Southern **Poultry** Research Group

SPRG

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WATKINSVILLE, GEORGIA 30677

Final Report

for

South Florida Farming, Corp.

Evaluation of the Effectiveness of SFF Endurance in Feed to Reduce *Salmonella enteritidis* Colonization in Broiler Chickens

Study Number: SFSE092023-112

Date: 12/22/2023

Study Certification

I, Charles L. Hofacre, DVM, MAM, PhD, certify this study was conducted according to procedures described in the protocol. The report and data I submitted to the sponsor are accurate in that they represent actual study results, were collected in a manner which represented the true effects, and complete in that all study data were submitted to the sponsor. No adverse reaction was observed to test treatments and no unanticipated events occurred during the study.

_____ _____

Charles L. Hofacre, DVM, MAM, PhD **Date**
Study Investigator
For Southern Poultry Research Group, Inc.

Performance Data Analysis

Means for pen weight gain, feed consumption, feed conversion (adjusted for mortality): feed consumed/ final live weight + mortality weight), and cause of mortality was calculated. The mortality was assessed by gross lesions on necropsy. Statistical evaluation of the data was performed using a STATISTIX for Windows program (Analytical Software, Tallahassee, FL). The procedures used were general linear procedures using ANOVA with a comparison of means using least significant difference (*t*-test) (LSD) (T)) at a significant level of 0.05.

Study Interpretation

This *Salmonella enteritidis* challenge study evaluated the South Florida Farming (SFF) product, Endurance, when added into the feed at 2.5 kg/metric ton or in the feed at 2.5 kg/mT and also a SFF probiotic continuously for the 43 day study in the birds drinking water at 5 grams/1000 liters. The broilers were challenged by the seeder method on day 3 where 13 chicks in each pen of 25 chicks were tagged and gavaged 1.0×10^7 CFU/chick S. enteritidis (S.E.). Litter environmental assessment of S.E. was performed with bootsock samples of each pen on days 21 and 42. On day 43, four (4) seeder (direct challenged) and four (4) indirect challenged were randomly selected and internal organs (liver/spleen) and ceca cultured.

Salmonella Results

There was no significant difference in the S.E. prevalence of the bootsock samples between treatments on days 21 or 42 (Table 1). However, the pens treated with both the Endurance in the feed and SFF probiotic in the water had a significant reduction in S.E. numbers in the bootsock samples at both day 21 and 42 (Table 2). This reduction is greatest by day 42 and is more readily observed in the dot plot of Figure 1. All six pens of the combined treatment #4 were below 2 $\log_{10}$ Salmonella per bootsock. The ceca samples on day 43 followed a similar trend. Although not significant, the combined Endurance + SFF probiotic had 42% S.E. positive ceca versus the challenge control at 69% (Table 3). Again, the dot plot of pen S.E. prevalence clearly highlights that most of the Endurance + SFF probiotic treatment pens had less than 50% of the ceca positive (Figure 2). The number of

Salmonella in the positive ceca was also clearly reduced by the combined Endurance and SFF probiotic at 0.33^{a} $\log_{10}$ MPN/g versus the control at 0.58^{a} $\log_{10}$ MPN/g (Table 4). This reduction was greatest in the chicks exposed horizontally (indirect). There was one birds' ceca of the Endurance alone with an exceptionally high number and when this birds' MPN is excluded, the Endurance alone mean $\log_{10}$ ceca MPN/g drops below the challenge control to 0.53^{a} $\log_{10}$ (Figure 3). Since culture methods are not able to detect extremely low numbers of Salmonella, it is possible that some of the results of MPN = 0 are positive. Therefore, to account for this all samples with an MPN = 0 are censored to a $\log_{10}$ MPN/g of -0.50. Then a Tobit regression analysis is applied to this censored data. This gives a truer analysis of the data. When this analysis is applied, the combined Endurance + SFF probiotic has a nearly significant reduction in S.E. number in the ceca –0.86 $\log_{10}$ MPN/g (P = 0.09) (Table 5). Again, there was an even greater reduction in the horizontal/indirect challenged at –1.29 $\log_{10}$/g.

Often internal organs are the indicators for ground chicken Salmonella positive. Both SFF treatments had numerical reductions in liver/spleen S.E. prevalence (Table 6). It should be highlighted that the combined Endurance + SFF probiotic had only 1/48 (2%) of the birds with internal organs culture positive (Figure 4).

Performance Results

On day 21, the combined Endurance + SFF probiotic had significantly lower body weight from the challenge control (Table 7). There was not a difference in feed intake or FCR at this age. However by day 36, the birds in the combined treatment had compensated in growth and there were no differences in body weight or FCR (Table 8). By day 43, the combined Endurance + SFF probiotic in the water had numerically heavier body weight from the challenge control (Table 9). There were no significant FCR or feed intake differences between treatments on day 43.

Overall, The South Florida Farming products did not negatively impact bird performance in this study. The Endurance alone in the feed appeared to have a numerical reduction on *Salmonella enteritidis* colonization in the internal organs of direct challenged birds. However, combining the Endurance with the SFF probiotic administered continuously in the water had consistent S.E. reduction in the birds ceca, litter and internal organs. It also appears that the combined Endurance + SFF probiotic may assist the negative birds from becoming as highly Salmonella colonized from the direct colonized birds. Future studies may investigate if the SFF probiotic needs to be in the drinking water continuously or if an early treatment (Day 0-7) and then late application (Day 39-42) would be just as effective. Also, it may be valuable to demonstrate that these products can be effective against other Salmonella serovars like *S. infantis*.

Summary of the South Florida Farming *Salmonella* Enteritidis Study (SFSE092023-112)

Prepared by: Roy D. Berghaus, DVM, PhD, Dip. ACVPM (Epidemiology)

Statistical Methods

Boot sock *Salmonella* prevalences were compared between treatment groups using Fisher's exact test, and boot sock MPNs were compared using linear regression. *Salmonella* prevalences in ceca and liver/spleen samples were compared between treatment groups using generalized estimating equations (GEE) logistic regression, and *Salmonella* MPNs in culture-positive ceca samples were compared using linear mixed models, to account for the correlation between responses of birds from the same pen. GEE models were estimated using robust standard errors and an exchangeable working correlation structure. Tobit regression models with a random pen effect were also used to compare treatments with respect to *Salmonella* MPNs in ceca samples while considering culture-negative samples to be censored at a lower limit of -0.50 $\log_{10}$ MPN/g. For the comparison of *Salmonella* MPNs, samples with a negative culture result by the MPN method but a positive result by primary or secondary enrichment were arbitrarily assigned an MPN equal to one-half the minimum detection limit of the MPN assay. MPNs were log-transformed prior to statistical analysis. Pairwise comparisons 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 18.0, StataCorp LLC, College Station, TX).

Results

Boot Sock Salmonella Prevalences. One environmental boot sock sample was collected from each of 6 pens per treatment on days 21 and 42. *Salmonella* prevalences in boot sock samples are summarized in Table 1. There was no significant difference between treatments with respect to boot sock *Salmonella* prevalences on day 21 (*P* = 1.00) or day 42 (*P* = 1.00). All *Salmonella* isolates obtained from boot socks were identified as belonging to serogroup D, which was consistent with the *S*. Enteritidis challenge strain.

Table 1. *Salmonella* prevalences in boot sock samples collected on days 21 and 42. At each time point, one boot sock was collected from each of 6 pens per treatment.

Day	*Treatment	No. samples	No. positive (%)	†*P*
21	T2 – Challenge Control	6	6 (100)[a]	1.00
	T3 – Endurance Dose 1	6	6 (100)[a]	
	T4 – Endurance SFF Water	6	5 (83)[a]	
42	T2 – Challenge Control	6	6 (100)[a]	1.00
	T3 – Endurance Dose 1	6	6 (100)[a]	
	T4 – Endurance SFF Water	6	6 (100)[a]	

*The unchallenged control group (T1) was excluded from statistical comparisons because *Salmonella* was not detected in any samples from the unchallenged controls.
†Fisher's exact test. Within days, percentages with a superscript in common do not differ with a level of significance of 5% over all comparisons.

Boot Sock Salmonella MPNs - Culture-Positive Samples. *Salmonella* MPNs in culture-positive boot sock samples are summarized in Table 2, and the distribution of *Salmonella* MPNs is illustrated in Figure 1. In a factorial analysis, there was a significant effect of treatment ($P = 0.048$), with the marginal mean of T3 being higher than that of T4. There was no significant effect of day ($P = 0.40$), and no significant interaction between the effects of treatment and day ($P = 0.64$).

Table 2. Mean (SE) $\log_{10}$ *Salmonella* MPN/boot sock for culture-positive samples. One boot sock was collected from each of 6 pens per treatment on days 21 and 42.

*Treatment	Day 21	Day 42	Total
T2 – Challenge Control	2.39 (0.38)	2.40 (0.38)	2.40^{ab} (0.27)
T3 – Endurance Dose 1	2.76 (0.38)	2.62 (0.38)	2.69^{b} (0.27)
T4 – Endurance SFF Water	2.06 (0.41)	1.36 (0.38)	1.70^{a} (0.28)
Total	2.42^{a} (0.22)	2.15^{a} (0.22)	2.27 (0.16)

*The unchallenged control group (T1) was excluded from statistical comparisons because *Salmonella* was not detected in any samples from the unchallenged controls.
Marginal means with a superscript in common do not differ with a level of significance of 5% over all comparisons.

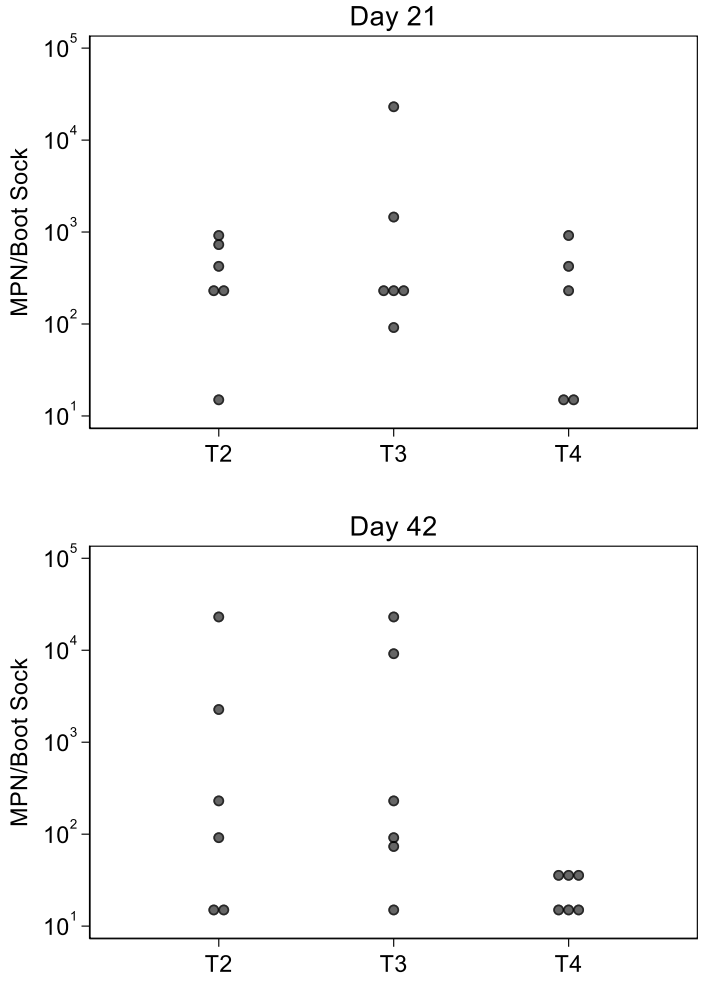

Figure 1. Dot plots of *Salmonella* MPNs for culture-positive boot sock samples by treatment and day. One boot sock was collected from each of 6 pens per treatment on days 21 and 42.

Ceca Salmonella Prevalences. *Salmonella* prevalences in ceca samples on day 43 are summarized in Table 3, and the pen-level distribution of *Salmonella* prevalences is illustrated in Figure 2. In a factorial analysis, there was no significant effect of treatment ($P = 0.073$), no significant effect of challenge status ($P = 0.40$), and no significant interaction between the effects of treatment and challenge status ($P = 0.97$). All *Salmonella* isolates obtained from ceca samples were identified as belonging to serogroup D.

Table 3. *Salmonella* prevalences (%) in ceca samples on day 43. Samples were collected from 4 indirect challenged birds and 4 direct challenged birds in each of 6 pens per treatment group.

	Challenge status		
*Treatment	Indirect	Direct	Total
T2 – Challenge Control	16/24 (67)	17/24 (71)	33/48 (69)[a]
T3 – Endurance Dose 1	18/24 (75)	19/24 (79)	37/48 (77)[a]
T4 – Endurance SFF Water	9/24 (38)	11/24 (46)	20/48 (42)[a]
Total	43/80 (54)[a]	47/80 (59)[a]	90/160 (56)

*The unchallenged control group (T1) was excluded from statistical comparisons because *Salmonella* was not detected in any samples from the unchallenged controls.
Marginal percentages with a superscript in common do not differ with a level of significance of 5% over all comparisons.

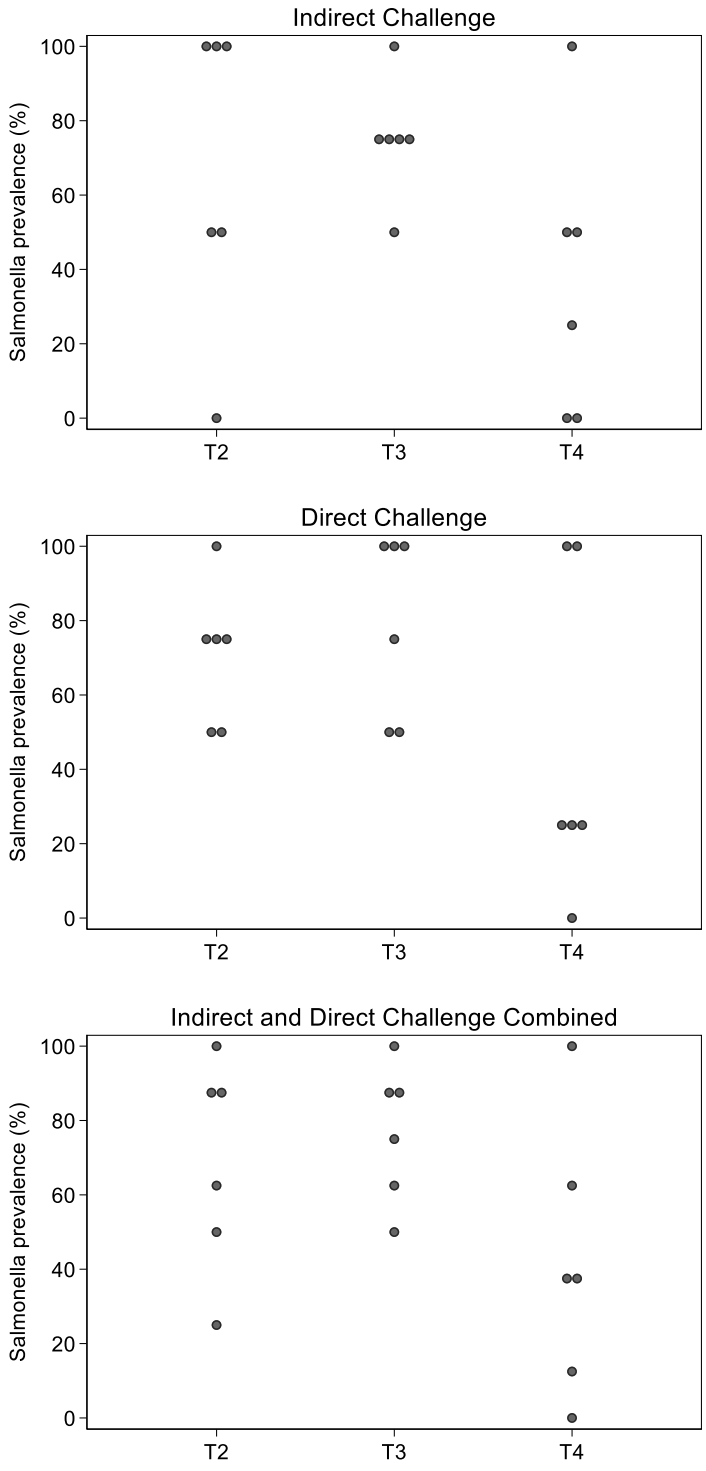

Figure 2. Dot plots of pen-level *Salmonella* prevalences in ceca samples on day 43 by treatment and challenge status. Samples were collected from 4 indirect challenged birds and 4 direct challenged birds in each of 6 pens per treatment group.

Ceca Salmonella MPNs - Culture-Positive Samples. *Salmonella* MPNs for the culture-positive ceca samples are summarized in Table 4, and the distribution of MPNs is illustrated in Figure 3. In a factorial analysis, there was a significant interaction between the effects of treatment and challenge status ($P = 0.033$). There was no significant difference between treatments for either the indirect challenged birds ($P = 0.13$) or the direct challenged birds ($P = 0.60$), but the relationship between treatment means varied by challenge status. T4 had the lowest mean for the indirect challenged birds, while T2 had the lowest mean for the direct challenged birds.

One of the direct challenged birds in T3 had an MPN that was approximately 1 $\log_{10}$ MPN/g higher than that of any other bird (Figure 3). If this observation was excluded, the mean (SE) of the direct challenged birds for T3 was reduced to 0.53 (0.31) $\log_{10}$ MPN/g, and the effect of treatment remained nonsignificant for both the indirect challenged birds ($P = 0.10$) and the direct challenged birds ($P = 0.70$).

Table 4. Estimated mean (SE) $\log_{10}$ *Salmonella* MPN/g for culture-positive ceca samples on day 43. Samples were collected from 4 indirect challenged birds and 4 direct challenged birds in each of 6 pens per treatment group.

	Challenge status		
*Treatment	Indirect	Direct	Total
T2 – Challenge Control	0.77[a] (0.34)	0.40[a] (0.32)	0.58 (0.29)
T3 – Endurance Dose 1	0.83[a] (0.32)	0.81[a] (0.31)	0.82 (0.28)
T4 – Endurance SFF Water	-0.22[a] (0.42)	0.83[a] (0.39)	0.33 (0.34)
Total	0.57 (0.20)	0.66 (0.20)	0.63 (0.17)

*The unchallenged control group (T1) was excluded from statistical comparisons because *Salmonella* was not detected in any samples from the unchallenged controls.

Within columns, means with a superscript in common do not differ with a level of significance of 5% over all comparisons. See Table 3 for the numbers of culture-positive samples.

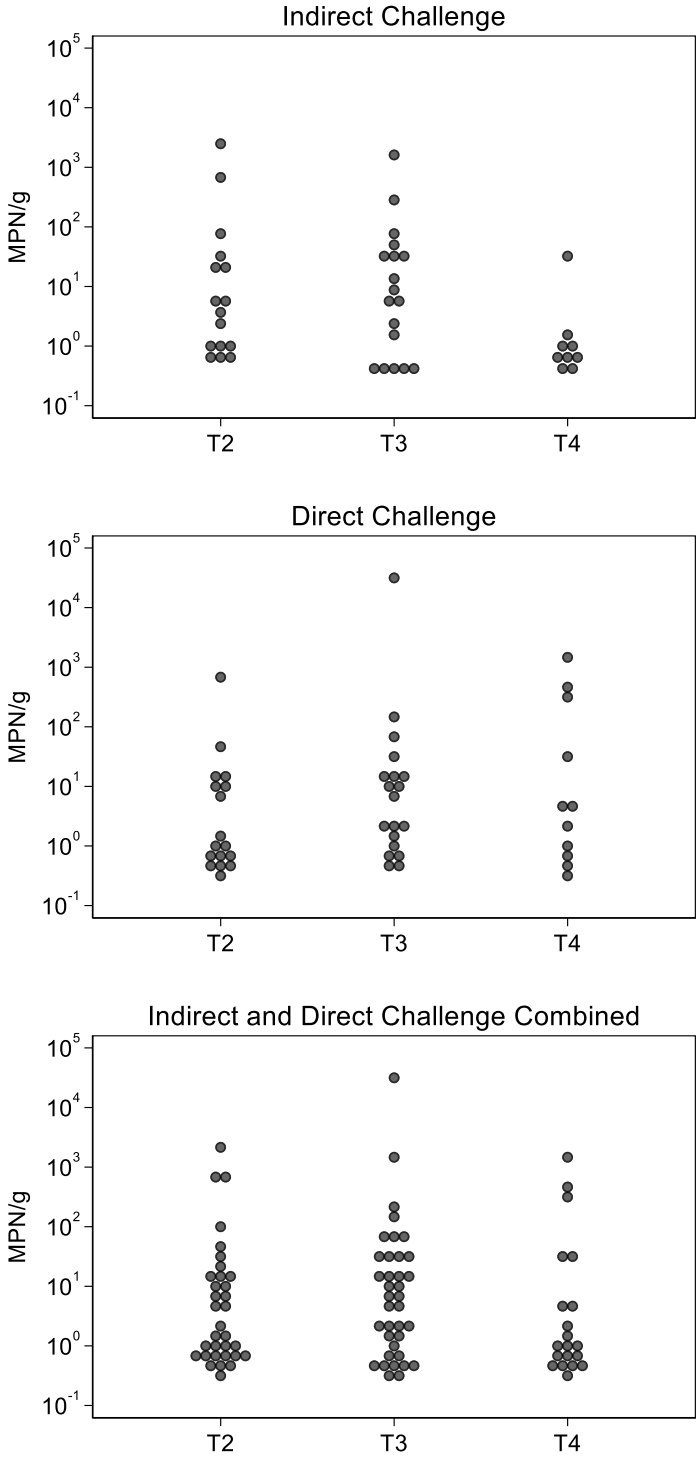

Figure 3. Dot plots of *Salmonella* MPNs for culture-positive ceca samples on day 43 by treatment and challenge status.

Ceca Salmonella MPNs - Taking the Culture-Negative Samples into Account. *Salmonella* MPNs for culture-negative samples are unknown. *Salmonella* may truly be absent from these samples (i.e., MPN = 0), or it may be present at a concentration that is below the culture method's lower limit of detection. To account for this uncertainty, a Tobit regression model was used to estimate the effects of treatment and challenge status on the *Salmonella* MPNs, while censoring the culture-negative samples at a concentration of -0.50 $\log_{10}$ MPN/g. Briefly, this model attempts to estimate the true mean MPNs based on the distribution of MPNs in the culture-positive samples as well as the proportions of culture-negative samples in the different treatment groups.
Salmonella MPNs based on the Tobit censored regression model are summarized in Table 5. In a factorial analysis, there was no significant effect of treatment (P = 0.09), no significant effect of challenge status (P = 0.21), and no significant interaction between the effects of treatment and challenge status (P = 0.11).

One of the direct challenged birds in T3 had an MPN that was approximately 1 $\log_{10}$ MPN/g higher than that of any other bird (Figure 3). If this observation was excluded, the estimated mean (SE) of the direct challenged birds for T3 was reduced to 0.22 (0.42) $\log_{10}$ MPN/g, and the effect of treatment remained nonsignificant (P = 0.14).

Table 5. Estimated mean (SE) $\log_{10}$ *Salmonella* MPN/g based on a Tobit regression model with culture-negative ceca samples censored at a lower limit of -0.50 $\log_{10}$ MPN/g. Samples were collected from 4 indirect challenged birds and 4 direct challenged birds in each of 6 pens per treatment group. There were 54 left-censored (culture-negative) observations and 90 uncensored (culture-positive) observations.

	Challenge status		
*Treatment	Indirect	Direct	Total
T2 – Challenge Control	0.12 (0.44)	-0.06 (0.44)	0.03[a] (0.40)
T3 – Endurance Dose 1	0.35 (0.43)	0.42 (0.43)	0.39[a] (0.40)
T4 – Endurance SFF Water	-1.29 (0.48)	-0.44 (0.46)	-0.86[a] (0.43)
Total	-0.27[a] (0.26)	-0.02[a] (0.26)	-0.15 (0.24)

*The unchallenged control group (T1) was excluded from statistical comparisons because *Salmonella* was not detected in any samples from the unchallenged controls.
Marginal means with a superscript in common do not differ with a level of significance of 5% over all comparisons.

Liver/Spleen Salmonella Prevalences. *Salmonella* prevalences in liver/spleen samples on day 43 are summarized in Table 6, and the pen-level distribution of *Salmonella* prevalences is illustrated in Figure 4. In a two-way analysis, there was no significant effect of treatment ($P = 0.10$), and no significant effect of challenge status ($P = 0.21$). It was not possible to evaluate the interaction between treatment and challenge status because *Salmonella* was not detected in any of the indirect challenged birds in T4. All *Salmonella* isolates obtained from liver/spleen samples were identified as belonging to serogroup D.

Table 6. *Salmonella* prevalences (%) in liver/spleen samples on day 43. Samples were collected from 4 indirect challenged birds and 4 direct challenged birds in each of 6 pens per treatment group.

	Challenge status		
*Treatment	Indirect	Direct	Total
T2 – Challenge Control	1/24 (4)	5/24 (21)	6/48 (13)[a]
T3 – Endurance Dose 1	2/24 (8)	3/24 (13)	5/48 (10)[a]
T4 – Endurance SFF Water	0/24 (0)	1/24 (4)	1/48 (2)[a]
Total	3/80 (4)[a]	9/80 (11)[a]	12/160 (8)

*The unchallenged control group (T1) was excluded from statistical comparisons because *Salmonella* was not detected in any samples from the unchallenged controls.
Marginal percentages with a superscript in common do not differ with a level of significance of 5% over all comparisons.

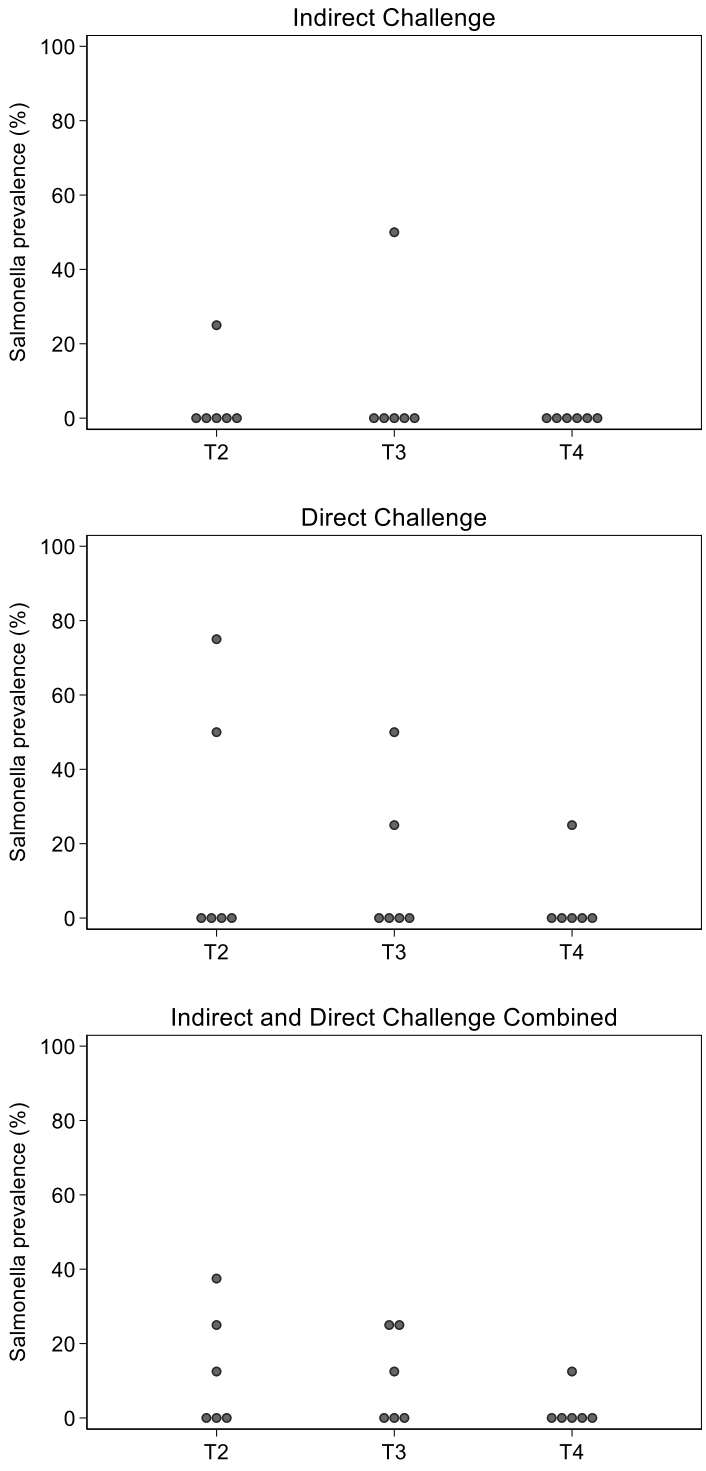

Figure 4. Dot plots of pen-level *Salmonella* prevalences in liver/spleen samples on day 43 by treatment and challenge status. Samples were collected from 4 indirect challenged birds and 4 direct challenged birds in each of 6 pens per treatment group.

Table 7. Day 0 to 21 Performance Results

Treatment	Feed Intake (Kg/Pen)	Adjusted FCR*	Non-Adjusted FCR	Weight Gain (kg)
1. No Treatment No Challenge**	22.000	1.387	1.456	0.710
2. Challenge Control	22.475A	1.288A	1.332A	0.792A
3. Endurance Dose 1	22.817A	1.345A	1.386A	0.763AB
4. Endurance SFF Water	21.708A	1.346A	1.386A	0.718B

*Adjusted FCR is adjusted for mortality
**Note: Since there were only 2 pens of no challenge, these pens were not statisitically evaluated.

Table 8. Day 0 to 36 Performance Results

Treatment	Feed Intake (Kg/Pen)	Adjusted FCR*	Non-Adjusted FCR	Weight Gain (kg)
1. No Treatment No Challenge**	60.150	1.528	1.622	1.826
2. Challenge Control	62.333A	1.511A	1.555A	1.918A
3. Endurance Dose 1	62.633A	1.544A	1.581A	1.856A
4. Endurance SFF Water	63.317A	1.496A	1.522A	1.917A

*Adjusted FCR is adjusted for mortality
**Note: Since there were only 2 pens of no challenge, these pens were not statisitically evaluated.

Table 9. Day 0 to 43 Performance Results

Treatment	Feed Intake (Kg/Pen)	Adjusted FCR*	Non-Adjusted FCR	Weight Gain (kg)	Percent Mortality
1. No Treatment No Challenge**	84.950	1.566	1.634	2.553	18.00
2. Challenge Control	86.258A	1.560A	1.626A	2.625A	18.67A
3. Endurance Dose 1	88.508A	1.569A	1.612A	2.607A	15.33A
4. Endurance SFF Water	89.217A	1.553A	1.604A	2.627A	14.67A

*Adjusted FCR is adjusted for mortality
**Note: Since there were only 2 pens of no challenge, these pens were not statisitically evaluated.

Objective

Salmonella enteritidis isolation in broiler processing plants is a problem in the U.S. The objective of this study was to evaluate the effectiveness of SFF Endurance administered continuously through the feed as a means to control *Salmonella enteritidis* colonization in broiler chickens.

Personnel

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Timeline

Start: 10/23/2023 **Finish:** 12/05/2023
Study Duration: 43 Days

Test Facility Location

Southern Poultry Research Group, Inc.
Sanford House 2, Back
529 Sanford Nicholson Road
Nicholson, GA 30565

Experimental Animals

Species: Chicken **Type:** Broiler
Breed/Strain: Ross x Ross **Sex:** Male
Origin: Aviagen Hatchery, Blairsville, GA **Initial Age:** Day-of-Hatch

Test Facility Design

The test facility diagram was included in final report source data files. This document provided test facility illustrations, descriptions, and information such as pen layout, size, watering, and feeding equipment.

Experimental Design

Number of Pens – 20
Number of Chicks – 500
Treatment Groups – 04 (3 treatments-6 pens and 1 treatment-2 pens)
Replicate Blocks – 06
Birds Per Pen – 25
Location – House 2, Back

Table 10: Experimental Design*

Treatment Number	Treatment Description	Number of Pens	Dosage Feed			S.E. Challenge	Water Treatment
			Starter	Grower	Finisher		
1	No Treatment No Challenge	2	———	———	———	No	No
2	Challenge Control	6	———	———	———	Yes	No
3	Endurance Dose 1	6	2.5 kg/mT	2.5 kg/mT	2.5 kg/mT	Yes	No
4	Endurance SFF Water	6	2.5 kg/mT	2.5 kg/mT	2.5 kg/mT	Yes	Yes 5 gm**/1000 liters of water

*Challenge was 13 birds/pen *S. enteritidis* on Day 3

** SFF Probiotics

Animal Welfare Practices

Animal care practices adhered to the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2020). All birds were euthanized by methods approved by the American Veterinary Medical Association (AVMA).

Materials and Methods

1. BIRDS. Five hundred (500) day-of-hatch Ross x Ross male broiler chicks were obtained from Aviagen Hatchery, Blairsville, GA. Birds were sexed, received routine vaccinations (HVTSB1) with no in ovo or S.Q. antibiotic, and breeder flock number information recorded at the hatchery.
   NOTE: All chicks were vaccinated with 1 dose per chick of a commercial coccidia vaccine.

2. BIRD ALLOCATION AND PEN RANDOMIZATON. Five hundred birds (500) were assigned to four (4) treatment groups with six (6) replicate pens per treatments 2-4 and 2 pens treatment 1 with 25 birds per pen. Southern Poultry Research Group completed randomization and assignment of treatment groups to pens using Random Permutation Tables (Cochran and Cox, 1992). The study began when birds were placed (day-of-hatch; DOT 0), at which time birds were allocated to experimental pens. Only healthy birds were selected. On DOT 0, group body weights were recorded by pen. No birds were replaced during the course of the study.

3. HOUSING AND ENVIRONMENTAL CONTROL. At study initiation, twenty-five (25) broiler chicks were allocated to twenty (20) floor pens measuring 5' x 5' (1.00 ft^2 /bird stocking density) in a modified conventional poultry house with solid-sides. The house had concrete walkways and the pens had concrete floors. The facility was fan-cooled. Thermostatically controlled gas heaters were the primary heat source. Birds were raised under ambient humidity and were provided a lighting program as per the primary breeder recommendations. At placement, each pen contained *approximately four (4) inches* ***new shavings.*** Litter was not replaced during the study course unless a drinker malfunction occurred. Each pen contained one (1) tube feeder and one (1) Plasson drinker resulting in a twenty-five (25) bird/feeder and drinker ratio.

4. FEED AND WATERING METHOD*. ad libitum*

5. DIETS. Rations were fed as follows: starter DOT 0 through DOT 21, grower DOT 21 through DOT 36 and finisher DOT 36 to DOT 43. Diets were fed as crumbles (starter feed) or pellets (grower and finisher). Feed formulations for this study consisted of un-medicated commercial-type broiler starter and grower diets compounded with commonly used United States feedstuffs representative of local formulations, calculated analyses to meet or exceed NRC standards. ***NO*** antibiotics were added to any feed unless specifically stated as a treatment protocol component. Treatment feeds were prepared from a basal starter feed with quantities of all basal feed to prepare treatment batches documented. Treatment feeds were mixed at the Southern Poultry Research Group (SPRG) feed mill and pelleted in a California Pellet mill at 80°C. After mixing was completed, feed was transferred to Sanford Research House and distributed among pens of designated treatment groups.

6. BODY AND FEED WEIGHT. All birds were weighed by pen on DOT 0, 21, 36, and 43. Feed added to each pen's feeder was weighed at the beginning of each formulation period on DOT 0 (starter), 21 (grower) and 36 (finisher). Any additional bags of feed were weighed (and documented) for each pen (as required) during each formulation period. Feed was distributed as needed to feeders from pre-weighed bags (assigned to each pen) throughout each period. Feed remaining in feeders (and feed bags if applicable) were weighed and disposed of on DOT 21, 36, and 43. Empty pan feeder weights were recorded prior to study initiation. The trial was terminated on DOT 43.

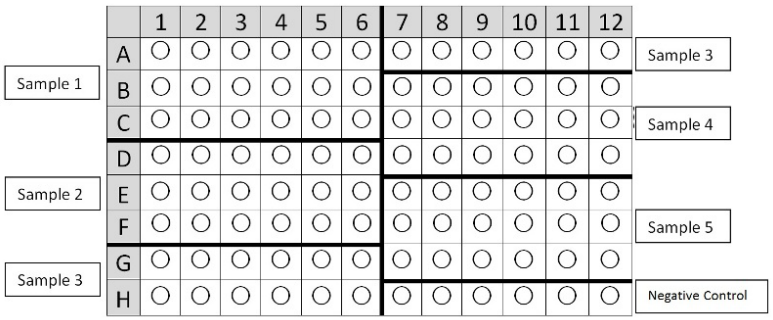
7. *Salmonella* INOCULATION**.** On DOT 3 thirteen (13) seeder chicks orally dosed (gavaged) with a $1x10^7$ CFU per chick *Salmonella enteritidis.* **The direct challenged (seeder chicks) were the tagged and dyed chicks.**

8. *Salmonella* SAMPLING BOOTSOCK. On DOT 21 and 42, bootsocks swab samples were collected for *Salmonella* environmental contamination determination from all pens. Gloves were changed between completion of each swab to reduce potential sample cross contamination. A pre-moistened bootsock swab (Solar Biologicals, Inc., Cat# BT SW-001) was removed from sterile bag, placed onto foot covered with a clean new plastic boot, the perimeter and interior of pen walked, bootsock removed, and placed into sterile bag labeled with pen number. After repeating procedure for each pen, samples were appropriately stored and delivered to the Southern Poultry Research Group Laboratory for *Salmonella* analysis.

9. CECAL and LIVER/SPLEEN *SALMONELLA* CULTURES**. Liver/spleen pooled into 1 bag, then cecal sampling (four [4] direct and four [4] horizontal) was completed on DOT 43.** Liver/spleen, ceca were collected aseptically. After removal, the **liver/spleen pool** sample was placed in **one** sterile plastic sample bag (Fisher Scientific), labeled, and immediately taken to SPRG lab for analysis of Salmonella prevalence and number for *Salmonella* analysis. The ceca sample was in a separate bag and taken to laboratory for analysis.

10. *SALMONELLA* ISOLATION AND IDENTIFICATION. All samples submitted for *Salmonella* isolation and identification were taken to the onsite Southern Poultry Research Group Laboratory on ice in sterile Whirl Pack bags. Upon arrival tetrathionate broth was added to bootsock samples while ceca plus liver/spleen were weighed, sterile saline added, and the sample stomachered. A one (1) ml aliquot was removed for MPN analysis, a 10X tetrathionate broth (Difco) solution added, and samples were incubated overnight at 41.5°C. A loopful of sample was struck onto xylose lysine tergitol-4 agar (XLT-4, Difco) plates which were incubated overnight at 37°C. Up to 3 (three) black colonies were selected and confirmed as *Salmonella* positives using Poly-O *Salmonell*a Specific Antiserum (MiraVista, Indianapolis, IN). (Berghaus et al., 2013; Alali et al., 2013)

11. *Salmonella* ENUMERATION PROCEDURE (MPN METHOD). For all ceca and bootsock samples, a one (1) ml sample of stomachered peptone broth was transferred to three (3) adjacent wells in the first row of a 96-well two (2) ml deep block. A 0.1 ml aliquot of sample was transferred to 0.9 ml of tetrathionate broth in the second row, process repeated for remaining rows (to produce five (5) ten-fold dilutions), and blocks incubated (24 hours at 42°C). Transferred one (1) µl of each well onto XLT-4 agar (containing nalidixic acid) with a pin-tool replicator, incubated plates (37°C for 24 hours), recorded final dilution of each sample, and entered in MPN calculator (to determine sample MPN). Suspect *Salmonella* isolates were confirmed by Poly-O *Salmonella* Specific Antiserum (MiraVista, Indianapolis, IN). (Berghaus et al., 2013; Alali et al., 2013)



12. DISEASE & COCCIDIA CONTROL. All birds were administered on DOT 0, one (1) dose/chick commercial coccidia vaccine. ***NO*** concomitant drug therapy was used during the study. To prevent cross-contamination, plastic disposable boots were worn when entering pens and changed between each pen.

13. BIRD IDENTIFICATION. The pen was the unit of measure. Pen security prevented bird migration.

14. MONITORING. All birds were monitored for general flock condition, temperature, lighting, water, feed, litter condition, and unanticipated house conditions/events. Findings were documented twice daily during the regular working hours (one [1] observation recorded on final study day). One (1) observation was recorded Saturday, Sunday, and observed holidays.

15. MORTALITY. Pens were checked daily for mortality. Birds were culled only to relieve suffering. The date and removal weight (Kg) were recorded for any bird culled (or found dead), gross necropsy was performed on all culled (or dead) birds, and the following information recorded: gender and probable cause of death.

16. BIRD AND FEED DISPOSITION. All birds were disposed of by appropriate methods. All mortalities and remaining feeds (including mixer flushes) were buried in the Southern Poultry Research Group disposal pit.

17. SOURCE DATA CONTROL AND HANDLING. Data was recorded in indelible ink with legible entries, each source data sheet signed (or initialed), and dated by individual recording entry. All source data errors (and/or changes) were initialed, dated, and a brief explanation statement or error code written directly on form.

Data Management

Data management and statistical analysis of weight gain, feed consumption, feed conversion, and *Salmonella* results were performed.

Source Data Location

Original source data sheets and final report were sent to South Florida Farming. An exact copy of the file and final report will be retained at Southern Poultry Research Group, Inc. for a period of three (3) years from study termination.

References

Berghaus, R., S. G Thayer, B. F. Law, R. M. Mild, C. L. Hofacre and R. S. Singer, 2013. Enumeration of *Salmonella* and *Campylobacter* in environmental farm samples and processing plant carcass rinses from commercial broiler chicken flocks, Appl. Environ. Microbiol. 1-37.

Cochran, W. G., and G. M. Cox. 1992. Experimental Design. 2nd Ed. John Wiley & Sons, New York, NY. Pg 582-583.

CALENDAR OF EVENTS

DOT	Date	General Description of Events
0	10/23/2023	⇒ Issued starter feed ⇒ Picked up male chicks from hatchery and group weighed ⇒ Sprayed chicks with 1 dose of coccidia vaccine ⇒ Grouped into sets of four (4) with three (3) treatments having six or two (2) replicates for treatment group 1 ⇒ Started feed treatments per Table 10 ⇒ Placed birds by pen and in appropriate pens ⇒ Began water treatment
0 - 43	10/23/2023 - 12/05/2023	⇒ Water treatment T4 only at 5 gm/1000 liters of water
3	10/26/2023	⇒ Thirteen (13) seeder birds per pen orally gavaged with a dose of $1x10^7$ CFU per chick *Salmonella enteritidis* ⇒ ***Tagged and dyed these direct exposed chicks***
21	11/13/2023	⇒ Weighed birds per pen ⇒ Discarded all non-consumed starter feed and replaced with grower feed ⇒ Bootsock swabbed all pens for S.I. enumeration
36	11/28/2023	⇒ Weighed birds per pen ⇒ Weighed and discarded all non-consumed grower feed and replaced with finisher feed
42	12/04/2023	⇒ Collected bootsock swab samples from all pens and cultured for enumeration by MPN
43	12/05/2023	⇒ Collected feces per pen for freezing for future analysis ⇒ Weighed all pens and feed ⇒ Collected liver/spleen and ceca from 8 birds/pen ⇒ Humanely euthanized remaining birds ⇒ Terminated trial