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The role of probiotic in the reduction of the colonization of *Campylobacter jejuni* in broiler chickens

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Abstract

Aim of study is to provide efficacy of probiotic to reduce colonization of *Campylobacter jejuni* in broiler chickens. A total of 150 samples from broiler collected at Qena Province. All samples subjected to bacteriological investigation for *C. jejuni* and purified colonies identified biochemically. Molecular identification done for isolated strains. Multispecies probiotic product (Durvet) tested in vitro experiment for its inhibitory effects against growth of *C. jejuni* strains. Seven-day-old Hubbard broiler chicks used in vivo experiment; 120 Chicks classified into six groups; each group 20 chicks; Group (1) negative control, Gp2 infected with *C.jejuni*, Gp3 treated with probiotic, Gp4) treated with probiotic and infected with *C.jejuni*, Gp5 feed with supplement and Gp6 feed supplement and infected with *C.jejuni*. Chicks weighed; performance parameters weekly followed during experiment. (21.3%) *C. jejuni* identified from chicken by bacteriological examination and (**23.3%**) positive by molecular. The bacteriological analysis of swabs at end of experiment detected Gp4 and 6 had a pathogen colonization count below 2 log CFU/g, but infected group (Gp2) 7.2 log CFU/g. Weight of birds at 14 d and 28 d increased in Gp1,3,4,5 and 6 but slightly increased in Gp2. The performance was good at 7d in all groups, at 14d and 28d gp1,3,5 and 6 are good and active but depressed, lazy and rough feather in Gp2. In conclusion, the probiotics properties have been antimicrobial activities against *C. jejuni*.

Keywords: *Campylobacter* jejuni, probiotics, virulence genes, supplement.

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Introduction

The most prevalent zoonotic illness affecting people worldwide is campylobacteriosis, and *Campylobacter jejuni* (*C. jejuni*) is one of the major causes of enteric infections in people (Aguiar et al. 2013). According to the CDC (2018), *campylobacter* species are known to be a major contributor to acute bacterial diarrhea in humans and to cause gastroenteritis. The natural habitat of *Campylobacter* is the gastrointestinal system of birds and mammals. Humans can contract the disease by touching and eating tainted meat. Typically, two to three weeks after hatching, *Campylobacter* is introduced into the chicken production cycle, and it spreads quickly within the flock. (Truccollo, 2021).

Poultry meat, particularly raw or undercooked chicken, is the main cause of human infection (EFSA, 2017; CDC, 2018). *C. jejuni* and *C.coli* are the two most common Campylobacter species implicated in human illnesses out of the 25 discovered to far (Skarp et al., 2016). In order to prevent the spread of human campylobacteriosis, several authors have investigated the epidemiology of *Campylobacter* in broiler flocks (Allen et al., 2011; Agumos et al., 2014; and Ingresa-Capacciomi et al., 2016).

The digestive system of chicken and foodborne infection are both related to *C. jejuni* (Cean et al., 2015). According to Corcionivoschi and colleagues (2012), *C. jejuni* is the prevalent species. *C. jejuni* is thought to have a close interaction with chickens because there have only been a few numbers of research on the potential health effects in chickens brought on by gut colonization (Thibodeau et al., 2015).

The most frequent cause of infection for the spread of *C. jejuni* in the environment is probably contamination with bird droppings. According to Georgiev et al. (2017), campylobacter can be transmitted vertically by either being present on the surface of eggs or via transovarial transmission. Young birds may catch *C. jejuni* from contaminated water or aliment. Additionally, if exposed to at least 10% moisture, chicken litter can remain infectious for extended periods of time. Consider a shallow well with non-chlorinated water as a potential source. Houseflies can act as a source of transmission for flocks, and footwear and equipment contaminated with feces from an infected source can act as a means of infection. Young chicks can excrete *C. jejuni* for the rest of their lives and are quickly colonized when exposed to it (Ugarte-Ruiz et al., 2018).

C. jejuni regularly colonizes flocks of poultry without causing any obvious symptoms. eliminating *Campylobacter* in the chicken reservoir is an essential first step in the management of this foodborne infection, as risk assessment evaluations have identified handling and consumption of poultry meat as one of the most significant sources of human campylobacteriosis (Saint-Cry, et al. 2016).

Probiotic usage has so far shown good benefits in lowering *Campylobacter* colonization. In order to find probiotics with an anti-*Campylobacter* activity and learn more about the inhibitory mechanism at work, various eukaryotic epithelial cell lines are used. Investigating the processes of *C. jejuni* colonization in poultry in the presence of probiotics may begin with these in vitro pathogenicity models that use avian cell lines. Probiotics' impact on *C. jejuni* colonization is also the subject of in vivo tests (Reid, 2005).

An effective substitute for administering antibiotics to cattle to prevent bacterial contamination is the use of probiotics, which can enhance the natural defense of animals against pathogenic bacteria (Nothaft et al., 2017). The purpose of this study is to present the most recent findings about the effectiveness of probiotics in preventing *C.jejuni* colonization in broiler chickens.

Materials and Methods

150 samples of broiler chicken were gathered in Qena Province, including (50 cloacal swabs, 50 samples of intestinal content, and 50 samples of livers). All of the obtained samples were taken as quickly as possible to the lab where they underwent a bacteriological examination to check for *C. jejuni*.

Bacteriological examination

Isolation of *Campylobacter* species

A loopful of each sample was cultivated for 24 to 72 hours in sterile tubes on Thioglycollate broth medium before being transferred to a modified *Campylobacter* blood free selective medium with antibiotics. All inoculation plates will incubate in anaerobic jars with kits that generate CO2 (10%), O2(5%), and nitrogen (85%) at 37°C for 48 hours, and they will check the colonies every day to see if they have the desired characteristics. On blood agar plates with defibrinated blood sheep including *Campylobacter* growth supplement, the questionable colonies were then purified for 24 hours.

Identification of the isolates

The suspected colonies will be identified by:

Morphological identification According to Koneman et al. (1995), suspected growing colonies on particular agar plates were thoroughly scrutinized for their morphological characteristics. Gram's stain was used to reveal the morphology of the isolates by staining a single suspicious colony. Species of *Campylobacter* are Gram negative.

Motility

Direct smears from a culture of suspected *Campylobacter* colonies that had been in existence for three days were created and studied under a phase contrast microscope to show the corkscrew-like motion that is unique to *Campylobacter* species (Smibert, 1974).

Biochemical identification

The purified colonies were identified biochemically by the following tests: Catalase production test, nitrate reduction test, oxidase test, urease test, hydrogen sulphide production by using lead acetate paper, temperature tolerance test, glycine tolerance test, sodium chloride (NaCl) tolerance test and hippurate hydrolysis test (El-Gohary, 1998).

Molecular identification by Polymerase chain reaction (PCR)

Using the (Thermo Scientific Gene Jet Genomic DNA Purification Kit#K0721, #K0722), *Campylobacter* DNA was extracted from the culture.

Quantification of genomic DNA extracted

Using a UV-visible spectrophotometer, the amount of DNA isolated from *Campylobacter* isolates was measured. In sterile distilled nuclease-free water, tenfold of the DNA was extracted and prepared in glass cuvettes. At wavelengths of 260 nm and 280 nm, the optical density (OD) of the diluted samples was measured using sterile distilled nuclease-free water as a blank (Sambrook et al., 1989). DNA concentration in the solution was calculated using OD at 260 nm. Using the rule that an OD of 1 equal around 50 mg/ml of double-standard DNA,

the amount of DNA was calculated. The purity of the DNA extracted was also estimated using the ratio of the OD at 260 nm to that at 280 nm. Readings between 1.8 and 2.0 suggested that the DNA was largely pure.

PCR amplification of *Campylobacters* gene segment: (Linton *et al.*, 1997)

PCR Protocol : The following steps were used to carry out the amplification in a DNA thermal cycler: initial denaturation at 94°C for 5 minutes, followed by 25 cycles of denaturation at 94°C for 1 minute, annealing at a temperature specific to the primer pair, 66 °C for 1 minute, and extension at 72 °C for 1 minute. A final extension step was carried out for 10 minutes at 72 degrees. Following amplification, 10 l of each reaction product were collected for electrophoresis on a 1.2% (W/V) agarose gel using 1 x TAE buffer (0.01 m Tri's acetate, 0.002 M EDTA), and ethidium bromide (0.5 mg/ml). 35 minutes of electrophoresis at 100 volts in a device for electrophoresis. Visualization using UV light at wavelength 421 was used to identify specific amplified DNA bands, which were then contrasted with a ladder-style molecular size marker (MW 100–MW 1000 bp) to determine their presence.

Table (1): PCR tube master mix, primer, DNA template and nuclease free water were mixed as follow

Total reaction volume	25 µl
Master mix	**12.5 µl**
Primer	**0.5 µl**
Template DNA	**4 µl**
Nuclease free water	**8 µl**

Table (2): Target virulence-associated genes, primer sequences and amplicon sizes

Genes	Sequences	Amplicon
Campylobacter* spp. *16SrRNA	CB1 TATACCGGTAAGGAGTGCTGGAG CB2 ATCAATTAACC TTCGAGCACCG	**Frasao *et al.*, 2010**
Campylobacter jejuni hipO	F: ACTTCTTTATTGCTTGCTGC R: GCCACAACAAGTAAAGAAGC	**Frasao *et al.*, 2010**
CdtA	F: GGAAATTGGATTTGGGGCTATACT R: ATCAACAAGGATAATGGACAAT	**Bang *et al.*, 2003**
cdtB	F: CAGAAAGCAAATGGAGTGTT R: AGCTAAAAGCGGTGGAGTAT	**Nahar and Bin Rashid, 2018**
cdtC	F: TGGATGATAGCAGGGGATTTTAAC R: TTGCACATAACCAAAAGGAAG	**Bang *et al.*, 2003**
flaA	F: TCCAAATCGGCGCAAGTTCA R: TCAGCCAAAGCTCCAAGTCC	**Zheng *et al.*, 2006**

Experiment

Bacterial Preparation of *C. jejuni* and Oral Challenge

The Animal Reproduction Research Institute in Giza, Egypt provided the strain of *C. jejuni* used in this study. *C.jejuni* was grown in brain heart infusion (BHI; Oxoid) for 72 hours at 37°C utilizing a gas package (Oxoid) at microaerophilic conditions (5% O2, 10% CO2, 85% N2). A 10 mL layer of semisolid agar containing roughly 10^8 precultured *C. jejuni* strain cells was placed on top of the agar plate. Using a sterile syringe, birds were orally challenged at 7 days with 10^8 CFU/ml in the oral cavity.

Experiment Design

Hubbard broiler chicks of various sexes that were one day old were used. Ten haphazardly approaching chicks were slaughtered and later examined bacteriologically to demonstrate their health. 120 chicks were divided into six groups of 20, each of which contained the following characteristics:

Table (3): Different groups of broiler chicks in the experiment

Groups	No. of birds	Infected & Treated groups
1	20	Negative control (neither infected nor treated birds)
2	20	Infected non treated (*C.jejuni* infected birds)
3	20	probiotic treated birds.
4	20	Probiotic treated and *C.jejuni* infected birds
5	20	Feed supplement treated birds
6	20	Feed supplement treated and *C.jejuni* infected birds

Multispecies probiotic product

Probiotics (Durvet) 100 mg for broiler

Commercially prepared mixed probiotics concentrate ($1x10^{10}$ CFU) including *Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus bulgaricus, Bifidobacterium bifidum, Streptococcus thermophilus, and Enterococcus faecium* is available. It was administered via drinking water at one day of age for five days in a row at a dose of 0.5 grams per 25 liters of water, as directed by the manufacturer for Gp.3 and 4

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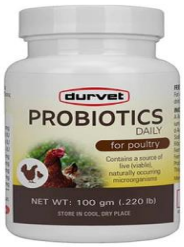

In vitro experiment

Tests are conducted in vitro to determine whether the multispecies probiotic product (Durvet) inhibits the growth of *C. jejuni* strains. In the test, 10 ml of sterile saline were used to dissolve 20 mg of the commercial product, and 10 l of this solution (equivalent to roughly $1 x10^5$ cells) was transferred to the middle of MHA plates using a sterile pipette. The agar plates were then incubated for 24 hours at 37°C in an anaerobic environment. After the agar plate had grown sufficiently, 1 ml of semisolid agar containing 10^8 cells of the *C. jejuni* strain was added. An inhibitory zone around the tested strain was looked for on the agar plate following 72 hours of co-cultivation at 37°C under anaerobic conditions. The tested strain's inhibition zone and growth zone diameters were measured and estimated.

In vivo experiment

Each chick in groups 2, 4, and 6 received an oral inoculation on the seventh day of life with 1 ml of saline suspension containing 10^7 CFU *C. jejuni*. 3 weeks after infection, the experiment's time frame was extended. The broiler chicks were used, kept in enclosures, and fed a typical beginning meal devoid of any medications. 3 weeks after infection, the experiment's time frame was extended. To make sure that all birds were free of *Campylobacter* spp., cloacal swabs from each bird were collected prior to the experimental infection and addition of probiotic (Mawas *et al.*, 2023).

Supplement (Vitamins & Electrolytes Plus 4 Oz by Agrilabs)

Measured parameters

The Performance parameters

The chicks were weighed when they arrived, and at the end of each week, their body weight (BW), feed intake (FI), and feed conversion ratio (FCR) were determined. These precautions were implemented up until the study's conclusion (4 weeks of age).

Clinical signs and postmortem lesions

Periodically, the experimental birds were observed for clinical symptoms, post-mortem examination of chicks who perished during the experiment, or scarification to view the gross lesions of the liver and intestine.

Collection of Samples for Bacteriology

At the end of the experiment's 14 and 28 days, each group's birds were put to death, and their ceca were taken for individual quantitative cultures of *C. jejuni*. All of the test birds were put to death after 35 days, and their ceca were taken for *C. jejuni* culture.

Quantitative Culture of Campylobacter spp.

All of the chicks were ethically slaughter at the ending of the experiment, and cecal samples were taken for quantitative *Campylobacter* spp. culture. The contents of the cecum were placed in centrifuge tubes, diluted 1:10 (wt/vol) in PBS, and homogenized using a vortex mixer. Each dilution was then directly plated on BHI agar after being 10-fold diluted. 48 hours of microaerophilic incubation at 42°C on the plates resulted in the confirmation of Campylobacter colonies. The log10 colony-forming units per gram CFU/gm of cecal contents were calculated from the direct counts.

Statistical analysis

According to Shott Statistical for health professionals (Shott, 1990), one-way analysis of variance (ANOVA) was performed.

Results

Identification of *Campylobacter jejuni*

The bacteriological examination identified 32 out of 150 (21.3%) *C. jejuni* from broiler chickens. The typical cork screw motility of *Campylobacter* was revealed by phase contrast microscopy, and biochemical tests revealed that suspect colonies were positive for oxidase, catalase, Nitrate reduction, growth at 37 ◦C, 43 ◦C, and 1% glycine, susceptible for Nalidixic acid, resist for Cephalothin and negative for urease test. These results were confirmed by PCR using the *16S rRNA* gene primers. (Tables 4).

Table (4): Incidence of *Campylobacter jejuni* by conventional method in broiler samples

Type of examined samples	Number of examined samples	*C. jejuni*	
		No.	**%**
Cloacal swabs	**50**	**15**	**30%**
Intestinal contents	**50**	**12**	**24%**
Liver	**50**	**5**	**10%**
Total	**150**	**32**	**21.3%**

Molecular identification of *C. jejuni* and virulence genes

Molecular identification of *C. jejuni* isolates indicated 35 (23.3%) positive samples and cytoxin genes (85.7%); the most abundant genes were *cdt*A, *cdt*B, and *cdt*C, followed by flagellar gene *fla*A (65.7%), as shown in table (5).

Table (5): prevalence of resistance and virulence genes

Detected genes		*C. jejuni* (n=35)	
		No.	(%)
C. jejuni	*16S rRNA*	35/150	**23.3%**
Cytoxin genes	*cdtA*	30/35	**85.7 %**
	cdtB	30/35	**85.7 %**
	cdtC	30/35	**85.7 %**
Flagellar gene	*flaA*	23/35	**65.7%**

In vitro antimicrobial probiotics against *C. jejuni*

The in vitro study utilizing Müller Hinton agar plate demonstrated that isolates generated from the broiler exhibited the inhibition of *C.jejuni* in vitro. The antimicrobial probiotics strains (Durvet) antagonized the growth of *C. jejuni*, displaying inhibition zone spanning from 12.5 to 15mm.

In vivo antimicrobial probiotics against *Campylobacter jejuni*

The broiler chicks used in the study did not naturally contain *Campylobacter* spp., according to a bacteriological investigation of cloacal swabs taken from the animals before the experiment. Cloacal swabs from the treated groups (Gp4 and 6) had pathogen colonization counts below 2 log CFU/g at the end of the experiment, but the infected group (Gp2) had a mean value of 7.2 log CFU/g. In comparison to controls negative Gp1, birds receiving the probiotic product Gp3 and birds receiving supplement alone Gp5 had no *C. jejuni* cecal colonization (Table 6). Performance was good at 7d in all groups, at 14d and 28d in gp1,3,5 and 6, but depressed, lethargic, and rough feather in Gp2 and 4 (Table 6). The weight of the birds grew in gp1,3,4,5 and 6 at these two time points, but it increased only little in Gp2 (the infected group).

Table (6). The effect of administration of antimicrobial probiotics and supplement on the weight, performance and on the cecal colonization of *C. jejuni* in broiler

Groups	performance				Cecal colonization of *C. jejuni*
	1st w	**2nd w**	**3rd w**	**4th w**	
Gp1	good	good	good	good	Nil
Gp2	good	depressed	lazy	depressed	7.2 CFU/g
Gp3	good	good	good	good	Nil
Gp4	good	Rough feather	good	good	1.9 CFU/g

Gp5	good	active	active	active	Nil
Gp6	good	good	good	good	1.6 CFU/g

Nil: Negative result

CFU: Count Forming Unit

Clinical signs and Postmortem of broiler chicks

The negative control group (Gp1)'s broiler chicks' clinical signs and PM appeared normal, devoid of any unusual clinical indications. The *C. jejuni* (Gp2)-infected group had restlessness, dullness, depression, and ruffled feathers; these clinical symptoms gradually progressed to diarrhea; no fatality rate was noted. The probiotic/supplement-treated groups (3 and 5) displayed good health, no signs of depression, and no unrest. Less clinical symptoms were present in the probiotic/supplement treated groups (Gp4 & Gp6) compared to the infected groups.

The performance

In comparison to the negative control and probiotics/supplement treated groups (1, 3, and 5, respectively), performance parameters in the infected groups (2, 4 & 6) were lower. According to Tables (6 and 7), the infected *C. jejuni* (GP2) at the third week had lower body weight (350 g/bird) weekly, feed consumption (700 g), and increased feed conversion rate (GP2) than the *C. jejuni* infected and probiotic treated group and the negative control group, which had body weight (550 and 730 g/bird) weekly, increased feed consumption (from 100 to 800 g during the four weeks and 1000 g), and lower feed conversion rate (1.05 and 1.6)(GP1).

Table (7). Feed intake, body weight gain and feed conversion rate of different experimental groups

Groups	Age/ week	F1 gm/bird	BWG/gm	FCR
Gp1	**1st w**	**100**	**95**	**1.05**
	2nd w	**400**	**250**	**1.6**
	3rd w	**800**	**550**	**1.45**
	4th w	**1000**	**950**	**1.05**
Gp2	**1st w**	**105**	**90**	**1.2**
	2nd w	**300**	**220**	**1.4**
	3rd w	**700**	**350**	**2**
	4th w	**850**	**750**	**1.13**
Gp3	**1st w**	**100**	**93**	**1.08**
	2nd w	**450**	**320**	**1.4**
	3rd w	**900**	**730**	**1.23**
	4th w	**1100**	**1200**	**0.92**
Gp4	**1st w**	**110**	**90**	**1.22**
	2nd w	**390**	**300**	**1.3**
	3rd w	**850**	**600**	**1.42**
	4th w	**1050**	**1150**	**0.91**
Gp5	**1st w**	**105**	**95**	**1.11**
	2nd w	**550**	**350**	**1.6**
	3rd w	**1000**	**780**	**1.3**
	4th w	**1200**	**1450**	**0.83**
Gp6	**1st w**	**100**	**93**	**1.07**
	2nd w	**450**	**320**	**1.41**
	3rd w	**950**	**700**	**1.4**
	4th w	**1100**	**1300**	**0.85**

FI: feed intake, BWG: body weight gain, FCR: feed conversion rate.

Discussion

Campylobacter is a human diarrheal infection that has been linked to irritable bowel syndrome and reactive arthritis (Mølbak and Havelaar, 2008). According to Kaakoush et al. (2015), *C. jejuni* infection can cause the autoimmune diseases as Miller Fisher syndrome and Guillain-Barré

syndrome (GBS). According to Abdi-Hachesoo et al. (2014), poultry meat is one of the main sources of human *Campylobacter* infection, as it contains organisms from the intestinal content.Due to the fact that *C. jejuni* (Vandamme, 2000) causes large amounts of *Campylobacter* to colonize on the cecum (10^6–10^8 CFU/g), and because *Campylobacter* is widely present in the environment, broiler chickens are an asymptomatic carrier of *Campylobacter* that is contaminated at the farm (Jacobs-Reitsma, 2000).

By conventional approach, the isolated strains of *C. jejuni* were (32 150) 21.3% in cloacal swabs, 24% in intestinal content, and 10% in liver (Table, 3). These findings concur with the findings of Salem et al. (2019). Virulence genes linked to the pathogen adhesion, colonization, and invasion, such as *fla*A and *cdt*, were commonly present, according to the molecular identification, which showed 35/150 (23.3%) by PCR (Table 5). Among the examined *C. jejuni* isolates, the cytolethal distending toxin (CDT) cluster genes *cdt*A, *cdt*B, and *cdt*C accounted for the majority of virulence genes (85.7%). These findings were published in multiple researches by Krutkiewicz et al. (2010) and Ahmed et al. (2019).

Table 5 shows the flagellar gene *fla*A (65.7%). Among *Campylobacter* isolates, the *fla*A gene is largely conserved. Flagella are essential for attachment to intestinal epithelial cells, motility, and chemotaxis. They are also secreted, autoagglutination, microcolony formation, innate immune response, and virulence protein production (Guerry, 2007).

Research has been done in recent years to examine the potential of probiotics to avoid *C. jejuni* from shedding during the production of poultry. Probiotic bacteria' antimicrobial properties and effectiveness also had an impact on the colonization of *C. jejuni* in broiler chickens. The reduction of *C. jejuni* colonization in both vitro and vivo is attributable to the antibacterial activity of probiotics against *C. jejuni*, according to the current study's in vitro inhibition test and in vivo experiment. This finding is very significant since eating poultry meat exposes humans to *C. jejuni*, which is the primary cause of campylobacteriosis (Keener et al., 2004). The outcomes align with the research conducted by Willis and Reid (2008), which revealed a reduced concentration of *C. jejuni* in broiler chicks given a regular diet plus a combination probiotic supplement (Table, 6).

Postmortem examination of a chicken infected with *C. jejuni* on its seventh day of life (G2) revealed hemorrhagic enteritis with evidence of inflammation of the mucosa, as well as a large and congested liver. Shah et al. (2003) reported the same conclusion. Since gram-negative bacteria, like *C. jejuni*, are more sensitive to organic acids, hydrogen peroxide, carbon dioxide, diacetyl, and bacteriocins have specific inhibition activity against them, it is likely that the bactericidal effect of probiotics against this type of bacteria is due to the production of organic acids (Ghareeb et al., 2012).

Here, Infected groups (2, 4 & 6) had worse performance parameters than probiotic/supplement treated groups (1, 3, and 5) and negative control groups (i.e. Comparing the infected *C. jejuni* group to the probiotic/supplement treated group, the Gp4 and Gp6 infected group, and the negative control group, showed that the former had body weights of 1300 and 1150 g/bird, respectively, increased feed consumption (from 100 to 1100 g over the course of four weeks), and lower feed

conversion rates (0.83 and 1.41). Feed conversion rate was lowered, and feed consumption increased with probiotic therapy. Growth capability was enhanced by probiotic administration techniques using water (Olnood et al., 2015).

Conclusion

Ultimately, probiotics have been shown to have antimicrobial activities against *C. jejuni*. They are a great way to reduce the colonization of *C. jejuni* in the cecum of broiler chickens and may also alter the gut microflora of the birds in a way that improves their health and lowers the risk of human campylobacteriosis. In addition, supplement product administration improves performance and lowers the quantity of possible food-borne infections in broiler chickens.

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