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Original Article

Production Layer Salmonella Enteritidis Control through Dry Fed Pre & Probiotic Products

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ABSTRACT

Increasing interest in multiple strain *Bacillus* probiotics and parietal yeast fractions as feed ingredients for egg laying hen diets has also led to food safety questions. This study was undertaken to evaluate the ability of these products to reduce *Salmonella* Enteritidis colonization. Sixty Hy-Line hens aged 56 weeks were placed in individual cages and fed a mash diet containing one of the following treatments, control, *Bacillus spp.* probiotic, yeast cell wall, or a combination of yeast cell wall and *Bacillus* probiotic. At 60 weeks of age all hens were challenged orally with 7 x 10^7 CFU/bird of *Salmonella* Enteritidis. At 61 weeks of age, birds were humanely euthanized, by cervical dislocation and the ceca aseptically removed and cultured for *S.* Enteritidis prevalence and number by the Most Probable Number method. There was no significant difference in prevalence of *Salmonella* Enteritidis between the control and any treatments. The control birds had 4.37 log10 MPN/g of *S*. Enteritidis detected in the ceca. The Probiotic group had 2.96 MPN/g, a reduction of 1.41(p<0.05) and the yeast cell wall group had 2.89 MPN/g a reduction of 1.48 (p<0.05). The combination had 3.60 MPN/g a numerical reduction of 0.78 (p=0.14). The yeast cell wall and *Bacillus* probiotic groups significantly reduced the amount of *Salmonella* Enteritidis in the ceca of the laying hens.

INTRODUCTION

Salmonella is commonly associated with poultry and poultry products, often resulting in highly publicized outbreaks of foodborne illnesses. Concerns over foodborne illnesses and the associated outbreaks have led to a focus on live animal pathogen control strategies. *Salmonella* annually causes an estimated 93 million enteric infections worldwide and 155,000 deaths (Majowicz, 2010). The Centers for Disease Control and Prevention estimate *Salmonella* is responsible for over 1.2 million illnesses in the United States, and that 1 million of these cases are the result of foodborne *Salmonella* infections (Galanis, 2006). *Salmonella enterica* serotypes Typhimurium and Enteritidis are the most common in human infections associated with animals worldwide (Herikstad, 2002; Afshari, 2018). Data from the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) shows that over 9% of all *Salmonella* positives are caused by *S*. Enteritidis (USDA-FSIS 2016). Current interventions used in live production for *Salmonella* control in the U.S. poultry industry consist of a mixture of biosecurity, nutritional and feed management, non-antimicrobial feed additives, and vaccines. The use of probiotics in poultry has been shown to alter microbial population and reduce the growth of pathogens (Fanning, 2018). It has been shown that *Bacillus spp.* probiotics can improve the efficiency of feed to gain nutrient utilization, and other production

parameters (Park, 2015). Poultry (Menconi, 2013), mice (O'Mahony, 2001), and human (Urdaci, 2004) models have all shown that *Bacillus* can influence the host immune system and compete for host attachment sites and nutrient utilization to detriment of *Salmonella* that may otherwise colonize the host. In poultry, *Bacillus spp.* delivered in feed has shown reduced *Salmonella* counts in the intestine, crop, and ceca. Some studies have shown reductions in prevalence as much as 72%, and increased reductions in number up to $1x10^3$ CFU/g (Knap, 2011; Adhikari, 2019). *Bacillus subtillus* and *Bacillus methylotrophicus* treatments showed reduction in the load of *Salmonella* positive layers by over $1x10^1$ CFU/g in a *S.* Gallinarum challenge (Upadhaya, 2016). *Bacillus subtillus* has also been effective in achieving reductions in *S.* Heidelberg in broiler chickens (Hayashi, 2018).

Yeast is a well-documented prebiotic source for poultry and previous work has demonstrated control over a variety of foodborne pathogens in poultry production (Hatoum 2012, Huff 2010, Roto 2015). The use of non-digestible oligosaccharide prebiotics has also been shown to affect intestinal and immune function through a variety of factors (Revolledo, 2006; Sheng, 2006; Alloui, 2013). Mannanoligosaccharides in particular are mannose-based oligomers that can influence cecal microbiota in broilers and layers due to their ability to reach the lower GI tract undigested (Pourabedin, 2015). Mannanoligosaccharide supplementation has shown reduced *Salmonella* Enteritidis shedding from broiler chickens (Lourenço, 2015). Mannose from *Saccharomyces cerevisiae* has shown consistent potential for the binding of pathogenic bacteria with type-1 fimbriae, such as *Salmonella*, which can in turn lower CFU counts and prevalence in intestinal and fecal content culture (Oyofo, 1989; Hooge, 2004; Cortés-Coronado, 2017). In the avian GI tract, the combination of mannanoligosaccharide and β-1,3 glucan in yeast cell can stimulate the epithelial cell lining junctions to strengthen and thereby reduce the flow of pathogens past the intestinal barrier (Shao, 2013). Shanmugasundaram *et al*. (2013) showed that the dietary addition of the whole yeast cell wall can reduce the incidence of *Salmonella* due to the impact on coccidiosis (Shanmugasundaram, 2013). The specific serovars, *S*. Typhimurium (Price, 2019), *S.* Heidelberg (Kiros, 2019), *S*. Enteritidis (Price, 2019b) have all been shown to have reduced numbers in poultry cecal by a commercially available yeast cell wall. This study focused on demonstrating the potential of a specific yeast cell wall, a multispecies probiotic, and their combination to reduce intestinal colonization of laying hens by *Salmonella* Enteritidis.

MATERIALS AND METHODS

Sixty, 56-week-old Hy-Line W-36 hens were purchased from a commercial layer company. Birds were provided with mash feed formulated to meet or exceed current NRC standards and water ad libitum throughout the duration of the trial. The unit for each treatment was fifteen (15) cages of a battery, therefore each cage became a replicate. Birds were randomly assigned to treatments: control, 500ppm yeast cell wall (YCW), 500ppm *Bacillus* spp. probiotic (PB), and a blend of 250ppm of YCW and 250ppm Probiotic (Combo). The YCW is a commercially available product with minimum guaranteed levels of mannan (20%) and β-glucan (20%). The Probiotic is a commercially available blend of *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, and *Bacillus pumilus*. The treatment diets were fed for 4 weeks prior to inoculation of *Salmonella*.

Inoculum preparation

A nalidixic acid/ novobiocin resistant strain of *Salmonella* Enteritidis was aseptically removed from -80C storage and grown onto tryptic soy agar II (TSAII) plates supplemented with 5% sheep blood. Cultures were grown aerobically for 24 hrs at 37C. A single colony from the TSAII agar plate was inoculated into a brain - heart infusion (BHI) broth and incubated in a shaker incubator (200 rpm) overnight at 37C. The culture was diluted into phosphate buffered saline (PBS) to the estimated desired CFU/mL prior to inoculation and confirmed retrospectively by serial dilution and culture.

Inoculation and sample collection

At 60 weeks-of-age each bird was orally challenged with 1mL of 7×10^7 CFU/bird of a nalidixic acid/ novobiocin resistant strain of *Salmonella* Enteritidis. On seven (7) days post-challenge all hens were humanely euthanized by cervical dislocation, ceca were aseptically removed and placed into sterile plastic sampling bags. The samples were placed on ice for transportation to the lab for *Salmonella* analysis. The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The US National Research Council's guidelines for the Care and Use of Laboratory Animals were followed.

Salmonella isolation, identification, and enumeration

Ceca samples were weighed and diluted in buffered peptone water BPW to give a 1:10 dilution. Each sample was then stomached for 1 minute ensure even mixing prior to serial dilution. Salmonella were enumerated using standard 10-fold serial dilution method. A 0.1mL aliquot was transferred to 0.9mL of PBS. This process was repeated creating (4) 10-fold dilutions. The dilution 10-1 was plated in triplicate using 0.1mL on a whole spread plate and the 10-2 to 10-4 dilutions were plated in triplicate using a 10uL micro drop technique onto Xylose Lysine Tergitol-4 (XLT-4) plates containing 100µg of nalidixic acid/mL and 15ug novobiocin/mL. Additionally, 1 ml of the ceca and BPW solution was placed into tubes containing 9 mL tetrathionate broth (TTB). These plates and tubes were incubated for 24 hrs at 37°C. After that time Salmonella was enumerated from the plates. To determine prevalence, for any samples that were negative for Salmonella in the enumeration step, one 10µL loop of the corresponding enrichment TTB tubes was streaked onto XLT-4 100µg of nalidixic acid/mL and 15ug novobiocin/mL. These plates were incubated for 24 hours at 37°C. After that time Salmonella prevalence was determined from the plates.

Statistics

Salmonella prevalence in ceca samples were compared between treatment groups using Fisher's exact test. A Tobit censored regression model was used to compare treatment groups with respect to *Salmonella* MPNs in ceca samples while considering culture-negative samples to be left-censored at 4.477 $\log_{10}$ MPN/g (because the culture method's lower limit of detection is 30 CFU at first dilution). For the comparison of *Salmonella* MPNs, samples with a negative culture result by the MPN method but a positive result by 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. 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 15.1, StataCorp LLC, College Station, TX) for Fisher's exact test and R software for Tobit model (version 3.6.1., R Foundation for Statistical Computing, Vienna, Austria) with packages AER (Kleiber 2008) and lmtest (Zeileis & Hothorn, 2002).

RESULTS AND DISCUSSION

The prevalence of SE in the ceca was similar between all treatments. The ceca in the control and Probiotic group were 93% positive for SE (14/15), the YCW group was 87% positive 13/15, and the Combo group 100% positive (15/15). The level of SE in the ceca, measured in log10 MPN/g, in the control group was 4.37. The level of SE was reduced by 1.41 logs in the Probiotic and 1.48 logs in the YCW group ($p<0.05$). The load of SE was numerically reduced compared to the control in the Combo group by 0.78 logs ($p=0.14$). These data are displayed in Figure 1. *Salmonella* spp. can bind to mannose via the type-1 binding fimbriae. The cell wall fraction of *S. cerevisiae* has been shown to bind a variety of gram negative organisms (Posadas, 2017). Reduction in *S.* Enteritidis levels in the ceca of layers will reduce the overall load in the environment leading to reduced risk of egg-shell contamination and transmission of foodborne illness. A feed additive reducing the level of Salmonella by 1 log is often viewed as a threshold of biological significance when cecal prevalence is near 100% (Hofacre, 2018). A previous study with the YCW product in this study showed not

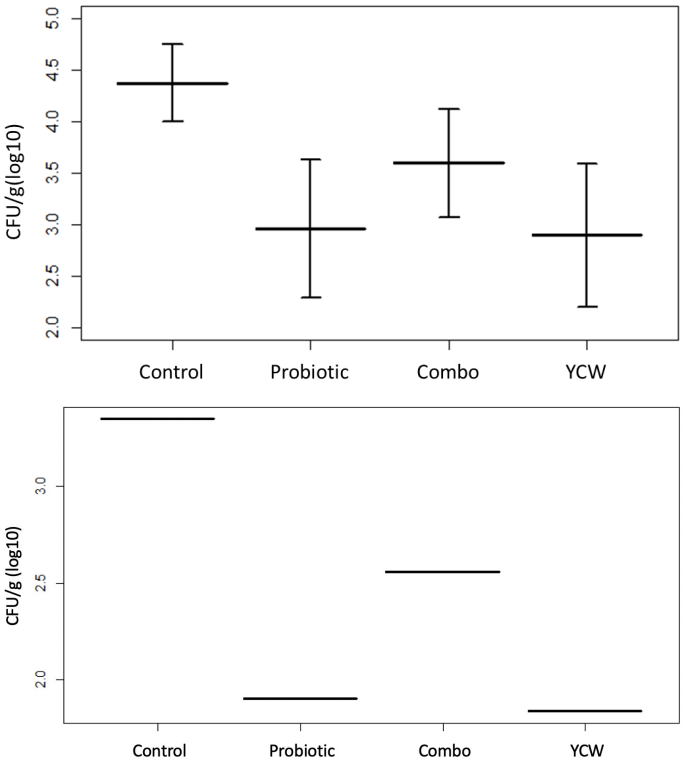

Figure 1 – *Salmonella* Enteritidis predicted means in the ceca displayed as CFU/g log10 of 61 weeks of age hens challenged orally with 7 x 10^7 CFU/bird of *Salmonella Enteritidis* at 60 weeks of age. The predicted means were obtained using Tobit censored regression model left-censored at 4.477 log10 MPN/g on the 52 enrichment-positive samples. Error bars represent the SEMStandard Error of the Mean (SEM). Treatments are untreated control (Control), 500ppm *Bacillus* spp. probiotic (Probiotic), 500ppm Yeast cell wall (YCW), blend of Probiotic and YCW each at 250ppm (Combo).

only a 1 log reduction in CFU/g of *Salmonella* than control (p<0.015), but also 20% lower prevalence (Price, 2019). A previous study with the same species of *Bacillus* as used in this study showed a significant reduction in the number of S. Enteritidis in layer ceca (Price, 2019b). The use of YCW as a prebiotic in layer diets and the multispecies *Bacillus* probiotic may decrease the level of *S.* Enteritidis in the ceca leading to lower contamination of the environment effectively reducing the risk of transmission of *S.* Enteritidis.

CONCLUSIONS

The use of YCW and Probiotics in layer diets can be part of a multi-hurdle approach to reduce the load of SE in layer chickens. Reducing the load of SE in the ceca of hens reduces the total load of SE in the environment likewise reducing the risk of contamination of eggs and eggshells entering the market.

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