

Research Note: In-feed Bacteriophage does not impact efficacy of live *Salmonella* vaccine

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ABSTRACT Dietary bacteriophages potentially can serve as a step to reduce *Salmonella* contamination of feed through direct lysis of the bacteria. However, poultry producers commonly vaccinate with live *Salmonella* vaccines, which could potentially be lysed by dietary bacteriophages. The objective of this study was to evaluate if dietary bacteriophages impacted the colonization of a live *Salmonella* vaccine. A total of 210 day-of-hatch Ross male broiler chicks were divided into 3 treatments consisting of 2 replicate per treatment. Each replicate contained 35 birds. T1 was the challenge control, given no *Salmonella* vaccine, T2 was challenged and given *Salmonella* vaccine and T3 was challenged, given *Salmonella* vaccine as well as dietary bacteriophage. *Salmonella* vaccine was administered day of hatch. On d 3, four birds/pen were sampled for *Salmonella* vaccine colonization of ceca and liver/spleen. The remaining birds were challenged with 5×10^7 CFU of nalidixic acid-resistant *Salmonella enteritidis* (**S.E.**). On d 28, ten birds/replicate were sampled via cloaca swabs to cul-

ture for S.E. On d 42, the trial was terminated, birds were weighed, and performance was calculated. In addition, 15 birds/replicate were sampled for cecal cultures of S.E.

On d 3, T1 had 0% vaccine strain isolated, and significantly lower ($P = 0.009$) cecal prevalence compared with T2 (75%) and T3 (38%) being intermediate. T1 (0%) had significantly lower liver/spleen vaccine strain prevalence ($P = 0.002$) compared with T3 (88%) and T2 (63%) being intermediate. No significant differences ($P > 0.05$) were observed among treatments in *Salmonella* prevalence in d 28 cloacal swabs. No significant differences ($P > 0.05$) were observed in d 42 cecal *Salmonella* prevalence between all treatments. No significant differences in bird weight were observed between treatments d 0 to 42 ($P > 0.05$). However, T2 and T3 had lower mortality and adjusted feed conversion ratio (**FCR**; $P < 0.05$) compared with T1. Therefore, the dietary bacteriophage did not interfere with colonization or protection afforded by the live *Salmonella* vaccine.

Key words: *Salmonella*, *Salmonella* vaccine, bacteriophage, ABF, food Safety

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INTRODUCTION

In the United States, foodborne non-typhoidal salmonellosis affects approximately one million individuals, results in 378 deaths and costs approximately \$3.3 billion USD annually, with most of these infections being associated with poultry and poultry products (Scallan et al., 2011). Therefore, reduction of *Salmonella* in the production cycle of poultry and in processing plants is important for food safety. *Salmonella* is

ubiquitous and can infect poultry through numerous routes such as: hatcheries, broiler houses, feed production facilities and feed ingredients (Jones, 2011).

One potential solution to feed contamination with *Salmonella* is bacteriophages (**BP**). Lytic BP bind to target bacteria, inject and replicate DNA within the bacteria and then lyse the bacteria when the progeny are released (Joerger, 2003). Unlike antibiotics, which kill both pathogenic bacteria and normal flora in the gut, BP is highly selective for their host bacteria (Joerger, 2003). BP has been used in a wide variety of applications such as in the treatment of live birds, poultry products, and processing equipment (Joerger, 2003). Previous research has demonstrated in chickens the efficacy of an in-feed bacteriophage for reducing the prevalence of *Salmonella* (Kimminau et al., 2020).

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Because there are a multitude of points in which of *Salmonella* can enter poultry production, comprehensive programs utilizing synergies have been reported between different management strategies to reduce *Salmonella* contamination (El-Shall et al., 2019). Some researchers have reported a lack of synergy with bacteriophages and competitive exclusion feed additives in *Salmonella*-challenged broilers (Toro et al., 2005). To date, there hasn't been research to evaluate the potential impact that an in-feed bacteriophage could have on a live *Salmonella* vaccine. Therefore, we sought to determine if inclusion of dietary bacteriophage impacts colonization and protection to S. E. challenge of a live *Salmonella* vaccine.

MATERIALS AND METHODS

Animals and Experimental Design

Two hundred and ten (210) day of hatch Ross broilers were obtained from a local hatchery (Baldwin, GA). Animal care practices conformed to the Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2020). IACUC number was PMB102020. The facility had unidirectional airflow with HEPA filtered exhaust and was thermostatically controlled with a heat pump. Birds were raised under ambient humidity and were provided a lighting program as per the primary breeder recommendations. The floor space per animal was 1.00 sq. foot/bird, with one tube feeder and one Plasson drinker in each pen. Feed and water were given ad libitum. Birds were monitored twice daily for mortality, general flock condition, changes in environmental conditions and all observations were recorded.

Treatments

Birds were placed in 2 replicate pens of 3 treatments: T1) unvaccinated control T2) *Salmonella* vaccine alone and T3) *Salmonella* vaccine and bacteriophage (1kg/metric ton). Each pen had 35 birds, for a total of 70 per treatment. Treatments 2 and 3 were given Megan Vaccine (Elanco Animal Health, Greenfield, IN) via coarse spray (0.25 mL/chick) at day of hatch. Basal diets were based off corn-soy commercial-type broiler diets in accordance with the current commercial broiler breeder recommendations (Aviagen, 2012). All diets contained Amprolium (Huvepharma, Peachtree City, GA) at 113.5 g/ton.

Feed periods were as follows: Starter d 0 to 21 (crumble), Grower d 21 to 35 (pellet), and Finisher d 35 to 42 (pellet).

Bacteriophage

The dietary BP used in this experiment (CTCBIO Inc., Seoul, Republic of Korea) was a lytic phage that specifically targeted *Salmonella* Enteritidis (KCTC 12012BP). BP was present at a concentration of 10^8

plaque-forming unit (pfu) per gram. These pfu were quantifiable plaques formed when live viral particles infect host cells within a cell monolayer.

Infection

Four (4) days post *Salmonella* vaccination. All birds were given oral gavage of 5×10^7 nalidixic acid-resistant *Salmonella* enteritidis.

Salmonella Vaccine Sampling

On d 3, four (4) birds from each pen (8 birds/treatment) were sampled for *Salmonella* vaccine strain prevalence in the ceca, and liver/spleen. On d 28, cloacal swabs from 10 birds from each pen (20 birds/treatment) were taken. On d 42 fifteen (15) birds from each pen (30 birds/treatment) were sampled for *Salmonella* prevalence in the ceca.

Salmonella Enumeration and Prevalence

All samples taken were placed on ice in sterile bags. Organ samples were weighed and tetrathionate broth was added to each ceca and combined liver and spleen at 1 part to 9 parts broth (1:10 wt/vol dilution). Samples were mixed with a stomacher. A tetrathionate broth solution was added, a 1 mL aliquot was removed for MPN analysis, and samples were incubated overnight at 41.5°C. A loopful of sample was struck onto xylose lysine tergitol-4 agar (XLT-4) plates which were incubated overnight at 37°C. Up to 3 black colonies were selected and confirmed as *Salmonella* positives using Poly-O *Salmonella* Specific Antiserum.

Salmonella Enumeration Via Most Probable Number Method

Salmonella in ceca and internal organ (liver and spleen) samples were enumerated using the Most Probable Number Method (MPN) of Berghaus et al. (2013). Briefly, a 1 mL aliquot of the homogenized organ/tetrathionate broth sample was transferred to 3 adjacent wells in the first row of a 96-well 2-mL deep block. A 0.1 mL aliquot of sample was transferred to 0.9 mL of tetrathionate broth in the second row, repeated process for remaining rows to produce 5 ten-fold dilutions, and incubated blocks for 24 h at 42°C. One (1) μ L of each well was transferred onto XLT-4 agar containing nalidixic acid, and were incubated at 37°C for 24 h. Final dilutions were recorded, and MPN calculations were performed (Berghaus et al., 2013 #6). Suspect *Salmonella* isolates were confirmed by Poly-O *Salmonella* specific antiserum.

Statistical Methods

S.E. prevalences in cloacal swabs, liver/spleen samples, and ceca were compared between treatment groups

using generalized estimating equations (**GEE**) logistic models adjusted for clustering by pen. SE MPNs in cloacal swabs and ceca were compared between treatment groups using a mixed-effects Tobit censored regression models to account for the distribution of samples that were above or below the limits of the MPN assay. MPN values were log-transformed prior to statistical analysis. Post hoc pairwise comparisons between treatments were performed using the Bonferroni procedure to limit the type I error rate to 5% over all comparisons. All statistical testing assumed a two-sided alternative hypothesis, and $P < 0.05$ was considered significant. Analyses were performed using commercially available statistical software (Stata version 17.0, StataCorp LLC, College Station, TX).

RESULTS AND DISCUSSION

Salmonella Prevalence

D 3 prevalence of *Salmonella* vaccine strain in ceca were significantly ($P = 0.009$) lower in T1 (0%) birds compared to T2 (75%) with T3 (38%) prevalence being intermediate. Prevalence of *Salmonella* vaccine strain in livers and spleens of T1 (0%) was significantly ($P = 0.002$) lower than T3 (88%) with T2 (63%) prevalence being intermediate (Table 1). All *Salmonella* isolates were identified as belonging to serogroup B, which is consistent with the vaccine strain. *Salmonella* vaccine strain prevalence in the ceca of T2 and T3 birds were 75 and 38%, respectively, while the unvaccinated control

was 0%, indicating successful application of the live vaccine.

No significant differences in d 28 cloacal swab *Salmonella* prevalence were observed between any of the treatments ($P = 0.189$; Table 1). No significant differences were observed between treatments with respect to the mean \log_{10} MPN in culture-positive samples ($P = 0.251$).

No significant differences in day 42 cecal *Salmonella* prevalence were observed between treatments ($P = 0.181$; Table 1). No significant differences in cecal *Salmonella* \log_{10} MPN/g based on a Tobit censored regression model were observed between treatments ($P = 0.625$).

Because of the low number of pens per treatment in this trial, expanding to a larger scale with more replications may help to elucidate the differences that were observed in this trial.

Growth Performance

No significant differences between any of the treatments were observed d 0 to 35 period. Both vaccinated treatments (with or without bacteriophage) had significantly ($P < 0.05$) lower mortality-adjusted feed conversion ratio (**FCR**) compared to the unvaccinated control during d 0 to 42 period (Table 2). Feed intake and weight gain were similar among all treatments and no significant differences were observed in mortality.

Previous research has shown broilers vaccinated with live *Salmonella* vaccine had decreased fecal shedding or

Table 1. *Salmonella* vaccine strain prevalence (%) in ceca and liver/spleen samples by treatment group. Four birds were sampled in each of 2 pens per treatment.

Treatment	No. positive / No. sampled (%)	
	Ceca	Liver/Spleen
Day 3		
T1. Unvaccinated control	0/8 ^a	0/8 ^a
T2. <i>Salmonella</i> vaccine alone	6/8 (75) ^b	5/8 (63) ^{ab}
T3. <i>Salmonella</i> vaccine + Bacteriophage	3/8 (38) ^{ab}	7/8 (88) ^b
<i>P</i> -value	0.009	0.002
Day 28		
T1. Unvaccinated control	7/20 (35) ^a	Cloaca
T2. <i>Salmonella</i> vaccine Alone	6/20 (30) ^a	
T3. <i>Salmonella</i> vaccine + Bacteriophage	3/20 (15) ^a	
<i>P</i> -value	0.189	
Day 42		
T1. Unvaccinated control	19/30 (63) ^a	
T2. <i>Salmonella</i> vaccine alone	20/30 (67) ^a	
T3. <i>Salmonella</i> vaccine + Bacteriophage	12/30 (40) ^a	
<i>P</i> -value	0.181	

Bolded values represent P -values > 0.05 and therefore considered significant.

^{ab}Within columns, percentages with a superscript in common do not differ with a level of significance of 5% over all comparisons.

Table 2. Influence of dietary bacteriophage and *Salmonella* vaccine on growth performance of *Salmonella*-challenged broiler chickens (D 0–42).

Treatment	Feed intake (Kg/Pen)	Adjusted FCR*	Non-adjusted FCR	Weight gain (kg)	Percent mortality
1. Challenge Control	135.48 ^A	1.568 ^A	1.573 ^A	2.823 ^A	1.43 ^A
2. <i>Salmonella</i> Vaccine alone	139.18 ^A	1.532 ^B	1.532 ^B	2.931 ^A	0.00 ^A
3. <i>Salmonella</i> Vaccine + Bacteriophage	139.23 ^A	1.519 ^B	1.519 ^C	2.956 ^A	0.00 ^A

^{A-C} Percentages with a superscript in common do not differ with a level of significance of 5% over all comparisons.

*Adjusted feed conversion ratio (FCR) is adjusted for mortality.

S.E. isolation when simultaneously given dietary pro- or prebiotics (El-Shall et al., 2019). Other researchers showed that birds given probiotic and vaccine were negative for *Salmonella* at all tested time points (Redweik et al., 2020). In addition, researchers have also demonstrated an increase in *Salmonella*-specific IgA in birds simultaneously given a live *Salmonella* vaccine and dietary probiotic (Beirao et al., 2018). It should be noted that the bacteriophages used in our trial have specific activity against *Salmonella* Enteritidis. Other feed additives such as probiotics or prebiotics, have been demonstrated to have a variety of proposed mechanisms against *Salmonella* such as competitive exclusion, immune stimulation and bacteriocin production (Jones, 2011).

In conclusion, the addition of dietary bacteriophage did not interfere with colonization nor protection afforded by the live *Salmonella* vaccine and can potentially serve as another tool to a multicomponent pre-harvest food safety program.

DISCLOSURES

E. A. Kimminau, T. Karnezos and K. Russo work for Purina Animal Nutrition which sponsored the research trial conducted at Southern Poultry Research Group

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