.042%, butyric 0.048% and acetic 0.048% (designated 1X), or saline as a negative control. Two other concentrations of the OAM were also evaluated having half the final concentration (designated 0.5X) or double the final concentration of the above mixture (designated 2X). Each treatment was repeated 10 times and the entire assay was repeated in an additional trial. After administering the treatment, the tubes were vortexed and incubated at 37°C for 2 h. The tubes then were agitated and 1 mL of the content was serially diluted and plated on XLD agar containing novobiocin and nalidixic acid. Typical SE colonies were counted after 24 h of incubation.

Horizontal transmission assay

Experiment 1: Day-of-hatch broiler chicks obtained from Cobb-Vantress Inc. (Fayetteville, AR) were randomly assigned to 4 pens, each pen measuring 2 M^2 with 20 chicks per pen and provided medication-free chick starter feed and water *ad libitum*. The chicks in each pen received 1 of 4 treatments administered in the drinking water I) probiotic on days 3, 5 and 7; ii) the 1X OAM on days 3, 5 and 7; iii) the 1X OAM on days 3, 5 and 7 and the probiotic on days 4, 6 and 8; or iv) no treatment to act as a negative control. Treatment was given for 24 h, then the water was replaced with either fresh untreated water or freshly treated water. In an additional and separate pen, 20 chicks were individually tagged for identification and challenged by oral gavage with SE containing approximately 10^5 cfu mL-L to act as seeders. After 24 h, each treatment pen received 5 seeder chicks. After 10 d, all chicks were humanely killed and the cecal tonsils collected to culture for SE as previously described.

Experiments 2 and 3: To determine the lasting effect of the treatments, the entire experiment was repeated in two additional experiments. In these experiments, 80 chicks and 20 seeder chicks were in each pen and treatment was the same as in Experiment 1. After 10 d, 40 chicks were sacrificed and the other 40 chicks were sacrificed after 20 d.

Crop assay

Experiment 4: Market age broilers from the same flock aged 6-7 wk were obtained from the University of Arkansas Poultry Research Farm (Fayetteville, AR) and moved to isolation facilities. Broilers were randomly assigned to 4 pens, each pen measuring 2 M^2 with 20 birds per pen and provided finisher feeder and water *ad libitum*. Broilers were challenged by oral gavage with SE containing approximately 10^9 cfu mL-L. Treatment was immediately initiated to 2 pens by adding the OAM to the water at a final concentration of 1X. The 2 untreated pens acted as controls. At the time treatment was initiated, feed was removed from the 1 control and 1 treatment pen and water consumption was monitored in all pens. After 6 h of treatment, all broilers were humanely killed and the crops were removed for culture and enumeration of SE as described above.

Experiment 5 and 6: To determine if prolonged treatment was more effective, the experiment was repeated with modifications. Treatment began 48 h prior to challenge with SE and continued for an additional 48 h. Crops were obtained 96 h after treatment was initiated. In addition, feed was removed from 1 control and 1 treatment pen and water consumption was monitored in all pens for the last 8 h of the experiments.

Statistical analysis: Colony forming units of SE per gram of feed suspension were analyzed using Analysis of Variance (ANOVA) with further separation of significantly different means using Duncan's Multiple Range test using the statistical software JMP (SAS, 2000). Significant differences were reported at P = 0.05. Colony forming units of SE per g of crop contents were also analyzed using ANOVA and Duncan's Multiple Range test. The individual P-value comparisons (one-way ANOVA) were reported between the appropriate pairs of full feed and FW. *Salmonella* recovery from cecal tonsils was analyzed by Chi Square Test of Independence, with a significance level (") set at 0.05 using the statistical program JMP (SAS, 2000).

Results and Discussion

Initial *in vitro* results in this study showed the combination of OAM was effective at reducing *Salmonella* recovery from a feed suspension by at least 1 $\log_{10}$ unit (Fig. 1) after only 2 h of incubation. Similar reductions in SE were observed among the 0.5X, 1X and 2X concentrations. The organic acids used in this study

Table 1: Effect of an Organic Acid Mixture (OAM)[1] or probiotic (P) culture alone or in combination, on *Salmonella enteritidis* (SE) recovery from cecal tonsils of broiler chicks at 10 or 20 d-of-age

Experiment		Treatment	SE-positive/total	% Reduction
1.	d 10	C[2]	19/20 (95%)[a]	-
		OAM[3]	16/20 (80%)[a]	15
		P[4]	19/20 (95%)[a]	0
		OAM-P[5]	14/20 (70%)[b]	25
2.	d 10	C	38/40 (95%)[a]	-
		OAM	14/40 (35%)[d]	60
		P	22/40 (55%)[c]	40
		OAM-P	30/40 (75%)[b]	20
	d 20	C	30/40 (75%)[a]	-
		OAM	4/40 (10%)[c]	65
		P	10/40 (25%)[b]	50
		OAM-P	6/40 (15%)[c]	60
3.	d 10	C	29/40 (72.5%)[b]	-
		OAM	8/40 (20%)[d]	53
		P	36/40 (90%)[a]	-18
		OAM-P	17/40 (42.5%)[c]	30
	d 20	C	25/40 (62.5%)[a]	-
		OAM	19/40 (47.5%)[b]	15
		P	27/40 (67.5%)[a]	-5
		OAM-P	26/40 (65%)[a]	-3

[1]OAM consisting of tannic 0.024%, lactic 0.042%, butyric 0.048% and acetic 0.048% acids (final drinking water concentration), [2]Untreated control group, [3]OAM in the drinking water on d 3, 5 and 7, [4]Probiotic culture in the drinking water on d 3, 5 and 7, [5]OAM on d 3, 5 and 7 with P on d 4, 6 and 8, [a-d]Different letter superscripts indicate significant difference between means ($p \leq 0.05$)

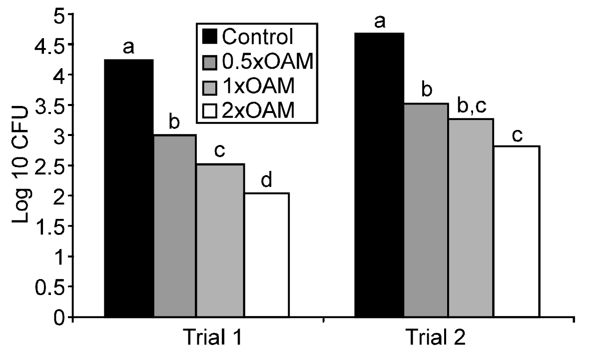

Fig. 1: Effect of selected concentrations of an Organic Acid Mixture (OAM) on *Salmonella enteritidis* (SE) recovery in a saline-feed suspension *in vitro*. A feed suspension containing approximately 10^5 cfu/mL SE was treated with an OAM and incubated for 2 h at 37°C. The 1X OAM contained 0.024% tannic, 0.048% acetic, 0.042% lactic and 0.048% butyric acids (0.5X and 2X OAM contained half or twice these concentrations, respectively). Means with different letters are significantly different ($p \leq 0.05$). The results are reported as means±SEM [n = 10 (number of replicates for each treatment)]

(tannic, butyric, lactic and acetic) as well as others have been shown to be individually effective at reducing *Salmonella in vitro* (Ricke, 2003; 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 (Thormar *et al*., 2006; Lawhon *et al*., 2002). Characteristics of organic acids such as chain length, side chain composition, pkA values and hydrophobicity could be factors that effect biocidal activity (Hsiao and Siebert, 1999). For these reasons, a mixture of organic acids was tested to reduce SE horizontal transmission and crop contamination. In addition, using four organic acids in low concentration, as opposed to a high concentration of a single acid, was hypothesized to alleviate reduced water consumption problems previously reported (Byrd *et al*., 2001).

In the preliminary 10 d experiment (Experiment 1) using the OAM, a reduction in horizontal transmission was not observed (Table 1). When the experiment was repeated using more chicks and lengthened to 20 d, statistically significant reductions resulted after 10 and 20 d (Experiments 2 and 3, P = 0.05). Taken together, the results of the experiments suggested the OAM was effective at reducing, but not completely eliminating horizontal transmission. Treatment with any antimicrobial may not always be completely effective, especially if infection pressure is high (Van Immerseel *et al*., 2006). Although the OAM was effective, perhaps greater prevention may have occurred if infection pressure was reduced by using fewer seeder chicks. In addition, research has shown susceptibility of chicks to infection with SE may vary by age and individual (Mead, 2000), which may have had an effect on the results of the experiments.

Treatment with organic acids can be effective at reducing SE contamination in the crop as previously reported (Byrd *et al*., 2001). However, treatment with OAM is probably not as effective at preventing SE colonization in the ceca, as the acids are most likely absorbed in the upper gastrointestinal tract (Hume *et al*., 1993). Conversely, probiotic cultures have been shown to prevent *Salmonella* colonization of the cecum (Rantala and Nurmi, 1973). For these reasons, a treatment of probiotic bacteria was combined with OAM to determine if a further reduction in horizontal transmission could be achieved. In the initial 10 d experiment (Experiment 1) and in the d 10 groups for Experiment 2 and 3, the combination of both the OAM and probiotics reduced ($p \leq 0.05$) SE recovery. The combination of both the OAM and probiotics at the 20 d treatment was inconsistent between trials. However, when comparing the results of OAM treatment alone with the combination of OAM and probiotics, no significant benefit of using the combination of treatments was observed.

Although the OAM treatment alone provided consistent reductions in SE, the probiotic treatment alone were

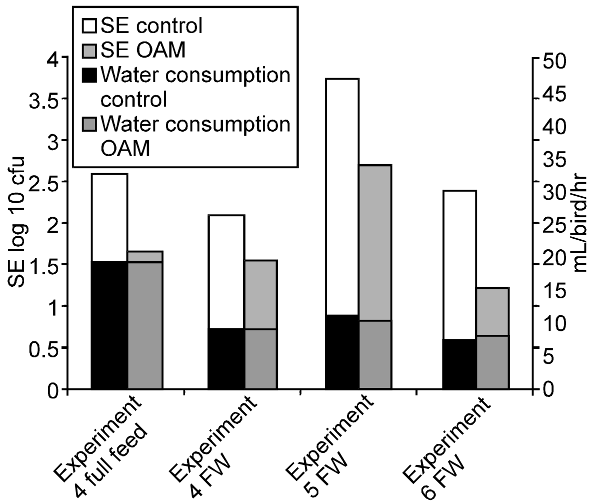

Fig. 2: Effect of an Organic Acid Mixture (OAM) on *Salmonella enteritidis* (SE) recovery from broiler crops aged 6-7 weeks. Broilers were challenged by oral gavage with SE containing approximately 10^9 cfu/mL and treated immediately (Experiment 4) or 48 h prior to challenge (Experiment 5 and 6) by adding the OAM to drinking water. After 6 h (Experiment 4) or 48 h (Experiment 5 and 6) of treatment, all broilers were humanely killed and the crops were removed for culture and enumeration of SE. Water consumption was recorded for the last 6 h (Experiment 4) or 8 h (Experiment 5 and 6). The OAM contained 0.024% tannic, 0.048% acetic, 0.042% lactic and 0.048% butyric acids. The results are reported as means±SEM [n = 20 (number of broilers for each treatment)]

variable. These inconsistencies may partially explain why the combination of OAM and probiotics was not more effective. Results from the preliminary 10 d experiment, showed the probiotic treatment alone had no effect compared to the control. However, this particular probiotic was found to be effective in turkey poults (data not shown) and was therefore used in the subsequent experiments. Probiotic treatment alone in Experiment 2 resulted in a 40% and 50% reduction after 10 d and 20 d respectively that was statistically significant ($p \leq 0.05$), while Experiment 3 showed a detrimental effect at d 10 and no effect at d 20 (Table 1). The wide variations seen in this study are not clearly understood. Variations of probiotic efficacy have been reported and some factors suggested to influence efficacy include route of administration and antibiotic residues in chicks gained from the maternal hens (Mead, 2000). Results from one antibiotic study showed residues may be present for up to 4 days after hatch (Donoghue and Hargis, 2005), which could have a negative impact on early administration of probiotic cultures. If this were the case, treatment with the OAM the first 4 d after hatch and then treatment at d 5 with the probiotic might be a better strategy.

The use of FW from broilers prior to processing allows partial clearance of the gastrointestinal tract and reduces fecal contamination of the carcass (May *et al*., 1990), but may increase the survival of *Salmonella* (Durant *et al*., 1999). FW has been shown to increase crop pH, decrease *Lactobacilli* numbers, decrease lactic acid concentrations and increase consumption of contaminated litter (Humphrey *et al*., 1993; Corrier *et al*., 1999a; Corrier *et al*., 1999b) all which favor *Salmonella* colonization. Evaluation of the OAM as a treatment to reduce *Salmonella* in the crop during FW was not statistically significant (Experiments 4-6, Fig. 2, p$0.05). Compared to previous reports, the OAM was not as effective as single acid treatments of lactic or formic acid (Byrd *et al*., 2001). However, unlike treatment with lactic or formic acid, the OAM treatment showed water consumption was not decreased (Fig. 2). Using a mixture of organic acids may be the key to avoiding decreased water consumption, but a different combination of acids will be needed to provide efficacy against SE.

Recently published data from our laboratory indicated that a combination of organic acids followed by a probiotic culture was effective for reducing environmental *Salmonella* recovery in commercial turkey flocks when administered 2 weeks prior to slaughter (Vicente *et al*., 2007). The present data similarly indicate that a combination of organic acids was helpful for controlling SE horizontal transmission in broilers.

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Research is funded by a grant from the Arkansas Biosciences Institute.