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Research Note—

Bacillus licheniformis Prevents Necrotic Enteritis in Broiler Chickens

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SUMMARY. Three studies were conducted using *Clostridium perfringens* as an intestinal challenge to produce necrotic enteritis (NE). The studies consisted of two battery screening studies and one production study in floor pens, which were used to test the effect of the addition of *Bacillus licheniformis* (DSM 17236) spores at different doses and feeding periods in comparison to birds fed diets with subtherapeutic levels of virginiamycin (15 g/ton feed). In all three studies the use of *B. licheniformis* (1.6×10^6–8×10^7 CFUs/g) or virginiamycin (15 g/ton feed) showed no difference in effect with regard to feed conversion ratio, weight gain, NE lesion score, and NE mortality. In the two battery studies, both treatments showed a significantly decreased feed conversion ratio, increased weight gain, reduced NE lesion score, and NE-reduced mortality compared to the nonmedicated *C. perfringens*–challenged group. In general, none of the treatments performed as well as the no-challenge group. The present data indicate that the use of *B. licheniformis* spores as a probiotic or direct-fed microbial could be an alternative to adding medication to the feed to overcome NE under commercial-like conditions and could therefore be of direct use in preventing antibiotic-resistant pathogens in chickens.

RESUMEN. *Nota de Investigación*—El *Bacillus licheniformis* previene la enteritis necrótica en pollos de engorde.

Se realizaron tres estudios utilizando *Clostridium perfringens* en un desafío intestinal para reproducir la enteritis necrótica. Los estudios consistieron de dos estudios de escrutinio en baterías y un estudio de producción en corrales en piso, los cuales se realizaron para probar el efecto de la adición de esporas de *Bacillus licheniformis* (DSM 17236) en dosis y períodos de alimentación diferentes en comparación con aves alimentadas con dietas que tenían niveles subterapéuticos de virginiamicina (15 g/tonelada de alimento). En los tres estudios, tanto el uso de *B. licheniformis* (1.6×10^6–8×10^7 unidades formadoras de colonias/g) o de virginiamicina (15 g/ton de alimento) no mostraron diferencia alguna en el efecto con respecto a la conversión alimenticia, ganancia de peso, grado de lesiones y mortalidad por enteritis necrótica. En los dos estudios en batería, ambos tratamientos mostraron una disminución significativa del índice de conversión alimenticia, aumento de la ganancia de peso, reducción en el grado de lesiones por enteritis necrótica y reducción en la mortalidad en comparación con las aves que no recibieron *C. perfringens* y que fueron desafiadas. En general, ninguno de los tratamientos mostró un desempeño similar al grupo no desafiado. Estos datos indican que el uso de esporas de *B. licheniformis* como probiótico o la alimentación directa con esta bacteria podría ser una alternativa a la adición de medicamentos en los alimentos para controlar la enteritis necrótica en condiciones comerciales y por lo tanto podría utilizarse directamente en la prevención de patógenos resistentes a los antibióticos de los pollos.

Key words: *Bacillus*, *Clostridium*, DFM, necrotic enteritis, probiotic

Abbreviations: CFU = colony-forming unit; CP = *Clostridium perfringens*; Cys = cysteine; DFM = direct-fed microbial; LSD (T) = least significant difference (*t*-test); ME = metabolizable energy; Met = methionine; NCR = National Research Council; NE = necrotic enteritis

Necrotic enteritis (NE) is the most common and financially devastating bacterial disease in modern broiler flocks (20). In 1977 Al-Sheikhly and Truscott (1) found that the intestinal necrosis characteristic of NE was caused by the potent alpha-toxin produced by *Clostridium perfringens*. Recently it has been found that theta-toxin and other *C. perfringens* toxins may play a role in development of NE (4,12). The acute form of the disease leads to increased mortality in the broiler flocks (21). In the subclinical form of NE, damage to the intestinal mucosa caused by *C. perfringens* leads to decreased digestion and nutrient absorption, resulting in reduced weight gain and increased feed conversion ratio (21).

Certain antibiotics such as bacitracin methylene disalicylate, tylosin phosphate, and virginiamycin are fed at subtherapeutic doses in the poultry industry as a growth promoter. Part of the growth promotion properties results from the suppression of *C. perfringens* (21). However, several antibiotic-resistant strains of *C. perfringens* have been isolated from chicken and turkeys (6).

Because of concern about antibiotic resistance, the ban of feeding growth-promoting antibiotics to broilers in the EU, and the general increased awareness from consumers to select "antibiotic-free" or "naturally produced" poultry products, other methods to inhibit the proliferation of *C. perfringens* are needed.

Several factors influence the growth of *C. perfringens* in the intestine of the birds. Proliferation of *C. perfringens* or production of toxin is enhanced by the composition of the feed. NE caused by *C. perfringens* is more prevalent in broilers fed wheat or barley diets (2,5) compared to a corn-based diet. Protein source and the dietary amino acid composition could also influence the proliferation of *C. perfringens* (22). Several studies have been conducted showing that adding live healthy bacteria to birds influences the level of *C. perfringens* or reduces the toxin level in the small intestine of birds (8,9,5). Probiotic or direct-fed microbial (DFM) products can modulate the ileal bacterial population in birds. Knarreborg *et al.* (13) showed that the addition of *Bacillus* spores to broiler chicken

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Table 1. Calculated composition of the basal diet, battery studies 1 and 2.

Ingredient	NRC[A] starter	Nutrient
Corn	52.375	Crude protein %: 23.00
Soybean meal 48	38.34	Chicken ME[A] kcal/kg: 3200
Soy oil	5.32	Calcium %: 1.00
Salt	0.40	P %: 0.76
DL methionine	0.22	Na %: 0.22
Limestone	1.23	Fat %: 8.05
Dicalcium phosphate	1.79	Met %: 0.58
Vitamin premix	0.25	Cys %: 0.37
Mineral premix	0.075	Met+Cys[A] %: 0.95
		Lys %: 1.28
Total	100.00	

[A]NRC = National Research Council; ME = metabolizable energy; Met = methionine; Cys = cysteine.

feed increased the microbial diversity in the ileum and increased the growth of lactic acid bacteria in the birds fed *Bacillus* organisms compared to the control birds. *Bacillus* spores not only modulate the intestinal microflora, but also have the ability to protect the chicken against specific pathogens (14,15). Feeding *Bacillus* spores to newly hatched pathogen-free chicks prior to challenge with *Escherichia coli* O78:K80 suppresses all aspects of *E. coli* O78:K80 infection (14). One single dose of *Bacillus* (1E9 spores/bird) was sufficient to protect against colonization of *Salmonella enterica* and *C. perfringens* in young chickens (15).

The objective of this work was to determine if *Bacillus licheniformis* had the ability to prevent NE in broiler chickens. Therefore three *C. perfringens* challenged studies were conducted with the supplementation of *B. licheniformis* (DSM 17236).

MATERIALS AND METHODS

In this study a total of three *C. perfringens* challenge experiments were conducted containing two battery screening studies and one floor pen production study. The isolate of *C. perfringens* used as the NE infection model in the present study is susceptible to virginiamycin and is described in previous work by Hofacre and colleagues (9). The overall objective was to test if *B. licheniformis* (DSM 17236) spores can influence the development of NE and to identify dose and feeding period to overcome the *C. perfringens* challenge. In all three studies a medicated control group with virginiamycin (15/ton) was included in the trial design.

Battery screening studies 1 and 2. A total of 384, 1-day-old Cobb × Cobb male broiler chicks were allocated to the battery study 1 and 320 chicks to the battery study 2. Upon arrival, the chicks were raised in modified Petersime battery cages (raised wire floors) and administered the treatment feeds according to a randomized complete block design with eight blocks and six treatments per block.

Eight chicks per cage were placed in each battery with a floor space of 0.63 ft^2/bird (588 cm^2/bird). Lighting was provided 24 hr/day, and a thermostatically controlled gas furnace/air conditioner maintained uniform temperature throughout the study.

The study began when the birds were placed (day 0), at which time they were allocated to the experimental cages. An unmedicated corn-soybean meal–based commercial broiler starter ration was formulated (Table 1). Feed and water were available *ad libitum* throughout the trials. On day 14, all birds were orally inoculated with a coccidial inoculum containing approximately 5000 oocysts of *Eimeria maxima* per bird. Starting on day 19, all birds, except treatment 1, were given 1 ml of a broth culture of *C. perfringens* at approximately 10^8 CFUs (colony-forming units)/bird. The birds were administered a fresh broth culture once daily for 2 days (on days 19 and 20).

Treatments in battery studies. Battery study 1 had the following six treatments: 1) nonmedicated, noninfected; 2) nonmedicated, *C. perfringens* (CP) infected; 3) *B. licheniformis* spores (DSM 17236) at a dose of 8×10^5 CFUs/g of feed, CP infected; 4) *B. licheniformis* spores (DSM 17236) at a dose of 8×10^6 CFUs/g of feed, CP infected; 5) *B. licheniformis* spores (DSM 17236) at a dose of 8×10^7 CFUs/g of feed, CP infected; and 6) virginiamycin 15 g/ton, CP infected.

Table 2. Calculated composition of the basal diet, pen floor study.

	Starter (%)	Grower (%)	Finisher (%)
Ingredients			
Corn, yellow, ground	56.12	60.80	68.00
Soybean meal (48)	37.50	32.61	26.22
Fat, poultry	3.00	3.43	2.99
Dicalcium phosphate	1.75	1.56	1.32
Limestone	0.80	0.78	0.62
Salt	0.30	0.32	0.35
Vitamin premix[A]	0.25	0.25	0.25
DL-methionine	0.20	0.17	0.17
Trace mineral premix[B]	0.08	0.08	0.08
Contents by calculation			
ME[C], kcal/kg	3.096	3.14	3.191
Protein, %	22.3	20.6	18.1
Lysine, %	1.18	1.01	0.85
Methionine, %	0.53	0.48	0.45
Met + Cys, %[C]	0.89	0.76	0.70

[A]Vitamin mix provided the following (per kg of diet): thiamin mononitrate, 2.4 mg; nicotinic acid, 44 mg; riboflavin, 4.4 mg; D-Ca pantothenate, 12 mg; vitamin B_{12} (cobalamin), 12.0 µg; pyridoxine HCl, 4.7 mg; D-biotin, 0.11 mg; folic acid, 5.5 mg; menadione sodium bisulfite complex, 3.34 mg; choline chloride, 220 mg; cholecalciferol, 27.5 µg; trans-retinyl acetate, 1892 µg; all-rac-α-tocopheryl acetate, 11 mg; ethoxyquin, 125 mg.

[B]Trace mineral mix provided the following (per kg of diet): manganese ($MnSO_4 \cdot H_2O$), 60 mg; iron ($FeSO_4 \cdot 7H_2O$), 30 mg; zinc (ZnO), 50 mg; copper ($CuSO_4 \cdot 5H_2O$), 5 mg; iodine (ethylene diamine dihydroiodide), 0.15 mg; selenium ($NaSeO_3$), 0.3 mg.

[C]ME = metabolizable energy; Met = methionine; Cys = cysteine.

Battery study 2 had the following five treatments: 1) nonmedicated, noninfected; 2) nonmedicated, CP challenged; 3) *B. licheniformis* spores (DSM 17236) at a dose of 1.6×10^6 CFUs/g of feed for the whole feeding period, CP challenged; 4) *B. licheniformis* spores (DSM 17236) at a dose of 1.6×10^7 CFUs/g of feed for the first 21 days of feeding, CP challenged; 5) virginiamycin 15 g/ton, CP challenged.

All birds were weighed by cage on 0, 14, 22, and 28 days of age. Feed was weighed in on day 0 and remaining feed was weighed on 14, 22, and 28 days of age. The trial was terminated on day 28.

On day 22, three birds from each cage were selected, sacrificed, weighed, and examined for the degree of presence of NE lesions. The scoring was done blindly and based on a zero to three score, with 0 being normal and 3 being the most severe (8).

Means for cage weight gain, feed consumption, feed conversion (adjusted for mortality: feed consumed/[final live weight + mortality weight]), NE lesion scores, and NE mortality were calculated. The mortality was assessed by gross lesions on necropsy-enlarged dark-colored livers and the pseudomembrane appearance of classical NE in the small intestine. 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 significance level of 0.05.

Floor pen production study. One thousand seven hundred and fifty male Cobb × Cobb chicks were allocated to the study. Upon arrival at Southern Poultry Research, all birds were spray vaccinated with the recommended level of a commercial coccidiosis vaccine before placement. At study initiation 50 males were randomly selected and allocated to each treatment pen by blocks. Bird weights (kg) by pen were recorded at 0, 21, 35, and 42 days of age. Similar to the battery studies,

Table 3. Result from battery study 1 on feed conversion ratio, weight gain, NE lesion score, and NE mortality.[A]

Treatment	Feed conversion Day 0–28	Weight gain Day 0–28	Lesion score NE (0–3) Day 22	Mortality % NE
1. Nonmed., no CP	1.517 d	0.992 a	0.0 c	0.0 c
2. Nonmed., CP	1.952 a	0.539 c	0.8 a	48.4 a
3. DSM 17236 (8×10^5 CFUs/G), CP	1.768 b	0.779 b	0.4 b	34.4 b
4. DSM 17236 (8×10^6 CFUs/g), CP	1.634 c	0.896 a	0.3 b	28.1 b
5. DSM 17236 (8×10^7 CFUs/g), CP	1.606 c	0.931 a	0.1 bc	26.6 b
6. Virginiamycin (15 g/ton), CP	1.599 cd	0.934 a	0.2 bc	26.6 b

[A]Means in a column with no common letters (a, b, c, d) are significantly different ($P < 0.05$).

an unmedicated corn-soybean meal–based commercial broiler starter ration was formulated. The dietary composition is given in Table 2. The diet did not contain any coccidiostat or other antimicrobial growth promoter other than those included in the dietary treatments. The entire amount of each basal diet was produced the same day.

NE disease was induced on 18, 19, and 20 days of age for all birds, except treatment 1. Each bird was dosed with 1 ml (1.0×10^8 CFUs/ml) of a fresh broth culture of *C. perfringens*. A field isolate of *C. perfringens* known to cause NE was utilized as the challenge organism (9). Fresh inoculum was used each day. The inoculum was administered by mixing into the feed found in the base of the two feeders (two tube feeders per pen).

At 21 and 35 days of age broilers were switched to a grower and finisher diet, respectively. At each feed change, feeders were removed from pens by block, weighed back, emptied, and refilled with the appropriate treatment diet. On the final day of the study (42 days of age), feed was weighed. At 21 days of age, five birds from each pen were selected, sacrificed, group weighed, and examined for the degree of presence of NE lesions. The scoring was done blindly and based on a zero to three score, with zero being normal and three being the most severe (8).

The pen floor study had the following five treatments: 1) nonmedicated, noninfected; 2) nonmedicated, CP challenged; 3) *B. licheniformis* spores (DSM 17236) at a dose of 1.6×10^6 CFUs/g of feed from day 0 to day 42, CP challenged; 4) *B. licheniformis* spores (DSM 17236) at a dose of 1.6×10^7 CFUs/g of feed for the first 21 days of feeding, CP challenged; 5) virginiamycin 15 g/tons from day 0 to day 42, CP challenged.

Means for cage weight gain, feed consumption, feed conversion, NE lesion scores, and NE mortality were calculated. The mortality was assessed by gross lesions on necropsy-enlarged dark-colored livers and the pseudomembrane appearance of classical NE in the small intestine. Statistical evaluation of the data was performed using a STATISTIX for Windows program. The procedures used were general linear procedures using ANOVA with a comparison of means using LSD (T) at a 0.05 level of significance.

RESULTS

The results of the first battery study are presented in Table 3. The challenge study was a dose response study with three different doses of *B. licheniformis* spores (8×10^5 CFUs/g of feed, 8×10^6 CFUs/g of feed, 8×10^7 CFUs/g of feed) and compared to addition of virginiamycin (15 g/ton). All three doses reduced the NE mortality and NE lesion score to the same level as the virginiamycin-treated group and were significantly lower than the *C. perfringens*–challenged nonmedicated group. The two high doses of *B. licheniformis* spores and the virginiamycin group decreased feed conversion ratio significantly compared to the *C. perfringens*–challenged nonmedicated group. No significant differences were observed between the treatment groups and the nonchallenged-nonmedicated group.

Therefore the optimal dose of *B. licheniformis*, under the above stated conditions, is between the 8×10^5 CFUs/g and the 8×10^6 CFUs/g of feed dose.

In the second battery study a dose of $2 \times 8 \times 10^5$ CFUs/g of feed of *B. licheniformis* spores for the whole feeding period, $20 \times 8 \times 10^5$ CFUs/g of feed of *B. licheniformis* spores in the first 21 days of feeding, and virginiamycin (15 g/ton feed) for the whole feeding period were tested in the same challenge model as in the battery study 1. The result of the second battery study is summarized in Table 4.

No statistical difference ($P > 0.05$) in effect of the two treatments of *B. licheniformis* or the virginiamycin group was seen with regard to feed conversion ratio, weight gain, NE lesion score, and NE mortality. All three treatments showed a significant decrease in feed conversion ratio, increased weight gain, reduced NE lesion score, and reduced NE mortality compared to the nonmedicated *C. perfringens*–challenged group. To confirm these findings, a production-like trial mimicking mild outbreaks of NE under commercial condition in the form of a pen floor study was conducted with the same doses of *B. licheniformis* and virginiamycin as in the second battery study. The result of the pen floor study is presented in Table 5. Under commercial-like conditions with a mild NE outbreak, the effect of the low-dose whole feeding period and the high dose in the first 21 days gave the same performance with regard to feed conversion ratio, weight gain, lesion score, and mortality as the medicated group.

DISCUSSION

Until recently the phospholipase C enzyme called alpha-toxin was thought to be the key virulence factor in NE caused by *C. perfringens*. However, a recent study has shown that an isolate, which does not

Table 4. Result from battery study 2 on feed conversion ratio, weight gain, NE lesion score, and NE mortality.[A]

Treatment	Feed conversion Day 0–28	Weight gain Day 0–28	Lesion score NE (0–3) Day 22	Mortality % NE
1. Nonmed., no CP	1.729 c	0.862 ab	0.0 c	0.0 c
2. Nonmed., CP	2.313 a	0.652 d	1.4 a	46.9 a
3. DSM 17236 ($2 \times 8 \times 10^5$ CFUs/g) day 0–28, CP	2.102 b	0.762 c	0.6 bc	15.6 b
4. DSM 17236 ($20 \times 8 \times 105$ CFUs/g) day 0–21, CP	2.095 b	0.785 c	0.9 b	15.6 b
5. Virginiamycin (15 g/ton), CP	2.037 b	0.803 bc	0.4 bc	14.1 bc

[A]Means in a column with no common letters (a, b, c, d) are significantly different ($P < 0.05$).

Table 5. Result from pen floor study on feed conversion ratio, weight gain, lesion score, and mortality.[A]

Treatment	Feed conversion Day 0–42	Weight Gain Day 0–42	Lesion score NE (0–3) Day 22	Mortality% NE
1. Nonmed., no CP	1.862 c	2.444 a	0.0 c	0.0 b
2. Nonmed., challenged	2.069 a	2.300 b	0.31 a	8.0 a
3. DSM 17236 (2 × 8 × 106 CFUs/g) day 0–42, CP	1.958 bc	2.386 ab	0.03 bc	3.4 b
4. DSM 17236 (20 × 8 × 106 CFUs/g) day 0–21, CP	1.972 ab	2.369 ab	0.20 ab	1.7 b
5. Virginiamycin (15 g/ton), CP	1.911 bc	2.364 ab	0.17 abc	1.7 b

[A]All birds were Coccivac-B vaccinated. Means in a column with no common letters (a, b, c, d) are significantly different ($P < 0.05$).

produce alpha-toxin, can still cause disease. In addition, a new toxin called NetB has been identified in disease causing *C. perfringens* isolates (12). Si and coworkers (16) found that the average *C. perfringens* count of 10^5 CFUs/g in ileal digesta appears to be the threshold for development of NE in chickens and that the production of toxins was positively correlated with the cell proliferation of *C. perfringens*. So the effect of the use of probiotics to prevent NE should be found either in preventing proliferation of *C. perfringens* or eliminating the effect of the toxins produced by *C. perfringens*.

The mode of action of probiotics in general and for *Bacillus* spp. in particular is not totally clear. *Bacillus* spp. are defined as gram-positive spore-forming organisms. The spore form is a dormant resistant stage that can transform into vegetative cells. *Bacillus* spp. are facultative aerobes but can, in the presence of nitrate or nitrite, grow anaerobically. Tam and colleagues (17) documented that *Bacillus* spp. are not transient passengers of the gastrointestinal tract but have adapted to carry out their entire life cycle within this environment, and therefore *Bacillus* spp. should also be categorized as part of the intestinal bacteria. These observations is supported by the current study where feeding a high dose of DFM the first 21 days of life had the same positive effect on feed conversion ratio and mortality as the group fed a lower dose of DFM for 42 days. This indicates that *B. licheniformis* can recover in the gastrointestinal tract for a period of time. Cartman and colleagues (3) showed that orally administered spores of *B. subtilis* germinate in the gastrointestinal tracts of the chicken; 20 hr after spores were administered, vegetative cells outnumbered spores throughout the GI tract. This demonstrated that spore-based probiotics may function in this host through metabolically active mechanisms.

The vegetative cell of *Bacillus* is known to be enzyme producing, and in many cases, is used as the production strain in the industrial production of enzymes, for example, proteases. As the purified alpha-toxin produced by *C. perfringens* has been shown to be a zinc-containing phosphalipase C enzyme (19), it may be hydrolyzed by the proteases produced by *B. licheniformis*. This could be one of the reasons for the NE prevention effect seen in chickens. Another explanation for the effect could be the production of bacterocin. Teo and Tan (18) indicated that *Bacillus*-produced bacterocin was active against various strains of *Clostridium* species so that a proliferation of *C. perfringens* could be reduced. Quorum sensing is an important mechanism of cell-to-cell communication that involves density-dependent recognition of signaling molecules, resulting in modulation of gene expression (11). Both *Bacillus* and *Clostridium* organisms possess the same *luxS* gene, and Kaper and Sperandio (11) reported that the *luxS*-mediated system enhances *C. perfringens* to express alpha-toxin. High levels of *B. licheniformis* as the consequence of its use as a probiotic may influence the response on *C. perfringens* in a way so that the expression of toxin is not activated.

Another approach could be related to the effect of probiotics on the birds' immune system. *Bacillus* spp. have been reported to stimulate the immune response in chickens. Inooka and colleagues (10) found that feeding *B. subtilis* for 27 days from hatch gave increased splendic T and B lymphocytes compared to the control group. *Bacillus* spores could also play a primary role in the development of the gut-associated lymphoid tissue. Haghighi and coworkers (7) reported that administration of probiotics enhanced alpha-toxin-reactive antibodies in serum and intestinal natural antibodies to several foreign antigens and could be important for defense against pathogens.

In conclusion, this study showed that *B. licheniformis* spores as a probiotic or DFM has the ability to prevent NE and could be an alternative to prophylactic use of antibiotics to overcome NE under commercial conditions. *Bacillus licheniformis* could therefore be of direct use in preventing antibiotic-resistant pathogens in chickens. However, future studies should investigate the lasting effects of DFM on the gut flora in order to clarify the potential effects with regard to other avian pathogens.

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