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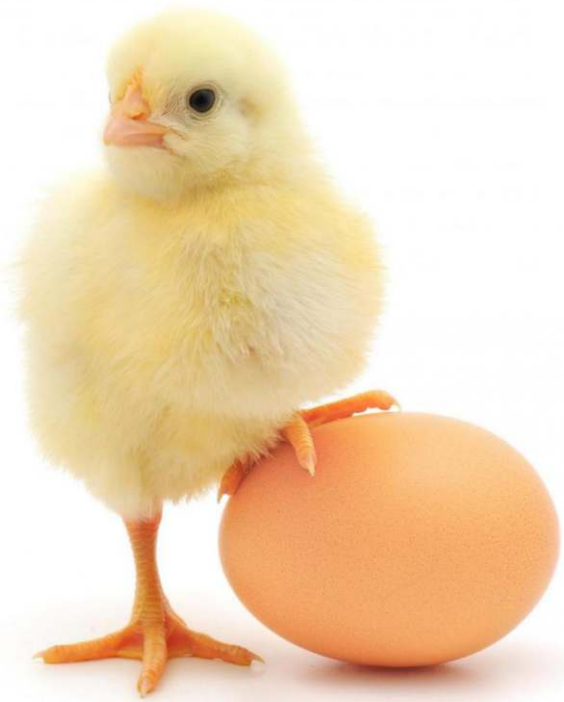

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Effect of Botanical Probiotic Containing *Lactobacilli* on Growth Performance and Populations of Bacteria in the Ceca, Cloaca, and Carcass Rinse of Broiler Chickens

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Abstract: This study was conducted to examine the effect of feeding a botanical probiotic (Feed Free™) containing *Lactobacillus* on growth performance of broiler chickens from 1 to 42 d of age. At 56 d, five broilers per pen were killed and processed to determine bacteria populations in the ceca, cloaca, and carcass rinse. The dietary treatments were the basal diet with coccidiostat and antibiotic (control), basal diet without coccidiostat and antibiotic (negative control) and basal diet supplemented with 0.10% probiotic. The results showed that body weights and average weight gain were not different ($P > 0.05$) due to treatment. Feed intake and feed to gain ratio from 22 to 42 d of age were lower ($P < 0.001$) for broilers fed 0.10% probiotic than broilers fed the control diets. The population of *Lactobacilli* recovered from cloaca contents was higher ($P < 0.002$) and the population of *Clostridium perfringens* recovered from cloaca contents was lower ($P < 0.02$) for broilers fed the 0.10% probiotic diet than for those fed the control diets. The population *C. jejuni* recovered from carcass rinses for broilers fed the diet supplemented with the probiotic tended ($P < 0.11$) to be lower when compared to the negative control. These results suggest that diets supplemented with the botanical probiotic containing *Lactobacillus* supports growth for broilers similar to the basal diet supplemented with antibiotic and coccidiostat, and with lower feed to gain ratio. Also, the botanical probiotic may reduce *C. perfringens* and *C. jejuni* in market-age broilers.

Key words: *Lactobacillus*, botanical probiotic, broiler, growth performance, bacteria population

Introduction

Historically, there has been widespread use of antibiotics in animal feed for improving growth rate and feed efficiency, as well as for the prevention and treatment of diseases.

However, the continued feeding of antibiotics at sub-therapeutic levels has created concerns about the extent to which usage increases the possibilities of antibiotic residue, the development of drug-resistant bacteria, and a reduction in the ability to cure these bacterial diseases in humans (Jensen, 1998). Increased awareness of the potential problems associated with the use of antibiotics has stimulated research efforts to identify alternatives to their use as feed additives. Probiotics (direct-fed microbial) have been suggested as alternatives to the use of antibiotics in food animals. Probiotics are characterized as live microorganisms (e.g., bacteria and fungi) that when ingested by animals have beneficial effects in the prevention and treatment of diseases (Fuller, 1989; Miles and Bootwalla, 1991; Havenaar and Huis in't Veld, 1992).

The composition of probiotics most frequently used contains strains of lactic acid bacteria (*L. acidophilus, L. casei L. helveticus, L. lactis, L. salivarius,* and *L. plantarum*), all of which originally were natural intestinal strains (Fuller, 1989). Plausible reasons for the selection of lactic acid bacteria are that they have been demonstrated to inhibit the *in vitro* growth of many enteric human pathogens, including *Salmonella* Typhimurium, *Staphylococcus aureus, Escherichia coli, Clostridium perfringens,* and *Clostridium difficile*. Lactic acid bacteria have also been used in both humans and animals to treat a broad range of gastrointestinal disorders (Silva *et al.,* 1987; Hinton *et al.*, 1992; Meurman *et al*., 1995).

Results from *in vitro* studies have shown that strains of *L. salivarius* have implications for use as a probiotic for pigs (Nemcova *et al*., 1997; Garriga *et al*., 1998). The attributes of *L. salivarius* offer promising possibilities as a probiotic, specifically, their ability to inhibit the growth of *Salmonella* Enteritidis and *E. coli*, their high adhesion efficiency to intestinal mucosal, and their resistance to bile salts and pH 3.0 (Nemcova, *et al*., 1997; Garriga *et al*., 1998; Murphy *et al*., 1999). According to Pascual *et al.* (1999), oral gavage and inclusion of *L. salivarius* (isolated from the crop of chickens) in the feed and the drinking water prevent colonization of salmonellae in the gastrointestinal tract of chickens within 21 days. Miyamoto *et al.* (2000) reported that *L. salivarius* (isolated from the cloaca of hens) inhibit the growth of *S.*

Table 1: Composition of basal diets for broilers

Ingredients and composition	0 to 21d Starter	22 to 49d Grower
	--- % ---	
Corn	56.12	60.79
Soybean meal, 48	37.50	32.61
Fat, poultry	3.07	3.43
Limestone	0.73	0.78
Defluorinated phosphate	1.75	1.56
Salt	0.29	0.32
Vitamin premix[1]	0.25	0.25
Mineral premix[2]	0.07	0.08
DL-98 methionine	0.20	0.17
Calculated composition		
Crude protein, %	22.50	20.50
Met. Energy, kcal/kg	3,080	3,150
Lysine, %	1.26	1.12
Calcium, %	0.95	0.90
Phosphorus (Avail.), %	0.45	0.41

[1]Vitamin premix provides the following per kilogram of diet: Vitamin A, 6614 IU from trans-retinyl acetate; cholecalciferol, 705 IU; vitamin E, 13 IU from all-rac-tocopherol acetate; riboflavin, 6.6 mg; Ca pantothenate, 12 mg; nicotinic acid, 39 mg; vitamin B12, 0.011 mg; vitamin B6, 1.9 mg; menadione, 1.3 mg (as menadione sodium bisulfate complex); folic acid, 0.72 mg; d-biotin, 0.055 mg; thiamine, 1.1 mg (as thiamine mononitrate); ethoxyquin, 125 mg. [2]Trace mineral premix provides the following in milligrams per kilogram of diet: Mn, 60; Zn, 50; Fe, 30; Cu, 5; I, 1.5.

enteritidis and *E. coli in vitro*. Also, Ehrmann *et al.* (2002) reported that *L. salivarius* (isolated from the crop and intestines of ducks) inhibit the growth of *S. enteritidis* and *E. coli in vitro*.

Another lactobacilli (*L. plantarum*) show promise for use as a probiotic. According to Vescovo *et al.* (1993) *L. plantarum* is a member of the facultative, heterofermentative group of *Lactobacilli* that are frequently isolated from plant material and is found in fermented foods of plant origin. Also, *L. plantarum* strains can adhere to human intestinal cell lines (Adlerberth *et al.*, 1996; Ahrne *et al.*, 1998). Efforts to examine the effectiveness of *L. plantarum* as a probiotic have only recently emerged. *L. plantarum* 299v, isolated from human intestine, has been used as a probiotic in foods for humans with irritable bile syndrome (Nobaek *et al.*, 2000). Results from a study by Mangell *et al.* (2002) revealed that pretreatment with *L. plantarum* 299v in the drinking water of rats protects against *E. coli*-induced increase in intestinal permeability. In addition, van Winsen *et al.* (2002) showed that feed fermented with *L. plantarum* reduced the population of *Enterobacteriaceae* in feces of pigs. Further, Heres *et al.* (2003) reported that feed fermented with *L. plantarum* reduced *S. enteritidis* in the ceca of chickens.

These findings, although providing support for the potential use of *L. salivarius* and *L. plantarum* as probiotics, are based on the utilizations of single strains of *Lactobacilli* isolated from the intestine of poultry, swine, or human. Although probiotic preparations may consist of single strains or more than eight strains of microorganisms, the attraction of multiple-strain preparation may offer greater benefits because they are active against a wider range of conditions and in a wider range of species (Fuller, 1989). Recently, Murry *et al.* (2004) isolated *Lactobacillus salivarius* and *Lactobacillus plantarum* from a botanical probiotic and reported that both strains inhibited ($P < 0.001$) the *in vitro* growth of *E. coli*, *S. typhimurium*, and *C. perfringens* for broilers starter and grower diets when compared to the control diet. However, there have been no published studies utilizing a botanical probiotic containing both *L. salivarius* and *L. plantarum* as a feed supplement for broilers. Therefore, the objective of this study was to investigate the effect of a commercial botanical probiotic on growth performance and bacteria populations in the intestinal tract and carcass rinse of broiler chickens.

Materials and Methods

Animals and Treatment: Four hundred fifty day of hatch high yield strain male broiler chicks (feather-sexed) were obtained from a local hatchery. All chicks were spray vaccinated for New Castle and Bronchitis at the hatchery and no other vaccinations were administered. Chicks were placed into 15 pens (1.5 by 4.3 M) with 30 broilers per pen. Each pen had wood shavings covering the concrete floor and contained a single tube feeder and nipple-drinker line that provided *ad libitum* access to feed and water. Brooder lamps provided supplemental heat in each pen and lowering or raising the side curtains adjusted house temperature. The broilers were fed a typical corn-soybean meal basal diet in mesh form (Table 1). The basal diet was formulated to meet or exceed NRC (1994) requirements for starter (1 to 21 d) and grower (22 to 42 d). Chicks were randomly assigned to one of three dietary treatments. The dietary groups were: (1) basal diet with coccidiostat (Coban 60 at 750 g/mtons) and antibiotic (bacitracin at 62.5 g/mton, positive-control), (2) basal diet without coccidiostat and antibiotic (negative control), and (3) basal diet without coccidiostat and antibiotic and supplemented with a 0.10% probiotic, (Feed Free™; Woonsuk Bio Food Co., Seoul, Korea). Broilers were observed twice daily and any mortality was removed and body weight recorded. On any day that unthrifty birds or birds with severe leg abnormalities were observed they were removed, body weight recorded and euthanized. Mortality and leg abnormality were recorded as they occurred and percentage mortality and leg abnormality were determined at the end of the study. Broilers were weighed at 21 and 42 d, and body weight gain and feed conversion calculated. Feed conversion was determined by difference from the weight of the feed placed into the feeder and that, which remained on day 21 and 42. Body weights from mortality and leg abnormality were used to adjust feed consumption. The University of Georgia Animal Care and use Committee approved all animal procedures and protocols used in this experiment.

Enumeration of Microorganisms in Feed Free™: A 25 g sample of Feed Free™ and 225 ml of 0.1% peptone water was blended for 2 min on high speed in a Waring Laboratory Blender, and serial dilutions of the suspension were made in 0.1% peptone water. The dilutions were plated on MRS Lactic Acid Agar (Difco, Detroit, MI 48232) or Acidified Potato Dextrose Agar (Difco, Detroit, MI 48232) for enumeration of lactic acid bacteria and yeasts, respectively. Inoculated MRS Lactic Acid Agar plates were incubated anaerobically at 35°C for 48 h in a controlled environment chamber (Coy Laboratory Products, Inc., Grass Lake, MI 49240). Inoculated Acidified Potato Dextrose Agar plates were incubated aerobically at 25°C for 3 days. Colony-forming units (cfu) were counted and morphologically distinct colonies were removed from the plates for identification with the MIDI Sherlock Microbial Identification (MIDI, Inc. Newark, DE 19713).

Challenge procedures: At 42 d, three broilers (seeders) from each pen were leg banded and orally gavaged with 1 mL suspension containing approximately 5 X 10^8 cfu of *C. jejuni* 2b. The *C. jejuni* 2b isolate was recovered from whole carcass rinse of commercially processed broilers by the Poultry Processing Unit at the Russell Research Center in Athens, GA.

Sample procedures: Feed was removed from each pen 12 h prior to processing. At 56 d, two challenged broilers and three randomly selected non-challenged pen mates were placed into a transport coop and transported to the pilot processing plant. Broilers were processed by pen number rotating through the treatment groups and a leg band applied to each carcass to indicate processing order. Each batch of five broilers (3 non-challenged followed by 2 challenged) were stunned at 12 volts pulse DC, bleed for 120 sec, and then scalded in series (48, 53, and 57°C) for a total of 120 sec. Carcasses were defeathered with a single picker adjusted to remove feathers but not the cuticle layer of the epidermis. To minimize leakage from the alimentary track during the collection of whole carcass rinses, upon exiting the picker the vents of each carcass were plugged by inserting a cotton tampon into the cloaca and a plastic cable tie was placed around the neck to occlude the esophagus. The head and feet were then removed, and the whole carcass was placed in a sterile plastic bag with 300 mL of sterile, 0.1% Bacto peptone (Difco, Detroit, MI 48232) solution. The carcass was shaken in the peptone solution for 1 min using a mechanical shaker. One hundred mL of carcass rinse were collected for bacteria analyses. Ceca and cloaca were removed aseptically and placed in sterile plastic bags with ten mL of 0.1% Bacto peptone solution. Each bag was blended in the solution for 2 min on high speed in a Stomacher 400 Laboratory Blender (Seward Medical Limited, London, UK).

Enumeration of bacteria: Selected bacteria in whole carcass rinse, ceca, and cloaca contents were enumerated. Lactic acid bacteria and *C. jejuni* were enumerated as described Hinton *et al.* (2002). *E. coli* was enumerated on 3M Petrifilm (3M Microbiology Products, St. Paul, MN 55144), and *C. perfringens* was enumerated on Perfringens Agar Base (Oxoid Limited, Basingstoke, Hampshire, England) supplemented with TSC selective supplement B and Egg Yolk and incubated in a controlled environment chamber at 35°C for 48 h.

Statistical analyses: Data were analyzed as a completely randomized design using the GLM procedures of SAS (1991). Treatment means were compared using the PDIFF statement of SAS (1991) when protected by a significant ($P < 0.05$) treatment effect. Significant differences among treatment means were determined using the F-statistic with results reported as least-square means ± pooled SEM. Mortality and leg abnormality percentage data were transformed to arcsine prior to analysis and approximate SEM values were obtained from actual percentages.

Results and Discussion

Growth performance: The body weight, weight gain, feed intake, gain to feed ratio, mortality, and leg abnormality of broilers from 1 to 42 d are presented in Table 2. There were no differences ($P > 0.05$) observed in body weights or weight gain due to treatment from 1 to 21 d, 22 to 42 d, or overall from 1 to 42 d. Furthermore, there were no differences ($P > 0.05$) due to treatment for mortality or leg abnormality of broilers from 1 to 42 d.

Little is known about growth performance and intestinal bacteria of broiler chicks fed diets supplemented with a natural botanical probiotic fermented from fruits and vegetables. Other probiotics used to evaluate growth performance and intestinal microorganisms of poultry have contained either *L. plantarum* or *L. salivarius* as a single strain or in combination with other *Lactobacillus* strains. For example, Balevi *et al.* (2001) used a commercial probiotic direct fed microbial containing 9 species of bacteria on performance of laying hens and for that study *L. plantarum* was one of the 9 bacteria in the commercial probiotic. Recently, Lan *et al.* (2003) studied the effect of two *Lactobacillus* strains (*L. salivarius* and *L. agillis*) isolated from chicken intestine on weight gain and fecal *Lactobacilli* levels in Leghorn chickens. Broilers in the present study consumed the diet supplemented with the probiotic readily, remained healthy throughout the experiment and their body weight

Table 2: Effect of botanical probiotic on growth performance in broiler chickens[1]

Parameters	Control (0.0+)	Control (0.0-)	Probiotic (0.10%)	SEM	P-value
	Diets				
Body weight, g					
d 1	46.00	46.00	46.00	0.10	0.59
d 21	732.69	711.80	723.57	14.12	0.59
d 42	2821.33	2830.47	2766.49	42.14	0.53
Weight gain, g					
1 to 21 d	686.69	665.80	677.57	14.12	0.59
22 to 42 d	2088.64	2118.67	2042.93	42.05	0.46
1 to 42 d	2775.33	2784.47	2720.49	42.12	0.53
Feed intake, g/bird					
1 to 21 d	1199.57[a]	1209.19[a]	1199.16[a]	14.49	0.86
22 to 42 d	4040.85[a]	3310.39[b]	3240.13[b]	46.42	0.0001
1 to 42 d	5240.42[a]	4519.59[b]	4439.29[b]	53.04	0.0001
Feed/gain					
1 to 21 d	1.74[a]	1.82[a]	1.77[a]	0.04	0.46
22 to 42 d	1.93[a]	1.56[b]	1.59[b]	0.03	0.0001
1 to 42 d	1.98[a]	1.62[b]	1.63[b]	0.02	0.0001
Leg abnormality, %					
1 to 42 d	5.06	5.12	3.80	1.16	0.14
Mortality rate, %					
1 to 42 d	4.50	7.02	4.76	2.00	0.71

[1]Data are means and SE of 5 replicates of 30 broilers per pen. [a,b]Means within a row with no common superscript differ ($P < 0.05$).

and weight gain were similar to those fed the control diets. Other studies have reported increased body weights in poultry fed with *Lactobacillus* supplemented diets in both the starter and grower periods (Mohan *et al.*, 1996; Jin *et al.,* 1998; Zulkifli *et al.*, 2000). Results from the present study suggest that the natural probiotic containing *L. plantarum* and *L. salivarius* as the major bacteria supplemented with the basal diet supported growth similar to that of the basal diet supplemented with a coccidiostat and antibiotic.

Feed intake and feed to gain ratio from 1 to 21 d of age were not different ($P > 0.05$) due to treatment. However, from 22 to 42 d and overall (1 to 42 d) feed intake was lower ($P < 0.001$) for broilers fed the probiotic and negative control diets when compared with control broilers. When compared with control broilers, feed intake from 22 to 42 d decreased 19.8 % (4040vs. 3240g) and 18.2% (4040vs. 3310 g) for broilers fed diets supplemented with the probiotic and the negative control diet, respectively. Overall (1 to 42 d) feed intake decreased 15.29% (5240 vs. 4439 g) and 13.8 % (5240 vs. 4519 g) for broilers supplemented with the probiotic and negative control diets, respectively. Also, feed to gain ratio from 22 to 44 d and overall (1 to 42 d) was lower ($P < 0.001$) for broilers fed the probiotic and negative control diet when compared with control broilers. Feed to gain ratio from 22 to 42 d decreased 17.6% and 19.2 % with the probiotic and negative control diets, respectively. From 1 to 42 d feed to gain ratio decreased ($P < 0.05$) 14.8% and 13.8% for broilers supplemented with the probiotic diet and negative control diet, respectively. Similar improvements in feed efficiency have been reported for broilers (Mohan *et al.,* 1996; Jin *et al.*, 1998; Zulkifli *et al.*, 2000), and Leghorn chickens (Balevi *et al.*, 2001) fed diets supplemented with probiotics.

In the present study, the improvements in feed to gain ratio of broilers fed the probiotic supplement was probably due to the strains of *Lactobacillus* present in the supplement.

According to Silva *et al.* (1987) and Meurman *et al.* (1995), to be effective, probiotics must have the following properties: (a) be a normal inhabitant of the gastrointestinal tract, (b) be able to resist gastric acid, bile salts and pancreatic enzymes, © be able to adhere to intestinal mucosa, and (d) readily colonize the intestinal tract so that their beneficial functions could be performed. The *Lactobacil1us* strains (*L. plantarum* and *L. salivarius*) have a strong ability to attach to the intestinal epithelium and are resistant to the bile and acidic conditions (Nemcova *et al.*, 1997; Garriga *et al.,* 1998; Murphy and 1999; Adlerberth *et al.*, 1996; Ahrne *et al.*, 1998).

Microbial activity: The effects of the natural botanical probiotic supplementation on the concentration of bacteria recovered from the ceca, cloaca and carcass rinse at 56 d of age are presented in Table 3. The population of *Lactobacilli* from ceca contents were not different ($P > 0.05$) due to treatment. This result is similar to the findings of (Mohan *et al.*, 1996) who reported no differences in the number of *Lactobacilli* in the ceca of broilers with or without *Lactobacillus* culture at 40 d of age. These results are, however, contrary to that of (Lan *et al.*, 2003) who reported that the count of *Lactobacilli* was significantly higher in broilers fed

Table 3: Effect of botanical probiotic on ceca, cloaca and carcass rinse microflora in broiler chickens

	Diets				
Parameters	Control (0.0+)	Control (0.0-)	Probiotic (0.10%)	SEM	P-value
Ceca, cfu/ml [a]					
Lactic acid bacteria	7.93[c]	8.05[c]	8.01[c]	0.29	0.90
E. coli	7.07[c]	7.12[c]	6.71[c]	0.29	0.55
Cloaca, cfu/ml[a]					
Lactic acid bacteria	7.52[c]	8.20[c]	9.00[d]	0.31	0.002
E. coli	8.36[c]	7.75[cd]	7.53[d]	0.26	0.17
C. perfringens	3.34[c]	3.07[cd]	2.65[d]	0.16	0.02
Carcass rinse, cfu/ml[b]					
Campylobacter	3.48[cd]	3.99[d]	3.02[c]	0.32	0.11
E. coli	5.02[c]	5.02[c]	4.90[c]	0.13	0.73

[a]Data are means and SE of 10 broilers per treatment. [b]Data are means and SE of 25 broilers per treatment.
[c,d]Means within a row with no common superscript differ (P < 0.05).

probiotic compared to controls. The concentration of *Lactobacilli* recovered from cloaca contents, however, of broilers fed the probiotic was higher (P < 0.002) than for those fed the control diets. *Lactobacilli* of broilers fed the probiotic diet was 8.9% higher than those fed the negative control and 16.4% higher than those fed the positive control diet. The reduction in *Lactobacillus* recovered from the cloaca of broilers fed the positive control diet may have been due to the use of the antibiotic bacitracin. It has been suggested that antibiotics used as feed additives may affect the stability of such bacteria groups as *Lactobacillus* in broilers (Ohya and Sato, 1983; Abdulrahim *et al.*, 1996; Abdulrahim *et al.*, 1999; Engberg *et al.,* 2000). The concentration of *C. perfringens* in the cloaca was also lower (P < 0.02) for broilers fed the probiotic than those fed the control diets. The concentration of *C. perfringens* of broilers fed the probiotic was 20.7 % lower than those fed the positive control diet but was not different from those fed the negative control diet. Murry *et al.* (2004) observed *in vitro* that media with cultures of *L. salivarius* and *L. plantarum* produced more (P < 0.001) acetic and lactic acid and the pH was lower (P < 0.001) than those of the controls. Murry *et al.* (2004) concluded that *L. salivarius* and *L. plantarum* contained in the botanical probiotic can ferment carbohydrates in poultry feed to produce pH levels and concentrations of lactic and acetic acid that inhibit the growth of *C. perfringens*. Other strains of *Lactobacilli* cultures have been used to control *C. perfringens* in poultry. A recent study reported by La Ragione *et al.* (2004) showed that when 20 d old chicks were dosed with *Lactobacillus johnsonii* and later challenged with *C. perfringens*, all aspects of colonization and persistence of *C. perfringens* were suppressed. According to Ficken and Wages (1997) and Van der-Sluis (2000a,b) although *C. perfringens* is part of the normal intestinal flora, high concentrations can cause necrotic enteritis in poultry. In the present study, the population of *E. coli* in the ceca and cloaca of broilers were not different (P > 0.05) due to treatment. The population *C. jejuni* recovered from carcass rinses for broilers fed the diet supplemented with the probiotic tended (P < 0.11) to be lower when compared to the negative control. *C. jejuni* was 24.3% lower for broilers fed the probiotic supplemented diet than for broilers fed the negative control diet, but was not different from those fed the positive control diet. It is known that *C. jejuni* in poultry has often been responsible for human gastroenteritis, and intestinal colonization of *C. jejuni* in the chicken plays a role in carcass contamination during slaughter (Genigeorgis, 1986). Morishita *et al.* (1997) reported also, that *Lactobacilli* cultures can reduce *C. jejuni* colonization and frequency of shedding in market-age broilers.

Natural botanical probiotics containing *L. plantarum* and *L. salivarius* supplemented diets may support growth for broilers similar to the basal diet supplemented with coccidiostat and antibiotic. Broilers fed botanical probiotics may consume less feed and the feed to gain ratio will be lower. Also, botanical probiotic supplementation may increase the population *Lactobacilli* and thus reduce the population of *C. perfringens* and *C. jejuni* in market-age broilers.

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