

Effect of dietary direct-fed microbial and yeast cell walls on cecal digesta microbiota of layer chicks inoculated with nalidixic acid resistant *Salmonella* Enteritidis

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ABSTRACT *Salmonella enterica* serovar Enteritidis (SE) has consistently been the most common serotype associated with the foodborne Salmonellosis worldwide. In this study, the effect of a dietary direct-fed microbial (DFM) and yeast cell walls (YCW) under a challenge of nalidixic acid resistant SE strain using layer chicks has been investigated. A total of 160 newly hatched Dekalb White female chicks were randomly assigned into 2 experimental groups (80 birds/treatment), control group (CON) and treatment group (DY). Chicks were fed ad libitum a non-medicated-corn-soy based diet and DY was supplemented with the combination of DFM and YCW. At 8 days of age, 2.1×10^9 CFU/bird of the SE was given to all chicks by oral administration. On 3 days postinoculation (dpi), 20 chicks/group were euthanized and all cecal contents were collected for analysis. On 6, 10, and 14 dpi, the cecal contents were sampled from 16 chicks per group. The number of SE in the

cecal contents was counted using culture-based methods. A 16S rRNA-based microbiota analysis was performed for additional microbial profiling. The CON and DY showed difference ($P \leq 0.05$) in β diversity throughout the trial. Prevalence of SE in cecal contents was lower ($P \leq 0.05$) in DY across all time-points. Lower abundance of *Salmonella* spp. was also shown in DY by liner discriminant analysis effect size (LEfSe). DY increased ($P \leq 0.05$) diversity of bacterial species in the cecal contents in DY at 10 and 14 dpi. For the SE challenged birds, SE reduction in DY was observed at 3 dpi and until the end of the trial at 14 dpi confirming a numerically larger difference between groups as well as an increase in bacterial species diversity in DY. It could be hypothesized that the SE reduction shown immediately after the challenge and the greater SE reduction shown after 10 dpi may be the synergistic effect of the combined feed additives.

Key words: cecal microbiota, layer chick, direct-fed microbial, yeast cell walls, *Salmonella* Enteritidis

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INTRODUCTION

Salmonella is one of the leading causes of foodborne illness in the United States (Scallan et al., 2011) as well as worldwide (Kirk et al., 2015). *Salmonella enterica* serovar Enteritidis (SE) has consistently been one of the most commonly reported serotypes causing foodborne illness (Boore et al., 2015; Tack et al., 2020). Laying hens can serve as SE reservoirs and eggs are at high risk of contamination (Arnold et al., 2014). In the European Union, 63% of all SE infections were attributed to laying hens (De Knegt et al., 2015). According to the investigation of outbreaks associated with *Salmonella* and food commodities between 1998 and 2008 in the United

States, eggs are the most common source of SE outbreaks (65% of all outbreaks; Jackson et al., 2013). Therefore, egg-related Salmonellosis is a common concern and unique countermeasures to control SE in eggs are implemented in each country or region (Chousalkar et al., 2018). To prevent SE contamination of eggs in the United States, the Food and Drug Administration (FDA) issued a final rule that required shell egg producers to implement measures including but not limited to routine flock testing, sanitation, biosecurity, vaccination, proper storing, washing, and processing procedures (U.S. Food and Drug Administration, 2009). In the European Union (EU), the National Control Programs (NCP) of *Salmonella* have been implemented in accordance with EU legislation targeting the SE serovar (European Union, 2003). In addition to measures currently in place, feed additives are also considered as possible key preharvest measures to minimize *Salmonella* infection in poultry production (Berge and Wierup, 2012; Alali and Hofacre, 2016), such as prebiotics

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(Ribeiro et al., 2007; Attia et al., 2012; Micciche et al., 2018), and Direct-Fed Microbial (DFM) products (probiotics) (Mountzouris et al., 2009; Knap et al., 2011; Neveling et al., 2020).

Bacillus species as DFM for poultry increase growth performance and their spore forming ability makes the product stable for a long time during pelleting of feed (Vazquez, 2016). *Bacillus subtilis* C-3102 (CALSPORIN[®], Asahi Biocycle Co., Ltd, Tokyo, Japan) has been approved as a zootechnical feed additive in the EU (European Food Safety Authority, 2007) and as a probiotic feed additive in Japan. In addition to body weight gain (Fritts et al., 2000; European Food Safety Authority, 2007; European Food Safety Authority, 2010; Jeong and Kim, 2014) and improved feed conversion ratio (Hooge et al., 2004; Marubashi et al., 2012), reduction of *Salmonella* in the gut was also reported with *Bacillus subtilis* C-3102 supplementation in broilers (Maruta et al., 1996a; Fritts et al., 2000; Jeong and Kim, 2014), in laying hens (Nishiyama et al., 2020), and in Japanese quail (Manafi et al., 2016).

Yeast cell walls (YCW), containing mannan-oligosaccharides (MOS), bind pathogens with type 1 fimbriae and prevents adhesion to the intestinal wall and subsequent proliferation (Spring et al., 2000). Previous studies have shown reduced levels of *Salmonella* when YCW is added to the feed of laying hens (Hofacre et al., 2018; Girgis et al., 2020), broilers (Bonato et al., 2020; Spring et al., 2000), and turkeys (Rahimi et al., 2019). A combination of *Bacillus* DFM and YCW was also evaluated with turkey poults, and shown to reduce *Salmonella* in feces (Rahimi et al., 2019). However, there is limited data available regarding the benefit of a DFM and YCW combination for laying hens in egg production to reduce *Salmonella* contamination of eggs. *Salmonella* infection in chicks of more than a few days old generally results in little or no clinical disease (Barrow, 2000). However, chicks exposed to SE shortly after hatching can remain infected until maturity, and might produce contaminated eggs or spread SE to uninfected hens (Gast and Holt, 1998). Therefore, it is important to adopt additional strategies against SE infection and spread from an early stage, even before the onset of egg production.

The objective of this study was to investigate the effect of the combination of DFM (*Bacillus subtilis* C-3102) and YCW (IMW50[®]) on SE colonization and cecal microbial composition in layer chicks under SE challenge.

MATERIALS AND METHODS

Animals and Model

During the study, behavior and health condition of chicks were evaluated daily by a qualified veterinarian. Care and use of procedures for chicks were approved by the Institutional Animal Care and Use Committee of Iowa State University. A total of 160 newly hatched Dekalb White female chicks (Hendrix Genetics, Grand Island, NE) without receiving *Salmonella* vaccine or antibiotics were provided for the trial. Chicks were fed

ad libitum a nonmedicated, commercial corn- and soybean meal-based diet containing the nutritional requirements for layer chicks (National Research Council, 1994). The chicks were randomly selected and assigned to 2 wire floor cages (size of 76.2 cm × 152.4 cm × 45.72 cm each) with sufficient feeders and drinkers in the AAALAC-accredited Laboratory Animal Resources isolation facility of Iowa State University during the trial. Chick paper was placed on the bottom of each cage during the first 7 d acclimatization period. Room temperature was controlled according to Dekalb White Commercial Product Guide North American Version (Hendrix-Genetics, 2019). Birds were kept under the following daily lighting schedule: 22 to 23 h in the first week, 20 h in the second week, and 18 h in the third week. One of the cages was assigned to Control group (CON) and the other was assigned for the DFM and YCW treatment group (DY). The CON was fed a basal diet and DY was fed a basal diet supplemented with a combination of DFM (250,000 CFU *Bacillus subtilis* C-3102/g of feed, CALSPORIN[®], Calpis America, Inc., Peachtree City, GA) and YCW (0.05% in feed, IMW50[®], ICC USA, Inc., Louisville, KY). Each cage was placed into an individual isolation unit to prevent cross-contamination. *Salmonella* negative status was confirmed at 5 days of age by environmental swab test. At 8 days of age, each chick was orally administered 2.1×10^9 CFU of a nalidixic acid resistant SE strain. Twenty chicks were randomly selected from each group at 3 days postinoculation (dpi), and 16 chicks were randomly selected from each group at 6, 10, and 14 dpi. At each sampling time point, the selected chicks were humanely euthanized by cervical dislocation and total cecal contents were aseptically collected.

SE Enumeration and Identification

Fresh cecal contents were individually weighed and were serially diluted (1:10 w/v) with diluent A (Mitsuoka, 1971). From each serial dilution, 50 μ L of suspensions was plated on XLT-4 plates (Thermo Fisher Scientific, Waltham, MT) with 25 μ g/mL of nalidixic acid. After 24 h incubation at 37°C, typical SE colonies were counted. Serological confirmation was performed with at least three randomly selected colonies from each positive sample to validate the accuracy of the visual counts. A miniature 3-tube most probable number (MPN) procedure developed by Berghaus et al. (2013) was also performed on each retained cecal contents solution.

The 16S rRNA Library Preparation and Illumina MiSeq Sequencing

The DNA was extracted from cecal samples following the manufacturer's protocol for Mag-Bind Blood & Tissue DNA HDQ 96 Kit (Omega Bio-tek, Inc., Norcross, GA). All extracted DNA samples were stored at -80°C until further analysis. The library preparations and Illumina

Table 1. Means \pm S.E. number (log CFU/g) of the inoculated SE strain in cecal contents of layer pullet at 3, 6, 10, and 14 days post-SE challenge (dpi).

Group	Days post-challenge (dpi)				Effect		
	3	6	10	14	Group	dpi	Group \times dpi
CON	7.63 \pm 0.10	8.37 \pm 0.10	8.08 \pm 0.11	8.10 \pm 0.08	0.000	0.000	0.1420
DY	7.02 \pm 0.28	7.88 \pm 0.11	7.63 \pm 0.14	6.85 \pm 0.35			

MiSeq sequencing were conducted by Zymo Research Corporation (Irvine, CA). The DNA samples were prepared for targeted sequencing with the Quick-16S NGS Library Prep Kit (Zymo Research Corporation). The V3-V4 region of 16S rRNA gene was amplified by using the primer set containing 341F and 785R proposed by Klindworth et al. (2013). The final library was sequenced on Illumina MiSeq with a v3 reagent kit (600 cycles). The sequencing was performed with 10% PhiX spike-in. Unique amplicon sequences were inferred from raw reads using the Dada2 pipeline (Callahan et al., 2016). Chimeric sequences were also removed with the Dada2 pipeline. Taxonomy assignment was performed using Uclust from Qiime v.1.9.1 (Caporaso et al., 2010) Composition visualization, alpha-diversity (Chao1 index), and beta-diversity analyses were also performed by Qiime with genus level (Caporaso et al., 2010). The 3-dimensional principal coordinate analysis (PCoA) plot of changes in cecal microbiota structure was created using the matrix of paired-wise distance between samples calculated by the Bray-Curtis dissimilarity, and were assessed with ZymoBIOMICS (Zymo Research Corporation).

Statistical Analysis

Statistical analysis was performed by SAS for Windows version 9.4 (SAS Institute Inc., Cary, NC) using Proc Mixed Procedure with fixed effects of treatment

and dpi. If a significant interaction was confirmed between treatment and dpi effects, Tukey HSD was applied for comparison between test groups, and differences between treatments means were considered significant at P value less than 0.05. For 16S rRNA-based microbiota analysis, Permutational multivariate analysis of variance (PERMANOVA) was conducted in R v4.0.2 (R Core Team, 2018), using the vegan package v2.5-7 and RVAideMemoire package v 0.9-79. Taxonomy that had significant differences in abundance among groups was identified by linear discriminant analysis effect size (LEfSe; Segata et al., 2011).

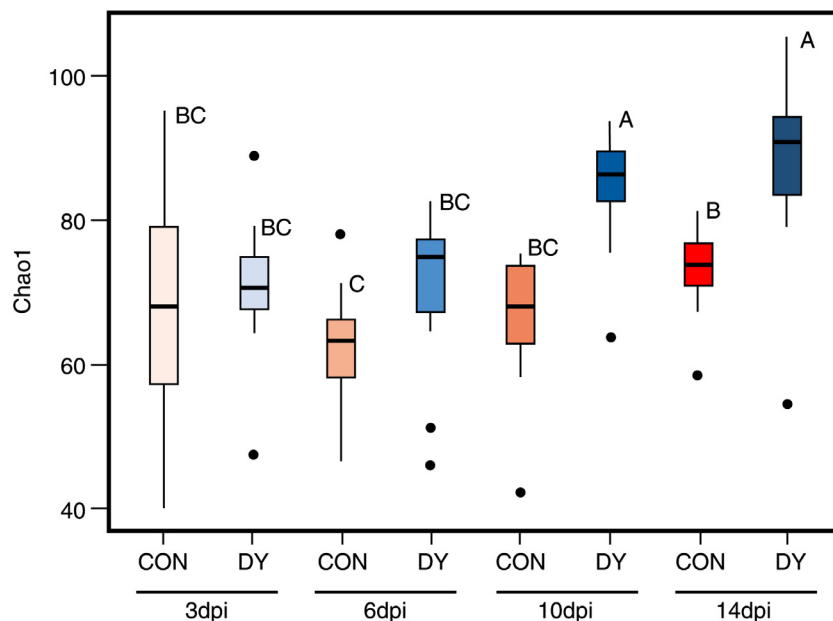
RESULTS

Enumeration of SE Strain

A total 132 cecal contents were collected and utilized for SE enumeration and microbial profiling. The number of challenged SE strains in the cecal contents was lower ($P \leq 0.001$) in the DY group throughout the trial (Table 1). The MPN method was not available because the SE number exceeded the designed target range (10^5 CFU/g upper limit, data not shown).

Microbiota Diversity of Cecal Contents

The alpha diversity (Chao1 index) of cecal microbiota is shown in Figure 1. No differences were observed between

**Figure 1.** Microbial alpha diversity (Chao1 index) of cecal microbiota in CON and DY at different dpi sampling time points. Values on each bar with no common letter differ ($P < 0.05$).

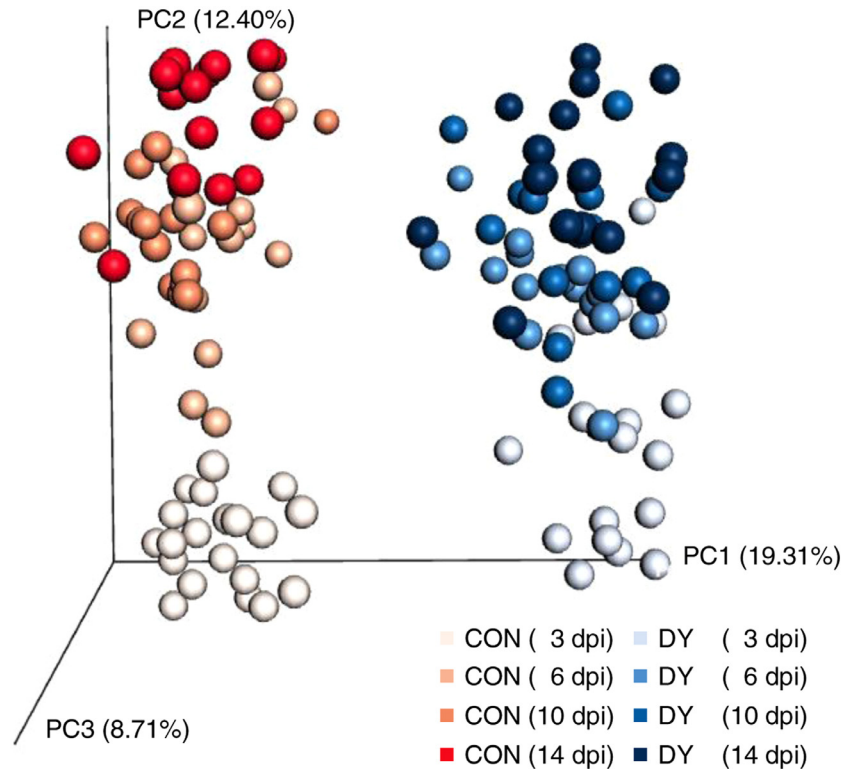


Figure 2. The principal coordinate analysis (PCoA) of the cecum microbial communities created using the matrix of paired-wise distance between samples calculated by the Bray-Curtis dissimilarity. Total 8 different colored dots indicates the relative distances between samples from CON and DY at 3, 6, 10, and 14 dpi. Differences ($P = 0.001$) were statically calculated between treatment groups using the PERMANOVA.

CON and DY at 3 dpi and 6 dpi. However, the alpha diversity in cecal contents appears to be higher ($P \leq 0.05$) in DY at 10 dpi and 14 dpi. The 3D PCoA of cecal microbiota (Figure 2) clear separation ($P = 0.001$) of PCoA scores between CON and DY treatments. A pairwise test for each dpi also showed differences ($P \leq 0.05$) in all combinations except for 6 dpi and 10 dpi in CON.

Cecal Bacterial Community Abundance

The composition of the cecal microbiota was evaluated based on a total of 15,405,388 sequence reads. Figure 3 shows the microbial community composition at the genera level, and *Salmonella* spp. appeared in the composition of each dpi for both groups. LEfSe identified several other taxonomic genera differences between CON and DY throughout the trial, and only genera above linear discriminant analysis (LDA) significant threshold of >2 are shown in Figure 4. The CON contained higher ($P \leq 0.05$) levels of *Proteus*, *Anaerotruncus*, *Blautia*, *Ruminiclostridium*, and *Salmonella* genera compared with DY. Higher levels ($P \leq 0.05$) of *Clostridiales*, *Bifidobacterium*, *Klebsiella*, *Peptoclostridium*, and *Pseudoflavonifractor* were observed in DY. Significantly higher abundance ($P \leq 0.05$) of *Salmonella* spp. in the CON was also supported by LEfSe.

DISCUSSION

Lower SE numbers in cecal contents of DY birds started to be observed at 3 dpi and the difference

between CON and DY was greatest at 14 dpi. This SE reduction in the ceca with DFM and YCW supplementation was also supported by less abundance of *Salmonella* spp. shown in LEfSe. Two hypotheses are proposed to explain this SE reduction in the ceca in the present challenge trial. Rubinelli et al. (2016) demonstrated that rapid reduction of *Salmonella* spp. by yeast fermentation product happens in an in vitro anaerobic mixed chicken cecal culture model after 24 and 48 h. MOS is a major component of YCW (Bychkov et al., 2010), which can bind pathogenic bacteria with type-1 fimbriae, such as *Escherichia coli* and *Salmonella* species (Spring et al., 2000; Ganner et al., 2011). Thus, MOS can limit *Salmonella* adhesion to host tissues by attaching to the supplemented MOS (Micciche et al., 2018). Reduction of SE attachment to the intestinal epithelial cells by cultured medium from *B. subtilis* NC11 was also reported by Thirabunyanon and Thongwittaya (2012). A recent study revealed that *Bacillus* strains have the potential to inhibit SE biofilm formation by producing the bacteriocin subtilisin A and subtilin (Tazehabadi et al., 2021). These direct SE exclusion mechanisms may contribute to the SE at 3 dpi in this trial.

In contrast to the first hypothesis, relatively long-term supplementation of DFM and YCW may be required for the second hypothesized mode of action and it may support SE reductions later in this trial. Interactions between SE and gut microbiota are not completely understood yet, but the gut microbiota has symbiotic ability to inhibit pathogen colonization in the gut via several mechanisms including direct killing, nutritional

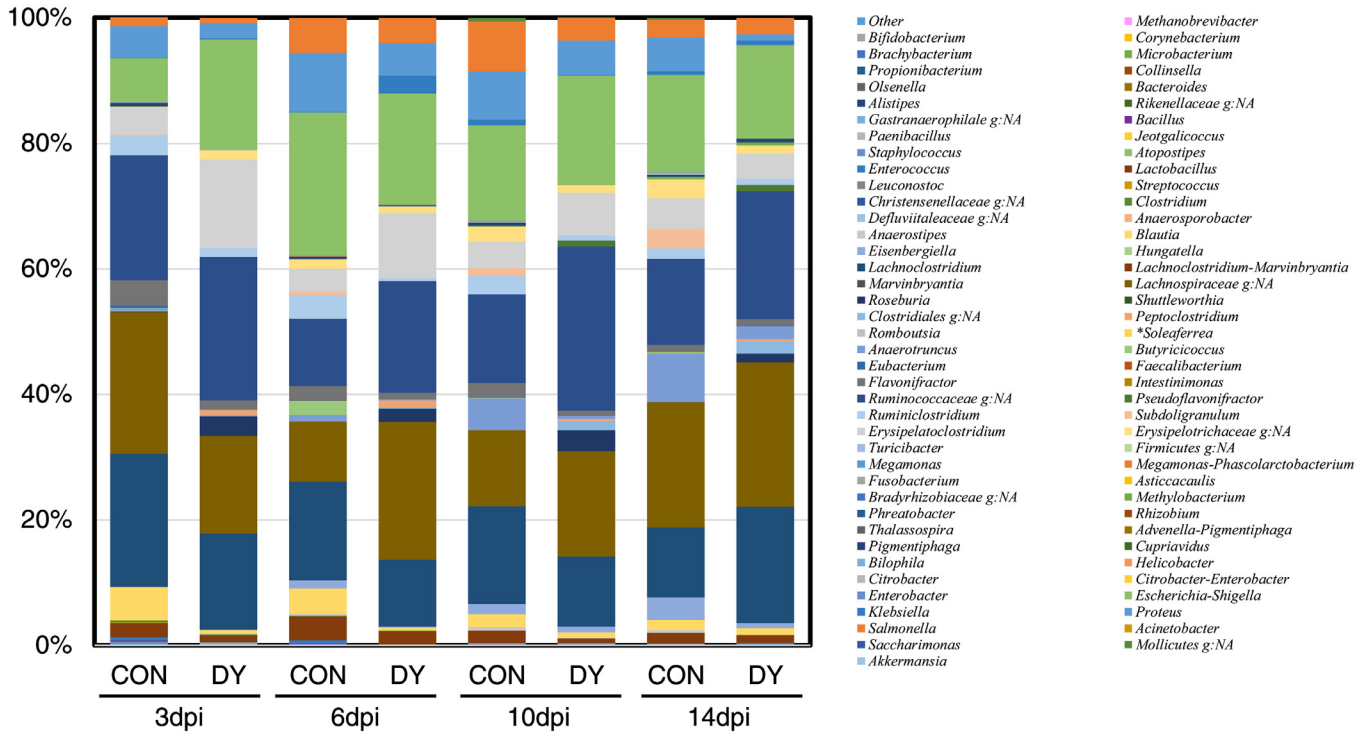


Figure 3. Abundance of microbial communities at genera level in cecal contents of SE challenged layer chicks in CON and DY at 3, 6, 10, and 14 dpi.

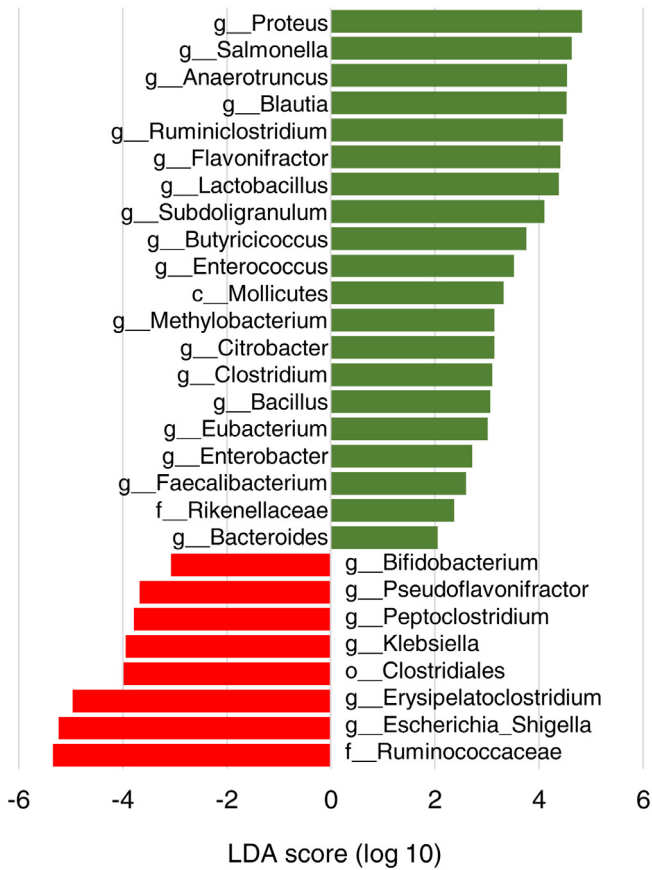


Figure 4. Linear discriminant analysis effect size (LEfSe) showing cecal microbiota differences between CON and DY throughout the trial at the genus level. Enriched genera in CON are indicated with a positive LDA score (green), and genera enriched in DY have a negative score (red). Only genera above LDA significant threshold of >2 are shown in the figure.

competition, and enhancing the immune system (Pickard et al., 2017). Furthermore, *Salmonella* infections cause dysbiosis by decreasing the abundance of specific microbial genera (Khan and Chousalkar, 2020) and microbial diversity in the fecal material of laying hens (Oh et al., 2017). The diversity recovered with *Bacillus* administration (Oh et al., 2017) and restoration of microbial abundance by *Bacillus* based probiotic (DFM) after a *Salmonella* challenge has been associated with the negative impact on *Escherichia/Shigella* and increase of *Lactobacillus* (Khan and Chousalkar, 2020). Maruta et al. (1996b) observed a decrease in *Salmonella* and increase of *Lactobacillus* in the broiler fecal samples 2 wk after *B. subtilis* C-3102 supplementation. Intestinal microbial composition alteration by *Bacillus* DFM supplementation resulted in increased *Lactobacillus*. Reduction of undesirable bacterial groups was also reported in several former studies (Jin et al., 1996; Jeong and Kim, 2014; Song et al., 2014). The latest molecular technologies show that previously reported shifts in microbial composition by *Bacillus* DFM can now be attributed to simultaneous SE reduction. In addition, MOS is also known as an influencer of microbiota in chicken gut (Teng and Kim, 2018). Rubinelli et al. (2016) reported that the presence of the cecal microbiota might be a prerequisite for a yeast fermentation product to provide its *Salmonella* reducing effect. Marzorati et al. (2020) suggested that 2 wk would be the minimum time requirement for observable benefits from *Bacillus* probiotic supplementation in the gut microbiome model. Furthermore, the effect of *Bacillus* DFM on the composition of gut microbiota was higher for the continuously supplemented group compared to

the intermittently supplemented group (Khan and Chousalkar, 2020). These data suggest that it may take a certain amount of time for DFMs to shift the balance of microbiota. In the present trial, increase of diversity of bacterial species ($P \leq 0.05$) appeared in DY at 10 dpi and 14 dpi, which may have contributed to the greater SE reduction observed in the latter half of this study. Therefore, 2 different pathways may be at play when chicks are exposed to a combination of DFM and YCW. SE reduction in the ceca shown in this trial between 3 dpi and 14 dpi could reflect the 2 different mechanisms.

In the nalidixic acid resistant SE strain trial, 8-day-old birds exposed to 2.1×10^9 CFU (9.3 log CFU) still had an average 8.10 log CFU/g of the SE strains recovered from cecal contents of CON birds at 14 dpi. This high SE colonization result is likely due to the immaturity of the intestinal microbiota and immune system in 8-day-old chicks. There is less microbial diversity in younger chickens compared with older chicken (Cui et al., 2017) and diversity of bacterial species in cecal contents of layer chicks drastically increased between d 0 to 24 (Xiao et al., 2021). In this trial, significant increase of bacterial species diversity was only confirmed in DY at 14 dpi. The SE challenge may have delayed the maturation of the gut microbiota and the combination of DFM and YCW may have accelerated maturation or eliminated the negative impact of SE in DY. Girgis et al. (2020) challenged 16-wk-old pullets with a similar concentration of the same SE strain (1.7×10^9 CFU; 9.2 log CFU), and recovered 3.9 log CFU/g of the strain from cecal contents at 7 dpi. This 4 log CFU/g difference between the two SE challenge trials may be due to differences in maturation of gut microbiota or host immune system in pullets from d 0 to 16 wk.

The high colonization level of the challenged SE strain that occurred in 8-day-old chicks suggests the importance of SE control during the early growing period of laying hen production. Chicks exposed to SE shortly after hatching can apparently remain infected with SE until maturity, as nearly half of challenged hens continue shedding SE in the feces at 24 wk of age (Gast and Holt, 1998). Field investigation also indicated SE strain could be delivered and shared between pullet house and commercial laying house receiving the birds (Davies et al., 2003). SE control in the early life-stages of laying hens could be one of important factor to avoid causing SE contaminated eggs.

The present trial also indicated effectiveness of the combination of DFM and YCW products in reducing *Salmonella* infection in ceca, both by culture-based method and modern molecular techniques. SE colonization level in the ceca at 14 dpi was still higher than acceptable limit for the MPN method, but the higher colonization result allowed to the detection of *Salmonella* spp. abundance by 16S rRNA-based microbiota analysis. However, knowledge regarding the benefits of DFM and YCW combination or relationship between complex gut microbiota and *Salmonella* for laying hen production remains limited. The life cycle of laying hens

for egg production is more than a year. In keeping with the original goal of protecting eggs from *Salmonella* contamination, further studies may be needed with longer term and/or different ages to optimize the effect of the combination treatment.

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DISCLOSURES

Authors affiliated with Calpis America are employed by the company. Calpis America is the manufacturer of CALSPORIN®.

REFERENCES

- Alali, W. Q., and C. L. Hofacre. 2016. Preharvest food safety in broiler chicken production. *Microbiol. Spectr.* 4:1–13 PFS-0002-2014.
- Arnold, M. E., F. Martelli, I. McLaren, and R. H. Davies. 2014. Estimation of the rate of egg contamination from *Salmonella*-infected chickens. *Zoonoses Public Health* 61:18–27.
- Attia, Y., H. Ellakany, A. E. El-Hamid, F. Bovera, and S. A. Ghazaly. 2012. Control of *Salmonella enteritidis* infection in male layer chickens by acetic acid and/or prebiotics, probiotics and antibiotics. *Arch. Geflügelk.* 76:S239–S245.
- Barrow, P. A. 2000. The paratyphoid salmonellae. *Rev. Sci. Tech. Off. Int. Epiz.* 19:351–375.
- Berge, A. C., and M. Wierup. 2012. Nutritional strategies to combat *Salmonella* in mono-gastric food animal production. *Animal* 6:557–564.
- Berghaus, R. D., S. G. Thayer, B. F. Law, R. M. Mild, C. L. Hofacre, and R. S. Singer. 2013. Enumeration of *Salmonella* and *Campylobacter* spp. in environmental farm samples and processing plant carcass rinses from commercial broiler chicken flocks. *Appl. Environ. Microbiol.* 79:4106–4114.
- Bonato, M., L. L. Borges, M. Ingberman, C. Fávoro, D. Mesa, L. F. Caron, and B. C. B. Beirao. 2020. Effects of yeast cell wall on immunity, microbiota, and intestinal integrity of *Salmonella*-infected broilers. *J. Appl. Poult. Res.* 29:545–558.
- Boore, A. L., R. M. Hoekstra, M. Iwamoto, P. I. Fields, R. D. Bishop, and D. L. Swerdlow. 2015. *Salmonella enterica* Infections in the United States and assessment of coefficients of variation: a novel approach to identify epidemiologic characteristics of individual serotypes, 1996–2011. *PLoS One* 10:e0145416.
- Bychkov, A. L., K. G. Korolev, and O. I. Lomovsky. 2010. Obtaining mannanoligosaccharide preparations by means of the mechanoenzymatic hydrolysis of yeast biomass. *Appl. Biochem. Biotechnol.* 162:2008–2014.
- Callahan, B. J., P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson, and S. P. Holmes. 2016. DADA2: High resolution sample inference from Illumina amplicon data. *Nat. Methods.* 13:581–583.
- Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Peña, J. K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, J. Reeder, J. R. Sevinsky, P. J. Turnbaugh, W. A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, and R. Knight. 2010. QIIME allows

- analysis of high-throughput community sequencing data. *Nat. Methods*. 7:335–336.
- Chousalkar, K., R. Gast, F. Martelli, and V. Pande. 2018. Review of egg-related salmonellosis and reduction strategies in United States, Australia, United Kingdom and New Zealand. *Crit. Rev. Microbiol.* 44:290–303.
- Cui, Y., Q. Wang, S. Liu, R. Sun, Y. Zhou, and Y. Li. 2017. Age-related variations in intestinal microflora of free-range and caged hens. *Front. Microbiol.* 8:1310.
- Davies, R., E. Liebana, and M. Breslin. 2003. Investigation of the distribution and control of *Salmonella enterica* serovar Enteritidis PT6 in layer breeding and egg production. *Avian Pathol.* 32:227–237.
- De Knecht, L. V., S. M. Pires, and T. Hald. 2015. Attributing food-borne salmonellosis in humans to animal reservoirs in the European Union using a multi-country stochastic model. *Epidemiol. Infect.* 143:1175–1186.
- European Food Safety Authority. 2007. Scientific Opinion of the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on the safety and efficacy of the product Calsporin[®], a preparation of *Bacillus subtilis*, as a feed additive for chickens for fattening in accordance with Regulation (EC) No 1831/2003. *EFSA J.* 5:43:1–8.
- European Food Safety Authority. 2010. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Scientific Opinion on the safety and efficacy of Calsporin[®] (*Bacillus subtilis*) for turkeys for fattening, ducks, geese, pigeons and other game birds for meat production, ducks, geese, pigeons, game birds, ornamental and sporting birds for rearing to point of lay, turkeys reared for breeding and chickens reared for laying. *EFSA J.* 8:1867 [13 pp.].
- European Union. 2003. Regulation (EC) No 2160/2003 of the European Parliament and of the Council of 17 November 2003 on the control of *Salmonella* and other specified food-borne zoonotic agents. *Off. J. Eur. Un. L* 325/1: 12.12.2003.
- Fritts, C. A., J. H. Kersey, M. A. Motl, E. C. Kroger, F. Yan, J. Si, Q. Jiang, M. M. Campos, A. L. Waldroup, and P. W. Waldroup. 2000. *Bacillus subtilis* C-3102 (Calsporin) improves live performance and microbiological status of broiler chickens. *J. Appl. Poult. Res.* 9:149–155.
- Ganner, A., C. Stoiber, D. Wieder, and G. Schatzmayr. 2011. Quantitative *in vitro* assay to evaluate the capability of yeast cell wall fractions from *Trichosporon mycotoxinivorans* to selectively bind gram negative pathogens. *J. Microbiol. Methods.* 83:168–174.
- Gast, R. K., and P. S. Holt. 1998. Persistence of *Salmonella enteritidis* from one day of age until maturity in experimentally infected layer chickens. *Poult. Sci.* 77:1759–1762.
- Girgis, G., M. Powell, M. Youssef, D. E. Graugnard, W. D. King, and K. A. Dawson. 2020. Effects of a mannan-rich yeast cell wall-derived preparation on cecal concentrations and tissue prevalence of *Salmonella* Enteritidis in layer chickens. *PLoS One* 15:e0232088.
- Hendrix-Genetics. 2019. Accessed Sep. 2019. <https://www.dekalb-poultry.com/en/product/dekalb-white/>
- Hofacre, C. L., R. D. Berghaus, S. Jalukar, G. F. Mathis, and J. A. Smith. 2018. Effect of a yeast cell wall preparation on cecal and ovarian colonization with *Salmonella enteritidis* in commercial layers. *J. Appl. Poult. Res.* 27:453–460.
- Hooge, D. M., H. Ishimaru, and M. D. Sims. 2004. Influence of dietary *Bacillus subtilis* C-3102 spores on live performance of broiler chickens in four controlled pen trials. *J. Appl. Poult. Res.* 13:222–228.
- Jackson, B. R., P. M. Griffin, D. Cole, K. A. Walsh, and S. J. Chai. 2013. Outbreak-associated *Salmonella enterica* serotypes and food commodities, United States, 1998–2008. *Emerg. Infect. Dis.* 19:1239–1244.
- Jeong, J. S., and I. H. Kim. 2014. Effect of *Bacillus subtilis* C-3102 spores as a probiotic feed supplement on growth performance, noxious gas emission, and intestinal microflora in broilers. *Poult. Sci.* 93:3097–3103.
- Jin, L. Z., Y. W. Ho, N. Abdullah, and S. Jalaludin. 1996. Influence of dried *Bacillus subtilis* and lactobacilli cultures on intestinal microflora and performance in broilers. *Asian-Australas. J. Anim. Sci.* 9:397–403.
- Khan, S., and K. K. Chousalkar. 2020. *Salmonella* Typhimurium infection disrupts but continuous feeding of *Bacillus* based probiotic restores gut microbiota in infected hens. *J. Anim. Sci. Biotechnol.* 11:29.
- Klindworth, A., E. Pruesse, T. Schweer, J. Peplies, C. Quast, M. Horn, and F. O. Glöckner. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41:e1.
- Kirk, M. D., S. M. Pires, R. E. Black, M. Caipo, J. A. Crump, B. Devleeschauwer, D. Döpfer, A. Fazil, C. L. Fischer-Walker, T. Hald, A. J. Hall, K. H. Keddy, R. J. Lake, C. F. Lanata, P. R. Torgerson, A. H. Havelaar, and F. J. Angulo. 2015. World Health Organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases, 2010: a data synthesis. *PLoS Med.* 12:e1001921.
- Knap, I., A. B. Kehlet, M. Bennedsen, G. F. Mathis, C. L. Hofacre, B. S. Lumpkins, M. M. Jensen, M. Raun, and A. Lay. 2011. *Bacillus subtilis* (DSM17299) significantly reduces *Salmonella* in broilers. *Poult. Sci.* 90:1690–1694.
- Manafi, M., S. Khalaji, and M. Hedayati. 2016. Assessment of a probiotic containing *Bacillus subtilis* on the performance and gut health of laying Japanese quails (*Coturnix Coturnix Japonica*). *Braz. J. Poult. Sci.* 18:599–606.
- Marubashi, T., M. I. Gracia, E. Esteve-Garcia, and M. Piskoriková. 2012. The efficacy of the probiotic feed additive Calsporin[®] (*Bacillus subtilis* C-3102) in broilers: combined analysis of four different studies. *J. Appl. Anim. Nutr.* 1:e5.
- Maruta, K., H. Miyazaki, S. Masuda, M. Takahashi, T. Marubashi, Y. Tadano, and H. Takahashi. 1996a. Exclusion of intestinal pathogens by continuous feeding with *Bacillus subtilis* C-3102 and its influence on the intestinal microflora in broilers. *Anim. Sci. Technol.* 67:273–280.
- Maruta, K., H. Miyazaki, Y. Tadano, S. Masuda, A. Suzuki, H. Takahashi, and M. Takahashi. 1996b. Effects of *Bacillus subtilis* C-3102 intake on fecal flora of sows and on diarrhea and mortality rate of their piglets. *Anim. Sci. Technol.* 67:403–409.
- Marzorati, M., P. V. D. Abbeele, S. S. Bubeck, T. Bayne, K. Krishnan, A. Young, D. Mehta, and A. DeSouza. 2020. *Bacillus subtilis* HU58 and *Bacillus coagulans* SC208 probiotics reduced the effects of antibiotic-induced gut microbiome dysbiosis in An M-SHIME[®] model. *Microorganisms* 8:1028.
- Micciche, A. C., S. L. Foley, H. O. Pavlidis, D. R. McIntyre, and S. C. Ricke. 2018. A review of prebiotics against *Salmonella* in poultry: current and future potential for microbiome research applications. *Front. Vet. Sci.* 5:191.
- Mitsuoka, T. 1971. Technics for intestinal microbiota. *Kansenshokugaku Zasshi* 45:406–419.
- Mountzouris, K. C., C. Balaskas, I. Xanthakos, A. Tzivinikou, and K. Fegeros. 2009. Effects of a multi-species probiotic on biomarkers of competitive exclusion efficacy in broilers challenged with *Salmonella enteritidis*. *Br. Poult. Sci.* 50:467–478.
- National Research Council. 1994. Nutrient Requirements of Poultry: Ninth Revised Edition. *Natl. Acad. Press*, Washington, DC.
- Neveling, D. P., L. van Emmenes, J. J. Ahire, E. Pieterse, C. Smith, and L. M. T. Dicks. 2020. Effect of a multi-species probiotic on the colonisation of *Salmonella* in broilers. *Probio. Antimicro. Prot.* 12:896–905.
- Nishiyama, T., N. Ashida, K. Nakagawa, S. Iwatani, and N. Yamamoto. 2020. Dietary *Bacillus subtilis* C-3102 supplementation enhances the exclusion of *Salmonella enterica* from chickens. *J. Poult. Sci.* 58:138–145.
- Oh, J. K., E. A. B. Pajarillo, J. P. Chae, I. H. Kim, and D. K. Kang. 2017. Protective effects of *Bacillus subtilis* against *Salmonella* infection in the microbiome of Hy-Line Brown layers. *Asian-Australas. J. Anim. Sci.* 30:1332–1339.
- Pickard, J. M., M. Y. Zeng, R. Caruso, and G. Núñez. 2017. Gut microbiota: role in pathogen colonization, immune responses, and inflammatory disease. *Immunol. Rev.* 279:70–89.
- R Core Team. 2018. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
- Rahimi, S., S. Kathariou, O. Fletcher, and J. L. Grimes. 2019. Effect of a direct-fed microbial and prebiotic on performance and intestinal histomorphology of turkey poult challenged with *Salmonella* and *Campylobacter*. *Poult. Sci.* 98:6572–6578.
- Ribeiro, A. M. L., L. K. Vogt, C. W. Canal, M. R. I. Cardoso, R. V. Labres, A. F. Streck, and M. C. Bessa. 2007. Effects of prebiotics and probiotics on the colonization and immune response of broiler chickens challenged with *Salmonella* Enteritidis. *Braz. J. Poult. Sci.* 9:193–200.

- Rubinelli, P., S. Roto, S. A. Kim, S. H. Park, H. O. Pavlidis, D. McIntyre, and S. C. Rieke. 2016. Reduction of *Salmonella* Typhimurium by fermentation metabolites of diamond V original XPC in an *in vitro* anaerobic mixed chicken cecal culture. *Front. Vet. Sci.* 3:83.
- Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the United States—major pathogens. *Emerg. Infect. Dis.* 17:7–15.
- Segata, N., J. Izard, L. Waldron, D. Gevers, L. Miropolsky, W. S. Garrett, and C. Huttenhower. 2011. Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60.
- Song, J., K. Xiao, Y. L. Ke, L. F. Jiao, C. H. Hu, Q. Y. Diao, B. Shi, and X. T. Zou. 2014. Effect of a probiotic mixture on intestinal microflora, morphology, and barrier integrity of broilers subjected to heat stress. *Poult. Sci.* 93:581–588.
- Spring, P., C. Wenk, K. A. Dawson, and K. E. Newman. 2000. The effects of dietary mannaoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of *Salmonella*-challenged broiler chicks. *Poult. Sci.* 79:205–211.
- Tack, D. M., L. Ray, P. M. Griffin, P. R. Cieslak, J. Dunn, T. Rissman, R. Jervis, S. Lathrop, A. Muse, M. Duwell, K. Smith, M. Tobin-D'Angelo, D. J. Vugia, J. Z. Kufel, B. J. Wolpert, R. Tauxe, and D. C. Payne. 2020. Preliminary incidence and trends of infections with pathogens transmitted commonly through food — foodborne diseases active surveillance network, 10 U.S. Sites, 2016–2019. *MMWR Morb. Mortal. Wkly. Rep.* 69:509–514.
- Tazehabadi, M. H., A. Algburi, I. V. Popov, A. M. Ermakov, V. A. Chistyakov, E. V. Prazdnova, R. Weeks, and M. L. Chikindas. 2021. Probiotic Bacilli inhibit *Salmonella* biofilm formation without killing planktonic cells. *Front. Microbiol.* 12:615328.
- Teng, P.-Y., and W. K. Kim. 2018. Review: roles of prebiotics in intestinal ecosystem of broilers. *Front. Vet. Sci.* 5:245.
- Thirabunyanon, M., and N. Thongwittaya. 2012. Protection activity of a novel probiotic strain of *Bacillus subtilis* against *Salmonella* Enteritidis infection. *Res. Vet. Sci.* 93:74–81.
- U.S. Food and Drug Administration. 2009. Prevention of *Salmonella* Enteritidis in shell eggs during production, storage, and transportation; final rule. *Federal Register* 74:33029–33101.
- Vazquez, A. P. 2016. *Bacillus species* are superior Probiotic feed-additives for poultry. *J. Bacteriol. Mycol.* 2:57–59.
- Xiao, S.-S., J.-D. Mi, L. Mei, J. Liang, K.-X. Feng, Y.-B. Wu, X.-D. Liao, and Y. Wang. 2021. Microbial diversity and community variation in the intestines of layer chickens. *Animals.* 11:840.