# Short communication: Effects of a commercial triple-strain *Bacillus*-based probiotic on cecal colonization with *Salmonella* Enteritidis in commercial layer pullets

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**ABSTRACT**

A commercial triple-strain *Bacillus*-based probiotic was tested to determine its effect on the colonization of the ceca by *Salmonella* Enteritidis (SE) in commercial layer pullets. Two treatments were tested, each with containing 128 day-of-hatch LSL layer chicks. On top of a standard diet: 1) no supplement (Control, CON), and 2) Probiotic (GalliPro® Fit, 500 g/MT, $1.6 \times 10^6$ CFU/g of finished feed, PRO). Environmental swabs were collected from each treatment group and tested to ensure freedom from SE prior to challenge. At 21 days of age, the SE challenge strain was administered orally at a dose of $3.3 \times 10^8$ CFU/bird. Pullets from each treatment group (n=32) were euthanized at 6-, 10-, 14-, and 18-days post infection (dpi). Contents from the ceca were aseptically collected and assessed for presence and abundance of SE. No differences in the prevalence of SE positive ceca following oral inoculation ($P>0.05$) were observed between treatment groups at 6-, 10-, 14-, or 18-dpi. Counts of SE in the ceca of the PRO group were not significantly different ($P>0.05$) from those of CON at 6- or 10-dpi. However, significantly lower counts of SE in the ceca of the PRO group were observed at 14-dpi ($P<0.05$) and 18-dpi ($P<0.05$) compared with CON. SE counts were 1.24 and 1.34 logs lower than CON at 14- and 18-dpi, respectively. In conclusion, supplementation of the triple-strain *Bacillus*-based probiotic resulted in lower cecal counts of SE compared to those that did not receive an effective probiotic, thereby reducing the risk of foodborne pathogens prior to harvest through sustainable, natural methods.

## 1. Introduction

*Salmonella* continues to be a predominant cause of foodborne illnesses globally. *Salmonella* is a Gram-negative facultatively anaerobic, rod-shaped bacterium that exhibits flagellation (Chen et al., 2013). It belongs to the Enterobacteriaceae family, and is differentiated based on its antigenic properties, which include O, H, and Vi antigens (Brenner et al., 2000). Salmonellosis is on an infection triggered by the bacteria of the *Salmonella* genus. This exposure typically results from consuming contaminated food or water, or through interaction with infected animals or their environments. Common sources include undercooked meats, eggs, and unpasteurized dairy products. The Centers for Disease Control and Prevention (CDC) estimates that *Salmonella* is responsible for 1.35 million infections, 26,500 hospitalizations, and 420 deaths in the United States annually (CDC, 2014). Currently, more than 2,500

serotypes of Salmonella have been identified. Notably, over half of these serotypes are classified under *Salmonella enterica* subsp. *enterica*, which is responsible for most *Salmonella*-related infections in humans (Brenner et al., 2000). Non-typhoidal *Salmonella* infections are the most prevalent, and many being attributed to *Salmonella enterica* subsp. *enterica* serovar Enteritidis (SE); other notable serotypes include *S.* Typhimurium, *S.* Infantis, and *S.* Heidelberg (Luvsansharav et al., 2020)

Several strategies are employed to control *Salmonella* in poultry production. *Salmonella* Enteritidis has been widely acknowledged as a significant causative agent of foodborne illness, predominantly associated with the consumption of table eggs (Whiley and Ross, 2015). In response to this public health concern, the US Food and Drug Administration instituted the Egg Safety Rule (FDA, 2009). This regulatory measure mandates egg producers to adopt preventative strategies during the production phase in poultry houses. It also outlines specific

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requirements for environmental monitoring and established detailed testing protocols to ensure food safety. While these interventions have proven effective to a certain extent, the egg industry continues to seek additional pre-harvest food safety measures and technologies that can be implemented at the farm (De Cort et al., 2017). Consequently, pre-harvest food safety emerges as a critical aspect that plays a pivotal role in the overall quality and safety of poultry products (Van Immerseel et al., 2002). It encompasses a range of strategies and practices aimed at minimizing the presence of pathogens and contaminants in poultry flocks prior to processing. These measures include appropriate feed and water hygiene, pest management, biosecurity protocols, vaccines, and the use of veterinary drugs and feed additives, all of which contribute significantly to reducing the risk of foodborne illness. (Trampel et al., 2014).

Among various feed additives, probiotics offer a promising option for producers, presenting an effective strategy to mitigate *Salmonella* and improve food safety (Van Immerseel et al., 2002). As defined by the World Health Organization (WHO), probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host. These beneficial bacteria are widely employed in poultry production to support bird health, optimal performance, and overall well-being. Spore-forming bacteria such as *Bacillus* spp. have gained attention because of their thermotolerance, environmental stability, and adaptability to harsh conditions (Abd El-Hack et al., 2020). *Bacillus* spp. can exert their beneficial effects on the host via mechanisms such as competitive exclusion, improved intestinal barrier function, immune modulation, and digestion and nutrient absorption (Abd El-Hack et al., 2020). Furthermore, considering concerns regarding overuse of antibiotics and the subsequent risk of pathogenic antibiotic resistance, probiotics have garnered attention in animal agriculture. They are increasingly employed as alternatives to antibiotics, aiming to support normal gut functions and inhibit potentially harmful bacteria commonly implicated in foodborne illness (Van Immerseel et al., 2002). Given that consumption of eggs is a primary source of *Salmonella* outbreaks, effective control of SE is crucial. Therefore, the objective of the present study was to evaluate the effects of a commercial triple-strain *Bacillus*-based probiotic on cecal colonization with SE in commercial layer pullets.

## 2. Materials and methods

### 2.1. Experimental design

A total of 256 day-of-hatch LSL-Lite layer chicks (Hy-Line, North America) were obtained from the Hy-Line hatchery (Goldfield, IA) and randomly assigned to two dietary treatments. Birds were placed into cage units with wire floor raised on stainless steel decks at the Laboratory Animal Resources (LAR) isolation facility of Iowa State University, and bird care was provided throughout the study by the LAR staff in accordance with Institutional Animal Care and Use Committee (4th Edition, 2020). Each cage unit (76cm × 457cm × 46cm) was equipped with adequate feeder space and nipple drinkers. Birds were housed in an environmentally controlled room in which temperature, humidity, and lighting were maintained according to breed guidelines (Hy-Line International, 2019). A non-medicated mash control diet based on corn and soybean meal was formulated to meet the nutritional requirements of the birds (ME 2900 kcal/kg; CP 20.0%). Additionally, feed from both treatment groups was tested to ensure the absence of *Salmonella* prior to feeding. This culture method is based on EN 15784:2021 "Animal feeding stuff – Isolation and enumeration of presumptive *Bacillus* spp. used as feed additive". On top of a standard corn and soybean meal-based diet, the treatments were: 1) no supplement (Control, CON); and 2) probiotic (GalliPro® Fit, 500 g/MT, $1.6 \times 10^6$ CFU/g of finished feed, PRO). Birds were offered *ad libitum* access to feed and water throughout the duration of the study. Concentrations of *Bacillus* in the PRO group were verified to be at a minimum of $1.6 \times 10^6$ CFU/g using a

culture-based method. It was also ascertained that the CON contained only negligible amounts of *Bacillus*, limited to those that might naturally occur. This analysis was performed at the Chr. Hansen Animal and Plant Health & Nutrition Laboratory in Milwaukee, Wisconsin.

### 2.2. Salmonella challenge

At 21 days of age, following the acclimatization period, each bird received a 0.5 ml inoculum of nalidixic-acid-resistant strain of SE containing $3.3 \times 10^8$ CFU using a dosing syringe and gavage tube. Inoculum was prepared by transferring a loopful of the SE stock to Tryptic Soy Broth (TSB) and incubated overnight with shaking at 37°C. A 1:10 dilution was further incubated with shaking and concentration was adjusted by measuring optical density at 600 nm with a spectrophotometer (SpectroVis Plus, Vernier, OR). Inoculum was harvested at a concentration of $10^9$ CFU/ml. Bacterial cells were washed twice, resuspended in sterile deionized water, and used immediately. Concentration of challenge dose was verified by standard plate count method and adjusted to the target dose. At 14 days-of-age, environmental swabs were collected from each treatment group and tested to ensure freedom from SE prior to challenge. Collection of environmental swabs was repeated at 7 days post-infection (dpi) to verify SE shedding with the experimental infection. At day 6-, 10-, 14-, and 18-dpi birds from each treatment group (n=32) were euthanized via cervical dislocation. Both ceca were aseptically collected from each individual bird. All samples were maintained on ice and transported to Nevysta Laboratory for microbiological analysis.

### 2.3. Microbiological analysis

Environmental swabs were processed for SE isolation using pre-enrichment in buffered peptone water, enrichment in Tetrathionate Hajna (TTH) broth and plating on Xylose Lysine Teritol-4 (XLT-4) agar and Brilliant Green with Novobiocin (BGN) agar. Suspected colonies were further tested in Triple Sugar Iron (TSI) and Lysine Iron (LIA) slants followed by sero-grouping using appropriate O and H *Salmonella* antisera as well as group D specific antiserum. Contents of the ceca were aseptically squeezed into sterile conical tubes. Sterile saline was added to prepare a 1:10 weight per volume pipettable suspension. Ten-fold serial dilutions were prepared, and standard plate count method was conducted using XLT-4 agar plates containing 25 μg nalidixic acid/ml. Plates were incubated aerobically for 24 hr. at 37°C and typical *Salmonella* colonies were counted. Concentration of *Salmonella* was calculated by the following formula: CFU/g = (Number of colonies × Dilution factor) / Volume cultured. Randomly selected colonies from positive countable plates were serologically confirmed to be the SE strain to validate the accuracy of visual counts. Samples that were below the detection limit of the counting method were cultured to determine the presence or absence of SE. Positive SE samples were assigned the minimum detection limit of the counting method (100 CFU/g), and negative samples were assigned zero *Salmonella* count.

### 2.4. Statistical analysis

All data were analyzed by ANOVA using GraphPad Prism 10.0.2 (GraphPad Software LLC, San Diego, CA). Significant differences were identified by Tukey's HSD of log transformed SE counts. Prevalence of SE positive ceca were analyzed following digitization of culture results. Differences were considered statistically significant at $P<0.05$.

## 3. Results and discussion

*Salmonella* Enteritidis is broadly recognized as a major contributor of foodborne illnesses, primarily linked with the consumption of table eggs. Therefore, incorporating feed additives such as probiotics, which double as pre-harvest food safety interventions on farm, is crucial. In the

**Table 1**

Prevalence of SE positive ceca following oral inoculation of $3.3 \times 10^8$ CFU at 21 days-of-age.

| Treatment | 6-dpi | 10-dpi | 14-dpi | 18-dpi |
|-----------|-------|--------|--------|--------|
| CON | 32/32 (100%) | 31/32 (97%) | 32/32 (100%) | 30/32 (94%) |
| PRO | 32/32 (100%) | 30/32 (94%) | 28/32 (88%) | 26/32 (81%) |

[a-b] Means in the same column with no common superscripts are significantly different at $P<0.05$; Values represent the number of SE positive birds/total number of birds tested

present study, a commercial triple-strain *Bacillus*-based probiotic was tested to determine its effect on the colonization in the ceca by SE in commercial layer pullets. The extent of colonization of SE was assessed by prevalence and enumeration following oral administration of $3.3 \times 10^8$ CFU of a nalidixic-acid-resistant strain of SE. Environmental swabs collected from each treatment group prior to challenge were negative for SE. At 7-days post-challenge environmental swabs tested positive for SE, confirming the establishment of infection and occurrence of shedding. Effects of treatment on prevalence of SE are presented in Table 1. The ceca, when tested at all time-points, consistently presented countable loads of SE. No differences in prevalence of SE positive ceca following oral inoculation were observed between treatment groups at 6-, 10-, 14-, and 18-dpi ($P>0.05$). At 14-dpi, the birds fed PRO exhibited 12% fewer positive samples compared to the CON group. However, these observations did not reach statistical significance ($P>0.05$). Similarly, at 18-dpi, birds in the PRO group showed a 13% reduction in positive samples relative to the CON group, but these results were not statistically significant ($P>0.05$). Effects of treatment on enumeration of SE are presented in Table 2. *Salmonella* counts ($Log_{10}$ CFU/g) in the birds fed PRO were not significantly different ($P>0.05$) from the CON group at 6- or 10-dpi. At 14-dpi, SE counts were significantly lower ($P<0.05$) in the birds fed PRO compared to the CON. Specifically, the mean SE values in the CON group were recorded at 5.14 $Log_{10}$ CFU/g, while those of the PRO group were reduced to 3.90 $Log_{10}$ CFU/g (1.24 Logs lower). Similar trends were observed at 18-dpi, SE counts were significantly lower ($P<0.05$) in the birds fed PRO compared to the CON group. The mean values for the CON were 4.65 $Log_{10}$ CFU/g, whereas for PRO, they were reduced to 3.31 $Log_{10}$ CFU/g (1.34 Logs lower).

Our findings are consistent with previous studies which have shown the ability of *Bacillus*-based probiotics to reduce the prevalence and enumeration of SE in poultry (Tellez et al., 2012). Similar results were observed by Price et al. (2020), where the efficacy of a commercially available probiotic, comprising of *Bacillus amyloliquefaciens, Bacillus licheniformis,* and *Bacillus pumilus* was assessed in reducing the concentrations of SE in the ceca of laying pullets. There was no significant difference among treatment groups with respect to prevalence of SE in the ceca. However, the birds fed a probiotic exhibited a SE mean reduction of 0.79 $Log^{10}$ MPN/g compared to the control group. In another study conducted by Vila et al. (2009), the effect of continuous administration of a *Bacillus*-based probiotic to birds infected with a field-isolated strain of SE was investigated. In Experiment 1, which concluded at 42 days, it was observed that 42% of the broilers in the untreated control group remained *Salmonella*-positive, whereas no traces of *Salmonella* were detected in birds that received a probiotic.

Similarly, Experiment 2 demonstrated a significant reduction in prevalence of *Salmonella* in layers receiving the probiotic, with only 38% testing positive three weeks post-inoculation, compared to 63% in the untreated control group. Additionally, in a study conducted by Khan and Chousalkar (2020) to evaluate the impact of an in-feed *Bacillus*-based probiotic on the gut microbiota and *Salmonella* load in laying hens challenged with *Salmonella* Typhimurium (ST). It was observed that ST infection led to a decreased abundance of genera such as *Lactobacillus, Bifidobacterium,* and *Faecalibacterium*. The infection also resulted in an increase in the abundance of genera such as *Akkermansia, Escherichia Shigella,* and *Flavonifractor*. This suggests that *Salmonella* may play a significant role in disrupting the gut microbiota, leading to a state of dysbiosis. Supplementation with probiotics partly ameliorated the gut microbiota imbalances caused by the ST infection, and significantly reduced the mean load of *Salmonella* in the feces and internal organs (*e. g.*, ceca, liver, and infundibulum). These results, along with those from the current study, suggest that supplementation with effective probiotics could represent a valuable strategy for reducing colonization and shedding of *Salmonella*. This approach shows potential for improving the effectiveness of existing pre-harvest food safety programs. It is hypothesized that probiotics support the health and performance of layer pullets in combating *Salmonella* through multiple mechanisms of action (Ohashi and Ushida, 2009). These beneficial effects may be achieved by the production of antimicrobial peptides that directly antagonize *Salmonella* growth. Additionally, probiotics may promote the formation of protective biofilms within the gastrointestinal tract, which act as barriers to prevent the adhesion and colonization of *Salmonella*. Furthermore, probiotics are believed to play a role in modulating the immune response, enhancing the birds' ability to respond to *Salmonella* colonization. This multi-faceted approach underscores the potential of probiotics as a vital component in the management of *Salmonella* in poultry.

## 4. Conclusions

Overall, supplementation of the triple-strain *Bacillus*-based probiotic resulted in significantly lower cecal counts of SE compared to those birds not on an effective probiotic. *Salmonella* counts were reduced by 1.24 and 1.34 logs of the CON group at 14- and 18-dpi, respectively. This study outcome reinforces the findings of prior *in-vitro* and *in-vivo* research, affirming that effective probiotics contribute significantly to improving pre-harvest food safety in poultry production. Reducing the presence and concentration of *Salmonella* within commercial laying flocks may substantially lower the risk of contaminated eggs being produced and introduced into the consumer market.

**Ethical statement**

This study was conducted in accordance with guidelines set by the Institutional Animal Care and Use Committee (4th Edition, 2020) at the Laboratory Animal Resources isolation facility of Iowa State University.

**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

**Table 2**

Mean counts Log10 CFU/g of SE in cecal contents of pullets challenged at 21 days-of-age.

| Descriptive Statistics | 6-dpi | | 10-dpi | | 14-dpi | | 18-dpi | |
|---|---|---|---|---|---|---|---|---|
| | CON | PRO | CON | PRO | CON | PRO | CON | PRO |
| Mean | 5.37 | 5.84 | 4.96 | 4.96 | 5.14[b] | 3.90[a] | 4.65[b] | 3.31[a] |
| SEM | 0.17 | 0.15 | 0.25 | 0.34 | 0.21 | 0.38 | 0.30 | 0.34 |
| Number | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 |
| *P*-value | >0.05 | | >0.05 | | <0.05 | | <0.05 | |

[a-b] Means in the same column with no common superscripts are significantly different at P<0.05; SEM, standard error of the mean

the work reported in this paper.

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