

Effects of feeding plant-derived agents on the colonization of *Campylobacter jejuni* in broiler chickens

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ABSTRACT The aim of this work was to test the potential use of plant-derived extracts and compounds to control *Campylobacter jejuni* in broiler chickens. Over a 7-wk feeding period, birds were fed a commercial diet with or without plant extracts (*Acacia decurrens*, *Eremophila glabra*), essential oil [lemon myrtle oil (LMO)], plant secondary compounds [terpinene-4-ol and α -tops (including α -terpineol, cineole, and terpinene-4-ol)], and the antibiotic virginiamycin. Traditional culture and real-time quantitative PCR techniques were used to enumerate the numbers of *C. jejuni* in chicken fecal and cecal samples. In addition, BW and feed intake were recorded weekly for the calculation of BW gain

and feed conversion ratio. The mean log₁₀ counts of *C. jejuni* were similar ($P > 0.05$) across treatments. However, significantly lower levels of fecal *Campylobacter* counts ($P < 0.05$) were recorded at d 41 for the α -tops treatment by culture methods. No differences ($P > 0.05$) in BW gain were obtained for dietary supplementation, except for the *E. glabra* extract, which had a negative impact ($P < 0.001$) on BW, resulting in sporadic death. Results from this study suggest that supplemental natural compounds used in the current study did not reduce the shedding of *C. jejuni* to desired levels.

Key words: *Campylobacter jejuni*, chicken, plant extract, essential oil

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INTRODUCTION

Campylobacter jejuni is a gram-negative bacterium, well-recognized as a major etiologic agent for acute gastrointestinal diseases in humans, particularly in the elderly and in children (Zilbauer et al., 2008). The most common nonspecific symptoms are bloody diarrhea, abdominal cramps, fever, and vomiting (Black et al., 1988). This organism can be isolated from a variety of wild and domestic animals, especially poultry. Healthy chickens are normally carriers of *C. jejuni*, which is then transmitted to humans through the consumption and mishandling of contaminated poultry products (Mullner et al., 2010). This bacterium colonizes the gastrointestinal tract of chickens to high abundance, resulting in undesirable levels of chicken meat contamination. It has been reported that up to 90% of retail chicken meat in Australia are surface contaminated by *Campy-*

lobacter spp. (Pointon et al., 2008). In a risk modeling study conducted by Rosenquist et al. (2003), it was estimated that a 2 log₁₀ reduction of *C. jejuni* in the chicken gastrointestinal tract could potentially result in a 30% decrease in human campylobacteriosis. Unfortunately, however, there are no measures for effectively controlling *C. jejuni* in chickens. Furthermore, poultry industries around the world have been forced to reduce the use of antibiotics in feed, with the use of antibiotics as a growth-promoting agent being banned in European livestock (Castanon, 2007). Hence, new strategies to control *C. jejuni* in poultry are needed immediately.

In the area of phytotherapy, research efforts using in vitro assays have produced evidence on the antimicrobial effectiveness of a large number of plants along with their components. Different plant-derived agents have been explored for their usefulness in animal production systems to improve health and the quality of their products. It has been shown that the inclusion of these agents in the diets of broiler chickens leads to improvement in growth performance and reduced pathogen load. For example, it was demonstrated that a diet containing a blend of essential oils (EO) as a feed

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additive improved feed intake, BW, and carcass yield in poultry (Alçiçek et al., 2003). It was also reported that diets containing herbal extracts reduced the numbers of the foodborne pathogen *Escherichia coli* (Arshad et al., 2008), the animal pathogen *Clostridium perfringens* (Allen et al., 1997), and the parasite *Eimeria tenella* (Christaki et al., 2004) in the digestive tract of poultry. In addition, plant-derived agents also appeared to improve the food quality of animal products by enhancing the oxidative stability of lipids in meat (Jang et al., 2008) and by protecting meat from food spoilage organisms (Karagöz Emiroğlu et al., 2010). As a result, it was suggested that plant-derived agents such as EO could be used as potential growth promotants in poultry. Furthermore, commercial premixes (Avigro, RepaXol, and CRINA) containing blends of plant-derived components as animal feed additives are currently available (Newbold et al., 2004; Zhang et al., 2005).

Our previous in vitro screening results have shown that plant extracts derived from *Acacia decurrens* and *Eremophila glabra* were found to be antimicrobial activity against *C. jejuni* at concentration of 64 µg/mL (Kurekci et al., 2012). In addition, EO from lemon myrtle oil (LMO), and plant secondary compounds (terpinen-4-ol and α -tops including cineole, α -terpineol, and terpinen-4-ol) were also antimicrobial toward *C. jejuni* without affecting the in vitro fermentation at concentration of 0.05% (vol/vol; Kurekci et al., 2013). The main aim of this study was to further investigate the effect of dietary inclusion of these agents on the control of *C. jejuni* in poultry. Furthermore, the abundance of *C. jejuni* in the cecal and fecal samples was monitored by real-time quantitative PCR (RT-qPCR), and the profile of the bacterial community was examined by denaturing gradient gel electrophoresis (DGGE). Also, the effect of these agents on chicken performance was determined and compared with virginiamycin, an antibiotic registered as a feed additive by the Australian Pesticides and Veterinary Medicines Authority.

MATERIALS AND METHODS

Preparation of Plant Extracts/ EO/Compounds

Freeze-dried plant leaves (300 g) from *A. decurrens* and *E. glabra*, which were selected on the basis of their high antimicrobial activities against *C. jejuni* in vitro, were obtained from Jason Emms (Future Farm Industries CRC, SARDI, Adelaide, Australia). Plant leaves were ground to pass through a 1-mm sieve (Restech, Raymond Terrace, NSW, Australia) and extracted as outlined by Kemp and McSweeney (2010) with some modifications. Ground plant material (50 g) was mixed with 350 mL of 80% ethanol on a rotating shaker at approximately 110 rpm for 3 h at room temperature (20 $\pm$ 2°C). This mixture was centrifuged at 2,000 $\times$ g for 5 min at room temperature and the supernatant was collected into 1-L round bottom flasks. The resulting

pellet was resuspended in 350 mL of ethanol (80%) and the process repeated. The supernatant was mixed and ethanol was evaporated by the use of vacuum evaporator (Buchi Labortechnik AG, Flawil, Switzerland) for 48 h. Dried extract was weighed and stored at -20°C until use.

The LMO, terpinen-4-ol, and α -tops were obtained from BioAust, Brisbane, Australia. To control the release of test agents through the digestive tract, all plant extracts, LMO, and compounds (terpinene-4-ol and α -tops) were formulated with cyclodextrine for which the ratio was measured by mass spectrometry (BioAust). The components of LMO were given previously (Kurekci et al., 2013). Formulations consisted of 78.7% cyclodextrine and 21.3% LMO or compounds and 66.7% cyclodextrin and 33.3% plant extract.

Bird Management and Experimental Design

One-day-old male broiler chicks, obtained from Bond Enterprises (Grantham, Queensland, Australia), were grown over a 7-wk experimental period. At 4 d of age, 84 chicks were individually weighed and tagged and randomly assigned to treatment groups (7 treatments, 3 replicates/treatment, and 4 chicks per replicate). The chicks were kept in brooders with wire screen floors for the first 21 d of age. The room was illuminated continuously with ambient temperature being maintained at 34 $\pm$ 1°C for the first 4 d and gradually reduced to 22 $\pm$ 2°C at d 21 where it was maintained until the end of the experiment. Water and feed were supplied ad libitum during the experimental period. Procedures for bird management and care were approved by the Animal Ethic Committee of The University of Queensland (approval number; SAS/288/10).

The composition of the basal diets (BD) is shown in Table 1. All ingredients were prepared in a 1-t horizontal mixer (Benmic, Toowoomba, Australia), with the test agents being added at the appropriate concentration to the BD.

In the first 4-d adaptation period, birds were fed a commercial starter diet as crumbles (Riverina, Brisbane, Australia). Starter and finisher diets were fed from 4 to 21 and 21 to 49 d of age, respectively (as shown in Table 1). The 7 experimental groups included the following: 1) negative control (N-CTL) with cyclodextrine (1 g/kg of the starter diet and 2 g/kg of the finisher diet); 2) positive control (P-CTL) with virginiamycin (Riverina; 40 mg/kg in the starter diet and 20 mg/kg in the finisher diet according to the label information); 3) with *A. decurrens* extract (0.5 g/kg of the starter diet and 1 g/kg of the finisher diet); 4) with *E. glabra* extract (0.5 g/kg of the starter diet and 1 g/kg of the finisher diet); 5) with α -tops (100 mg/kg of starter diet and 200 mg/kg of the finisher diet); 6) with LMO (100 mg/kg of starter diet and 200 mg/kg of the finisher diet); and 7) with terpinen-4-ol (100 mg/kg of starter diet and 200 mg/kg of the finisher diet). The concentrations of the test agents chosen were based on

Table 1. Experimental starter and finisher diets fed to birds

Dietary constituent (kg/t)	Diet	
	Starter diet	Finisher diet
Canola	100	120
Wheat	150	150
Sorghum	427	439.6
Meat meal	80	100
Soybean meal	209	145
Soybean oil	19	35
Lysine	2.1	1.5
Methionine	2.3	2.1
Threonine	1.1	0.9
Salt	3.4	0.8
Vitamin and mineral premix ¹	3	3
Sodium bicarbonate	2	2
Choline chloride	1.1	0.1

¹Vitamin and mineral supplement provided (per kg of premix): 15 MIU of vitamin A; 6 MIU of vitamin D₃; 60 g of vitamin E; 4.5 g of vitamin K₃; 2.25 g of vitamin B₁; 7.5 g of vitamin B₂; 4.5 g of vitamin B₆; 0.03 mg of vitamin B₁₂; 18 g of D-calcium pantothenate; 1.5 g of folic acid; 75 g of niacin-vitamin B₃; 0.3 mg of biotin; 30 g of copper sulfate; 30 g of ferrous sulfate; 1.5 mg of iodine; 180 g of manganese; 0.15 mg of selenium; 150 g of zinc; 75 g of ethoxyquin.

reports of *Peganum harmala* extracts fed to poultry to minimize *E. coli* infection (Arshad et al., 2008) and the use of plant extracts and EO in poultry nutrition (Brenes and Roura, 2010). In these experiments, the chickens were naturally colonized by *C. jejuni*. To maximize this natural colonization, biosecurity procedures such as insecticide treatments, disinfectants, and changing footwear by farm workers in contact with poultry before entering the shed were not employed. At d 28 after collecting the fecal samples, the cage environment was also contaminated with turkey litter with feces that was previously shown to be *C. jejuni* positive.

The overall health of the birds and mortality were monitored and recorded daily during the experimental period. Body weight was individually recorded on d 4 and 7 and continued weekly thereafter. Feed intake measurement was also taken weekly to determine the feed conversion ratio (FCR). At the end of the experiment, each chicken was euthanized by cervical dislocation, and the cecum aseptically removed for bacteriological and volatile fatty acid (VFA) analyses.

Enumeration of *C. jejuni* by the Culture Method

Collection of fecal samples commenced on d 21 and continued on d 28, 35, 41, and 45. To collect the fecal droppings, the cage bottom was completely covered with blotting paper with fresh paper installed before each fecal sample collection. The number of droppings collected equaled the number of chickens in each cage, assuming one dropping for each bird. Fecal samples were pooled per pen and kept on ice and transported to the laboratory for analysis. The number of viable *C. jejuni* was determined from 1 g of composited fecal

sample and enumerated by plating duplicate dilutions on charcoal cefoperazone deoxycholate agar plates containing selective supplement (CCDA, Oxoid, Adelaide, Australia) and incubated for 48 h at 37°C under micro-aerophilic conditions (Bolton et al., 1984). The number of resulting colonies was expressed as cfu per gram of sample. At least one colony from each dilution series was selected for confirmation of presumptive *C. jejuni* by using Gram staining, biochemical tests including oxidase, catalase, and rapid hippurate hydrolysis and PCR method as described by Inglis and Kalischuk (2004).

After the birds were euthanized, cecal contents (1 g) from each bird were pooled for each cage and serially diluted for identification and quantification of *C. jejuni* as described above. The remaining pooled cecal samples were stored at -80°C for DNA extraction.

Quantification of *C. jejuni* and Total Bacteria by RT-qPCR

DNA Extraction. Genomic DNA from chicken fecal and cecal samples were extracted by a bead-beating procedure as described by Yu and Morrison (2004). Contaminating RNA and proteins were removed using the QIAamp mini kit (Qiagen, Crawley, UK), following the manufacturer's instructions. The DNA concentrations were measured using the Qubit dsDNA BR assay kit in the Qubit fluorometer (Invitrogen, Carlsbad, CA). The DNA samples were stored at -20°C until use.

RT-qPCR Conditions. The primers used to quantify *C. jejuni* and total bacteria by RT-qPCR were *C. jejuni* QCjmapANf (5'GGTTTGAAGCAAAGAT-TAAAGG3'), QCjmapANr (5'AAGCAATACCAGT-GTCT AAAGTGC3'; Inglis and Kalischuk, 2004), and 1114f (5'CGGCAACGA GCGCAACCC3'), 1275r (5'CCATTGTAGCACGTGTGTAGCC3'), respectively (Denman and McSweeney, 2006). The PCR product of the *mapA* gene of *C. jejuni* was used to construct the standard curve for *C. jejuni* quantification by RT-qPCR. Amplified PCR products were purified and ligated into a pGEM-T easy vector and transformed into *E. coli* cells. The copy number of DNA molecules was calculated by constructing a standard curve using 10-fold dilutions of known quantities of plasmid DNA (1×10^7 to 1×10^1 copies/ μ L). For RT-qPCR standard curves, functions describing the relationship between Ct and x ($\log_{10}$ DNA concentration in ng/ μ L) for the assays were as follows: Ct = -3.4171x + 34.578; with a correlation of determination (R^2) of 0.997 for *C. jejuni*; Ct = -3.4567x + 36.467; R^2 = 0.991 for total bacteria. The standard curve for total bacteria was generated by Carly Rosewarne (CSIRO, Livestock Industries, Brisbane, Australia) in the same way described above and kindly given for this experiment. Amplification efficiency (E) was estimated by using the slope of the standard curve and the formula $E = (10^{-1/\text{slope}}) - 1$. A reaction with 100% efficiency generated a slope of -3.32.

Amplification and detection of DNA by RT-qPCR were performed in an ABI prism (7900HT Sequencing Detection System, AB, Applied Bioscience, Mulgrave, Australia) using MicroAMP optical 384-well reaction plates (AB, Applied Biosystems, Singapore). Three technical replicates from 3 pooled biological replicates were routinely used, and mean values were calculated. Reactions were performed in a total volume of 5 μ L containing 2.5 μ L of the Platinum SYBR Green qPCR Supermix-UDG Master Mix (Invitrogen, Carlsbad, CA), 0.1 μ L of ROX reference dye, 1 μ L of template DNA (1 ng), and 300 nM of each of the forward and reverse primers. A mixture of all PCR reagents without any DNA was used as a negative control. The reaction conditions for amplification of *C. jejuni* mapA gene were 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s, and a final extension 72°C for 5 min (Inglis and Kalischuk, 2004). The RT-qPCR cycling conditions for total bacterial 16S rRNA gene were as follows: 50°C for 2 min, 95°C for 2 min, and 40 cycles 2 min at 50°C, 2 min at 95°C, and 40 cycles of at 95°C for 15 s and 60°C for 1 min. (Denman and McSweeney, 2006). Melting curve analysis for both was performed at the end of each amplification step at 95°C for 15 s and 60°C for 15 s. The RT-qPCR results were analyzed using the SDS 2.1 software (Applied Biosystems Inc., Mulgrave, Australia). The results of the analysis were the quantity of gene copy number per gram of sample, which is equal to cell numbers as each *C. jejuni* cell was reported to have single copy of this gene (Ahmed et al., 2009).

DGGE

Denaturing gradient gel electrophoresis was performed to profile the bacterial communities in experimental groups by using the Dcode universal mutation detection system (Bio-Rad, Madrid, Spain) as previously described (Muyzer et al., 1993). Briefly, PCR was performed to amplify the V3 region of the 16S rRNA gene by a touchdown PCR, and primers used were 341f + GC clamp and 518r (Muyzer et al., 1993). The PCR products (25 μ L) were run on gels consisting of 8% (wt/vol) polyacrylamide, a denaturing gradient from 30 to 60% [100% denaturant is 7 M urea and 40% (vol/vol) formamide], in 1 $\times$ Tris-acetate-EDTA (TE) at 60 V and 60°C for 17 h. After electrophoresis, the bands were visualized using silver staining. Selected DNA bands on the DGGE gel were picked for reamplification, cloning, and sequencing. The bands were eluted in 200 μ L of TE solution and washed with 50 μ L of TE solution 3 times and then subjected to 3 rounds of freeze-thaw, which included holding at -80°C for 15 min followed by thawing at 65°C 15 min for further DNA recovery into the solution. The eluted DNA was re-amplified with the primers 341f and 534r with the cycling conditions of 4 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C; and then 7 min at 72°C. The PCR product for each band was cloned

as described by (Sambrook and Russel, 2001). Of the 24 randomly selected clones (8 clones for each band), inserts of 21 clones were found to be useful for sequence analysis. The closest known relatives of the partial 16S rRNA gene sequences obtained were determined based on nomenclature taxonomy and Bergey's Manual by using the ribosomal database project (RDP) Naive Bayesian rRNA Classifier Version 2.2 (<http://rdp.cme.msu.edu/index.jsp>; Wang et al., 2007).

VFA

For VFA analysis, 0.5 g of cecal content was diluted in 1 mL of PBS and vortexed. This mixture was centrifuged at 10,000 $\times g$ at 4°C for 15 min. Then, supernatant was collected and filtered through 0.45 μ m-pore size filter (Millipore, Carrigtwohill, Co. Cork, Ireland), and analyzed for VFA concentration as previously described (Kurekci et al., 2013).

Toxicology

After the death of 7 birds in the *E. glabra* supplemented group between d 28 and 32, blood samples were collected from 4 birds randomly selected from the N-CTL group and the remaining 4 birds in the *E. glabra* group. Blood was collected from the wing veins into vacutainer blood collection tubes (serum and K₂EDTA, BD, Plymouth, UK) and then sent to the laboratory at the School of Veterinary Science, The University of Queensland, for biochemical and hematological analyses. Two chickens that died suddenly in the *E. glabra* group were evaluated via postmortem examination for pathological abnormalities of the heart, liver, spleen, kidneys, and gastrointestinal tract.

Statistics

Data on BW, BW gain, and VFA and biochemical parameters were based on the individual bird as the experimental unit. Cage was considered as the experimental unit for microbiology parameters. Because there were only 3 replicates in the groups, no statistical analyses were conducted for feed intake and FCR data for which only mean values with SE are given. Fecal and cecal cfu data were converted to log₁₀ cfu numbers before analysis. All values are expressed as means $\pm$ SE. The data were analyzed using ANOVA (GLM) by the use of the Minitab 16 (Minitab Inc., State College, PA) software. Differences were determined by the use of the Tukey test and considered significant at $P < 0.05$.

RESULTS

Growth and Feed Consumption

The BW, BWG, feed intake, and FCR for starter diet (between d 4 and 21) are given in Table 2. Inclusion of *E. glabra* extract at a rate of 0.5 g/kg diet reduced (P

Table 2. Effect of plant-derived agents on growth performance of broilers fed a starter diet (between d 4 and 21)

Treatment ¹	BW ² (g)	BW gain ² (g)	Feed intake ³ (g)	FCR ³ (g/g)
N-CTL (n = 12)	1,004 ± 15 ^a	895 ± 15 ^a	1,236 ± 27	1.38 ± 0.03
Virginiamycin (n = 11)	980 ± 17 ^a	872 ± 15 ^a	1,309 ± 15	1.50 ± 0.02
<i>Acacia decurrens</i> (n = 12)	1,031 ± 20 ^a	921 ± 19 ^a	1,246 ± 26	1.35 ± 0.02
<i>Eremophila glabra</i> (n = 11)	862 ± 15 ^b	752 ± 16 ^b	1,264 ± 14	1.65 ± 0.02
α-Tops (n = 12)	1,030 ± 19 ^a	921 ± 19 ^a	1,259 ± 20	1.37 ± 0.03
LMO (n = 11)	1,044 ± 90 ^a	933 ± 90 ^a	1,297 ± 23	1.39 ± 0.02
Terpinen-4-ol (n = 12)	1,006 ± 18 ^a	896 ± 18 ^a	1,271 ± 25	1.42 ± 0.01

^{a,b}Means with different superscripts in the same column for BW and BWG differ significantly (Tukey, $P < 0.05$).

¹N-CTL (negative control with cyclodextrine) and LMO (lemon myrtle oil).

²All figures are means ± SE (n).

³All figures are means of 3 replicates ± SE. Feed conversion ratio (FCR) was calculated using the following equation: $FCR = \text{feed intake (g)} / \text{BW gain (g)}$.

< 0.001) BW at 21 d of age compared with N-CTL and other treatments. However, dietary inclusion of virginiamycin, *A. decurrens*, LMO, and α-tops did not result in greater BW, BWG, and FCR in comparison with the N-CTR group at d 21 of age ($P > 0.05$). There were no differences ($P > 0.05$) in final BW (49 d of age) and BWG (Table 3) with the exception of *E. glabra*, which was no longer part of the experiment because of the side effects. In general, birds fed the N-CTL diet had the highest BW, although there were no differences ($P > 0.05$) detected between individual groups. The dietary addition of LMO in concentrations of 200 mg/kg feed resulted in the lowest BW (Table 3) compared with the other groups, but the differences were not significant ($P > 0.05$). In addition, there was a big BW variation among chickens in the LMO-supplemented group.

Numbers of *C. jejuni* and Total Bacteria in Fecal and Cecal Samples

Campylobacter jejuni population in feces and cecal contents of chicken detected by culture and RT-qPCR methods are presented in Figure 1. The concentrations of *C. jejuni* obtained by RT-qPCR among the treatments were in agreement with those obtained by the traditional culture method. Although the RT-qPCR method gave a higher mean value compared with the

traditional culture method, this variation occurred in all samples and did not change the overall results. There was no amplification signal from the fecal samples collected at 21 and 28 d of age, showing that these samples were either free of *C. jejuni* or the number was far less than 1×10^4 cfu/g (minimum detection limit). Seven days after contaminating the cages with poultry litter (at 35 d of age), *C. jejuni* was detected and enumerated in fecal samples by using RT-qPCR. However, no *C. jejuni* was detected from one of the cages in terpinen-4-ol supplemented group, showing that chickens in that cage were either not colonized or the number was below the detection limit as mentioned above. Inclusion of plant-derived agents into the diet showed a tendency to lower the *C. jejuni* population in the feces at d 35 ($P > 0.05$) compared with N-CTL and virginiamycin-treated groups (data not shown). During the rest of the study, there were no significant effects of *A. decurrens* and terpinen-4-ol on the *C. jejuni* counts. The RT-qPCR and traditional culture analysis showed that animals receiving α-tops tended to have less concentration of *C. jejuni* during the experimental period and this difference reached statistical significance on d 41 by culture method compared with N-CTL (Figure 1). In addition, the LMO-supplemented group had higher ($P < 0.05$) fecal concentrations of *C. jejuni* on d 45 by RT-qPCR method compared with N-CTL and α-tops. No signifi-

Table 3. Effect of plant-derived agents on growth performance of broilers fed finisher diet (between d 21 and 49)

Treatment ¹	BW ^{2,3} (g)	BW gain ³ (g)	Feed intake ⁴ (g)	FCR ⁴
N-CTL (n = 11)	3,541 ± 41 ^a	2,534 ± 40 ^a	4,796 ± 44	1.90 ± 0.02
Virginiamycin (n = 11)	3,424 ± 87 ^a	2,444 ± 87 ^a	4,131 ± 99	1.70 ± 0.06
<i>Acacia decurrens</i> (n = 12)	3,537 ± 92 ^a	2,517 ± 90 ^a	4,335 ± 133	1.73 ± 0.07
α-Tops (n = 11)	3,519 ± 79 ^a	2,480 ± 77 ^a	4,652 ± 190	1.87 ± 0.10
LMO (n = 10)	3,369 ± 121 ^a	2,324 ± 115 ^a	5,259 ± 133	2.08 ± 0.16
Terpinen-4-ol (n = 11)	3,476 ± 93 ^a	2,468 ± 86 ^a	4,739 ± 214	1.85 ± 0.11

^{a,b}Means with different superscripts in the same column for BW and BWG differ significantly (Tukey, $P < 0.05$).

¹N-CTL (negative control with cyclodextrine) and LMO (lemon myrtle oil).

²Final BW.

³All figures are mean ± SE (n).

⁴All figures are mean of 3 replicates ± SE. FCR = feed conversion ratio.

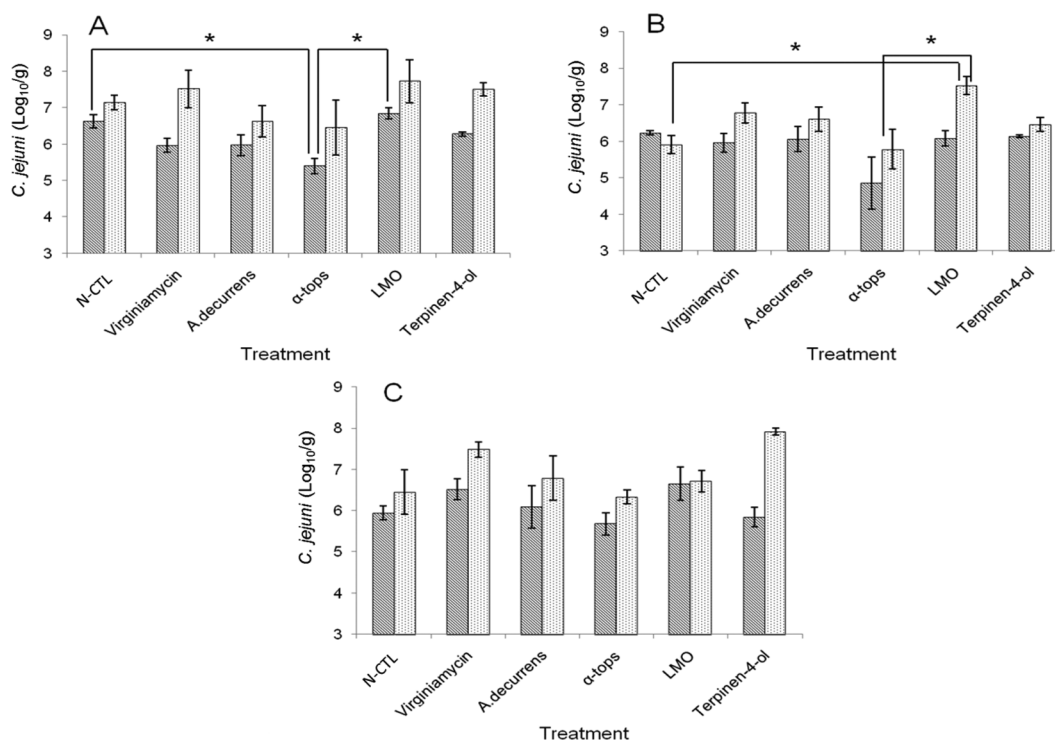


Figure 1. *Campylobacter jejuni* counts in fecal and cecal samples (*n* = 3) measured by culture and quantitative PCR. Hatched bars represent the real-time quantitative PCR results (log₁₀ copies per g of sample), and dark bars represent the culture-based results (log₁₀ cfu/g) for fecal samples [d 41 (A) and d 45 (B)] and cecal samples (C). Data are expressed as means ± SE. *Bars with an asterisk correspond to values that are significantly different (Tukey, *P* < 0.05). N-CTL (negative control with cyclodextrine) and LMO (lemon myrtle oil).

cant differences in the populations of *C. jejuni* (*P* > 0.05) related to experimental diets were found in cecal contents.

Additionally, the total microbial population was quantified using RT-qPCR and there were no differences noted among the treatments and between the treatments and the CTL groups. The gene copy number for total bacteria ranged from 1.52×10^{11} to 8.14×10^{11} per g of fecal samples and from 3.32×10^{11} to 1.67×10^{12} per g of cecal samples.

Bacterial Profile of the Cecal Contents of Chicken

Visual examination of the DGGE gel showed similarity between treatments with few exceptions. Two bands (A and B, Figure 2) were selected on the basis that they were absent in those groups fed a diet containing α-tops and *A. decurrens* while present in all other treatment groups. In addition, one more band (C, Figure 2) on the gel was also not present in samples obtained from α-tops treated group. Eight clones obtained from band A were assigned to 2 families in which 7 clone sequences affiliated to the family of *Flavobacteriaceae* with 37 to 70% homology and only one clone affiliated to the family of *Rikenellaceae* with 100% homology. Five out of 8 clones from band B gave good sequences for identification, and these were spread over 4 families including *Synergistaceae* (40% homologous), *Lachnospiraceae* (90% homologous), *Rikenellaceae* (99% homologous), and *Flavobacteriaceae* (32–33% homologous).

Furthermore, 5 clones from band C were affiliated with family *Lachnospiraceae* with 82 to 99% homology, 2 affiliated with family *Porphyromonadaceae* with 50 to 52% homology, and 1 clone was slightly similar (25% homologous) to the *Flammeovirgaceae* family.

VFA Concentration

Total VFA concentration was not influenced (*P* > 0.05) by treatments (Table 4). In the cecal contents of chicken in present study, acetate was the main VFA, followed by propionate and butyrate, and the concentrations of these were found to be consistent among the treatment groups.

Toxicology and Postmortem Findings

During the trial, 14 of the 84 birds died suddenly without showing any clinical signs. Eight of the mortalities occurred in the group fed a diet containing *E. glabra* with the remaining mortalities occurring in groups not associated with any one particular treatment. Because 7 chickens died within a short period of time (between d 28 to 32) in the *E. glabra* supplemented group, blood samples were collected from the remaining 4 chickens and evaluated for toxicological analysis. The remaining 4 birds in the *E. glabra* group remained clinically normal and were fed with BD only and survived to the end of the study.

Table 4. Effect of plant-derived agents on cecal volatile fatty acid (VFA) concentrations¹

Item	Treatment ²						SEM
	N-CTL (n = 11)	Virginiamycin (n = 11)	<i>Acacia decurrens</i> (n = 12)	α -tops (n = 11)	LMO (n = 10)	Terpinen-4-ol (n = 11)	
Total VFA (mM)	89.5	97.7	93.6	83.3	83.0	78.6	8.84
Acetate (mM)	58.1	63.6	59.9	52.8	51.5	50.3	5.72
Propionate (mM)	12.9	14.7	14.5	12.3	13.8	11.8	2.29
n-Butyrate (mM)	11.8	13.2	12.7	12.5	10.6	10.8	1.74
Iso-butyrate (mM)	1.5	1.2	1.1	1.1	1.6	1.1	0.27
Iso-valerate (mM)	1.5	1.1	1.2	1.0	1.8	1.1	0.32
n-Valerate (mM)	3.7	3.9	4.3	3.5	3.7	3.5	0.60

¹Values shown are means and were not statistically different (Tukey, $P > 0.05$).

²N-CTL (negative control with cyclodextrine) and LMO (lemon myrtle oil).

Laboratory results for the clinical chemistry parameters showed that there were no differences between the mean values (Table 5). Postmortem examination revealed internal bleeding in the abdominal cavity. Despite the internal bleeding, no pathological abnormalities were found on the organs.

DISCUSSION

There is a growing interest in investigating the beneficial effects of plant-derived agents in controlling diseases and improving animal performance. Evidence from in vitro assays suggests beneficial antimicrobial activities, but there are limited data that show similar action in vivo. We have previously reported the poten-

tial in vitro antimicrobial activity of Australian plant extracts toward *C. jejuni* (Kurekci et al., 2012). This study was undertaken to investigate if the in vitro activities can be confirmed in vivo and if the extracts have an effect on broiler performance.

The results reported herein showed that the inclusion of plant extracts and compounds did not result in BW improvement at concentration used in the starter and finisher diets. However, Cross et al. (2007) and Christaki et al. (2004) showed an increase in BW and better feed conversion ratio in broilers supplemented with herbal extracts. In agreement with these results, dietary EO were also reported to improve growth performance in broilers (Alçiçek et al., 2003; Tiihonen et al., 2010) and in pigs (Cho et al., 2006), although, in

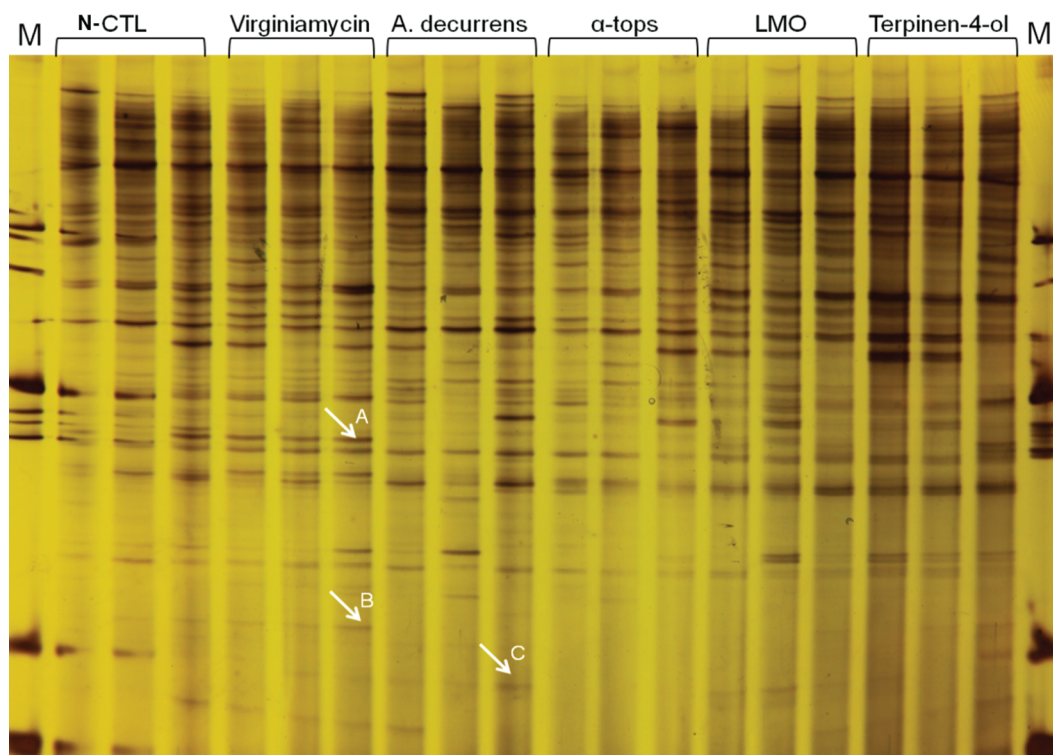


Figure 2. Denaturing gradient gel electrophoresis band pattern of chicken ceca. M: marker prepared from a mixture of pure culture of rumen bacteria. Triplicate samples for each treatment group, which are indicated above the gel. Arrows indicate bands (A, B, and C) that were excised from the gel and sequenced. N-CTL (negative control with cyclodextrine) and LMO (lemon myrtle oil). Color version available in the online PDF.

Table 5. Some biochemical parameters of blood from control and *Eremophila glabra* birds¹

Value	N-CTL ²		<i>E. glabra</i>	
	Mean	Range	Mean	Range
Sodium (mmol/L)	147	146–148	150	148–154
Potassium (mmol/L)	6.27	5.9–7.1	7.2	611–8.4
Chloride (mmol/L)	107	105–110	108	106–109
Calcium (mmol/L)	2.47	2.42–2.52	2.59	2.5–2.71
Aspartate aminotransferase (IU/L at 37°C)	214	186–234	258	220–250
Alanine aminotransferase (IU/L at 37°C)	3.75	2–5	3.25	2–4
Creatine phosphokinase (IU/L at 37°C)	3,669	2,538–4,785	2,818	2,061–4,232
Amylase (U/L at 37°C)	554	422–711	468	362–634
Protein (g/L)	29	23–33	28	26–31
Albumin (g/L)	11.8	9–13	11	11–12
Total bilirubin (μmol/L)	0.6	0–1	1.55	1.1–2
Glucose (mmol/L)	14.85	12.5–16.9	15.3	14–16.7
Uric acid (μmol/L)	313	264–362	269	212–355

¹Values represent the mean of 4 birds; no differences were found (Tukey, $P > 0.05$) between the 2 groups.

²N-CTL (negative control with cyclodextrine).

other reports (Botsoglou et al., 2002; Lee et al., 2003), the simultaneous administration of the blend of EO to poultry failed to improve growth performance. The possible mechanism of EO on growth performance could be attributed to the increased digestibility of feed by stimulation of endogenous enzymes and the regulation of the gut microbial flora (Lee et al., 2003; Jang et al., 2007).

Antimicrobial activities of plant extracts and EO were reported in a series of animal experiments in which fecal and cecal population of pathogens (*C. perfringens* and *E. coli*) were reduced when animals were fed a diet including a plant-derived agent. According to Siragusa et al. (2008), adding extracts of *Humulus lupulus* into drinking water decreased the number of *C. perfringens* in the broiler intestine. A blend of EO containing carvacrol and thymol appeared to be capable of decreasing the shedding of *E. coli* and *C. perfringens* in broilers (Jamroz et al., 2003; Mitsch et al., 2004; Jang et al., 2007). However, the plant-derived agents used in the present study, with the possible exception of α -tops, failed to show an inhibitory effect against the colonization of *C. jejuni* in poultry. There was also no significant decrease achieved in the cecal population of *C. jejuni* by the dietary supplementation of these agents. These results support other work (Hermans et al., 2011) that showed that dietary supplementation with a plant-derived antimicrobial compound, trans-cinnamaldehyde, had no protective effect on chicks infected with *C. jejuni*. However, it is noteworthy that the supplementation of α -tops in the feed tended to decrease the number of *C. jejuni* in all fecal and cecal samples. Although the mechanism by which dietary supplements may have reduced *C. jejuni* remains unclear at present, this study indicates that a blend of components (α -tops containing cineole, terpinen-4-ol, and terpinolene) is more effective than terpinen-4-ol alone in reducing *C. jejuni* population. Many factors that might influence the effectiveness of dietary supplementation of plant extracts such as dose of extracts in the mucus layer in the intestine as mentioned by

Hermans et al. (2011). The results obtained from the present study suggested that supplementation of plant-derived compounds (α -tops) in the diet to reduce *C. jejuni* population in chickens was more effective than other dietary strategy such as fermented liquid feed and acidified feed (Heres et al., 2003, 2004). Based on these findings, more work is needed to determine whether there is an effective dose of α -tops to control *C. jejuni* without a detrimental effect on the birds. Future bird experiments involving plant extracts must also ensure the solubility of additive in the mucus layer as *C. jejuni* colonizes in the mucus layers of the cecum of broiler chicken (Newell and Fearnley, 2003).

Chicken ceca harbors a range of microorganisms that have been proven to have a strong impact on the performance and health of the broiler chicken (Apajalahti et al., 2001). Therefore, the aim should be to create a favorable environment to maintain the integrity of gut microflora, while decreasing the pathogenic bacteria by the use of selective antimicrobial agents. The use of DGGE technique in this study has revealed some changes in bacterial profile of cecal contents of birds on α -tops and *A. decurrens* treatments. It was found herein that the family *Lachnospiraceae* was affected by α -tops. However, there are no data available on beneficial or detrimental effects of this bacterial group on chicken health or performance. Moreover, there were no differences observed in the concentration of VFA production in chicken ceca, suggesting that disappearance of this group of bacteria by the dietary treatment did not adversely affect cecal fermentation.

There was no evidence of adverse effects on the health and performance of broiler chickens when *A. decurrens*, LMO, α -tops, terpinen-4-ol, and cyclodextrin were included in the diet. However, inclusion of *E. glabra* extract in the diet had a negative impact on the growth of birds. In addition, inclusion of the same extracts at a higher rate (1 g/kg of diet) resulted in a 63.6% mortality rate in 10 d. There are no reports in the literature about toxic effects of *E. glabra* or its compounds in animals. Moreover, Wilson et al. (1975)

reported that the leaves of *E. glabra* in nature are eaten largely by goats, though not consumed by sheep. A review of Australian *Eremophila* spp. by Richmond and Ghisalberthi (1994) stated that many *Eremophila* spp. are considered as a fodder species for both sheep and cattle in Australia, though some poisonous effects are reported for several *Eremophila* species. Hutton et al. (2010) administered freeze-dried leaf material of *E. glabra* to sheep to prevent lactic acidosis subsequent to in vitro experiments, but no adverse effects were reported on this species. The same group reported that *E. glabra* contains mainly serrulatane diterpenes (Hutton et al., 2010). However, there is also no study reporting the adverse effects of these compounds. Results obtained from our study could be attributed to species-related differences in gut microflora, which could detoxify some of the plant toxic compounds (Reed, 1995). Toxicity of different herbal extracts in poultry was noted previously (Arshad et al., 2008; Naidoo et al., 2008). For example, blood biochemical alterations and reduced BW were noted by Arshad et al. (2008) when poultry were fed a ration containing *Paganum harmala* extract (150 mg/kg of BW) over 6-wk period. However, there was no significant elevation in blood biochemicals to indicate toxicity compared with the control group. High mortality might have also been resulted from the increased susceptibility of chickens to infections as chickens were found to be significantly smaller in that group. However, there is no evidence to support this hypothesis. Additionally, there was no postmortem alteration observed in the organs of birds that died as a result of feeding *E. glabra*. The mechanism by which chickens died warrants further investigation.

This is the first report on the adverse effects of feeding *E. glabra* extracts to poultry, which had reduced performance and high mortality. Although α -tops decreased the number of *C. jejuni*, the other treatments did not result in a decrease in *C. jejuni*. There are minor population differences created as a result of dietary inclusion of α -tops and *A. decurrens* in the diet, even though no changes in total VFA concentration or total bacterial counts were observed among treatments. Thus, further research is warranted to establish the potential beneficial effect of α -tops at increased concentrations.

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