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Effect of supplementing commercial broiler diets with a combination of natural thiosulfinate, cinnamaldehyde, and naphthoquinones on gut health

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Abstract
An experiment was conducted for a period of 42 days to study the effect of supplementing herbal extract combination of natural thiosulfinate, cinnamaldehyde and naphthoquinones (TCN) on gut health of broiler chickens. A total of 200, day-old commercial broiler chicks of uniform weight were selected and grouped into four treatments *viz.*, T_1 (Control- basal diet), T_2 (basal diet with standard antibiotic - 250mg/kg of feed), T_3 (basal diet with combination of natural thiosulfinate, cinnamaldehyde and naphthoquinones - [TCN] supplemented at 250 mg/kg diet) and T_4 (basal diet with TCN supplemented at 500 mg/kg diet). Diets and supplements were analyzed for nutrient composition as per AOAC (2005) method. Birds were reared on deep litter system under standard managemental practices. The results indicated that the gut microbial count (log_{10} CFU/g) of experimental birds fed with combination of natural thiosulfinate, cinnamaldehyde and naphthoquinones at 250 mg/kg (T_3) and 500 mg/kg (T_4) diet revealed significantly ($P \leq 0.05$) decreased *Escherichia coli* count compared with control group (T_1) whereas the gut microbial count was similar with antibiotic supplemented group (T_2). Herbal extract combinations at 500 mg/kg (T_4) revealed significantly ($P \leq 0.05$) increased *Lactobacillus spp.* count compared to control group (T_1), whereas similar with groups T_2 and T_3. While higher L*actobacillus spp*. counts were recorded in group supplemented with herbal extracts combination at 500 mg/kg diet (T_4). Based on the results of the experiment it was concluded that the supplementing herbal extracts combination of natural thiosulfinate, cinnamaldehyde and naphthoquinones {TCN} at 500 mg/kg broiler diets was beneficial with respect to gut health in broilers.

Keywords: Thiosulfinate, cinnamaldehyde, naphthoquinones, gut microbial count, broilers

Introduction
Poultry plays a vital role in enhancing food security and nutrition by supplying high-quality protein, energy, and essential micronutrients. Its short production cycles and ability to convert diverse agri-food by-products and waste into nutritious meat and eggs make it an efficient and sustainable food source. As the fastest-growing agricultural sub-sector particularly in developing nations poultry is poised for continued expansion. This growth is fueled by increasing global populations, rising income levels, and accelerating urbanization, all of which drive demand for affordable animal protein for human consumption.

In poultry production use of antibiotics at sub-therapeutic levels for growth promotion and disease prevention (antibiotic growth promoter, AGP) improved feed-to-weight ratio, meat quality and overall health of livestock. These benefits encouraged the heavy use of AGPs such that about 70% of global use of antibiotics was for food animals. Despite the numerous benefits of AGPs, the emergence of antibiotic resistance associated with their use on livestock health may affect animal and the environment. This scenario compelled the researchers to use other alternatives like herbs and their extracts as feed additives in poultry production (Gou *et al*., 2000) [11].

Phytogenic feed additives (PFA) are usually safe natural plant derivatives from herbs, spices, essential oils and oleoresins are drawing much attention because of ban on antibiotics in poultry industry. PFA helps to increase performance of birds by stimulating secretion of digestive enzymes, leading to enhanced digestion and absorption. The presence of active ingredients and phenolic compounds can reduce number of intestinal pathogens thus,

minimizing nutrient loss and improving performance and these effects may result in better gut health and may lead to more protein deposition in tissues (Geier and Oster, 2001) [10].

Garlic (*Allium sativum*) is a vegetable plant, a bulb belonging to the family *Liliaceae.* Garlic has anti-bacterial properties (Bayan *et al*., 2014), which is widely attributed to its major bioactive compound thiosulfinates (allicin) and the development of resistance to beta-lactam antibiotics is 100-fold easier than development of resistance to these thiosulfinates (Ariga and Seki, 2006) [1].

Dietary supplementation of garlic derived product propyl propane thiosulfinate (PTS-O) on pathogens in broilers fed at 45 and 90 mg/ kg diet, showed that, the inclusion of higher level of 90 mg of PTS- O/kg of diet had significantly ($P \leq 0.01$) lowered *Escherichia coli* numbers in the ileal and cecal contents (Peinado *et al*., 2012) [19]. The lycopene and allicin each added at 100, 200 and 300 mg/kg diet showed significantly ($P \leq 0.05$) decreased intestinal counts of bacterial and total Coliform compared with control group (Zweil *et al*., 2016) [32].

Cinnamon (*Cinnamomum verum*) belongs to the family *Lauraceae* consists of a variety of resinous bioactive compounds including cinnamaldehyde, cinnamate, cinnamic acid and other numerous essential oils (Senanayake *et al*., 1978) [26]. Studies have shown that cinnamon possesses antibacterial, anti-diabetic and anti-inflammatory properties (Jakhetia *et al*., 2010) [13]. Cinnamaldehyde has been reported to exhibit antimicrobial properties against laboratory media, animal feeds and human foods contaminated with disease-causing bacteria including *Bacillus cereus, Campylobacter jejuni, Clostridium perfringens, Escherichia coli, Staphylococcus aureus, Staphylococcus epidermis, Listeria monocytogenes* and *Salmonella enterica* (Chang *et al*., 2001; Fridman, 2017) [4, 8].

Effect of dietary cinnamon oil (CO) in broilers fed at 500, 1000 and 1500 mg/kg diet (cinnamaldehyde at 60-75%) indicated significantly ($P \leq 0.05$) decreased *Escherichia coli* and caecal total microbial count in chickens compared to control group whereas the count of *Lactobacilli spp*. was significantly ($P \leq 0.05$) increased in the caecum of chickens fed cinnamon oil in comparison with control group but both the counts were similar to antibiotic-treated group (Saied *et al*., 2022) [24].

Lawsonia inermis belongs to family Lythraceae commonly known as 'Henna', is a shrub grown in dry tropical and subtropical zones. Lawsone also called naphthoquinone is the main active compound present in the henna extract about 0.5-2% and possess biomedical uses due to its high cytotoxic activities against disease-causing bacteria, fungi and protozoa (Semwal *et al*., 2014) [25]. The henna extract had antimicrobial activity against both gram positive such as *Staphylococcus aureus*, *Bacillus subtilis* and *Staphylococcus epidermidis* and gram negative such as *Escherichia coli* and *Klebsiella* species indicates great significance as substitute antimicrobial agent in therapeutics (Gull *et al*., 2013) [12].

Research conducted by Kiavandani *et al*. (2021) [15] to evaluate the effect of *Lawsonia inermis* (henna) in broilers with 5 treatments supplemented with control diet, flavophospholipol, henna at 0.15%, 0.20% and 0.25% respectively, showed significantly ($P \leq 0.05$) reduced total aerobic, *Escherichia coli* count and significantly ($P \leq 0.05$) increased *Lactobacilli spp*. count than broilers fed with the control diet and flavophospholipol groups.

In broilers, data on effect of supplementing garlic, cinnamon and henna on gut health are available whereas the effect of extracts of these herbs like natural thiosulfinate, cinnamaldehyde and naphthoquinones (TCN) and their combination are not available, hence the present study was undertaken with the objective to study the effect of supplementing combination of natural thiosulfinate, cinnamaldehyde and naphthoquinones as growth promoter on gut health in broilers.

Material and Methods

A biological experiment was conducted to study the effect of supplementing combination of natural thiosulfinate, cinnamaldehyde and naphthoquinones (TCN) as growth promoter on gut health in broilers up to six weeks of age.

Procurement of chicks: A total of two hundred, day-old commercial broiler chicks of uniform body weight were procured from the Venkateshwara hatcheries private limited, Bengaluru. The chicks were wing banded, weighed and allocated randomly to four experimental groups each consisting of five replicates of ten chicks each.

Procurement of feed ingredients and herbal extract feed supplements: Feed ingredients required for the formulation of the experimental diet were procured from the reliable source. The herbal extract feed supplements used in the trial were a combination of natural thiosulfinate, cinnamaldehyde and naphthoquinones procured from Quadragen Vet Health Private Limited, Bengaluru. The herbal extract feed supplements were extracted by accelerated natural biotransformation (ANBiot) technology contained thiosulfinates -70%, cinnamaldehydes -15% and naphthoquinones -15%, respectively. The extraction and analysis of herbal extract feed supplements combination was carried out at Chemlife Innovations Private Limited, Bengaluru.

Description of experimental design and diets: The broiler chicks were randomly assigned to one of the four dietary treatments: T_1 (control group) - basal diet without growth promoters, T_2 - basal diet with antibiotic growth promoter (Zinc bacitracin) incorporated at 250 mg/kg in broiler diet, T_3 - basal diet added with herbal extracts combination of natural thiosulfinate, cinnamaldehyde and naphthoquinones (TCN) at 250 mg/kg in broiler diet and T_4 - basal diet added with herbal extracts combination of natural thiosulfinate, cinnamaldehyde and naphthoquinones (TCN) at 500 mg/kg in broiler diet.

Group	Description of the treatment	No. of replicates	Birds per replica	Total
T_1	Basal diet without growth promoter	05	10	50
T_2	Basal diet added with antibiotic growth promoter (AGP) supplemented at 250 mg/kg broiler diet	05	10	50
T_3	Basal diet added with herbal extracts combination of natural thiosulfinate, cinnamaldehyde and naphthoquinones at 250 mg/kg broiler diet	05	10	50
T_4	Basal diet added with herbal extracts combination of natural thiosulfinate, cinnamaldehyde and naphthoquinones at 500 mg/kg broiler diet	05	10	50
Total				200

Formulation of experimental diet: A standard pre-starter, starter and finisher rations of experimental diets were formulated as per BIS (2007) [3] specifications and the basal diet was prepared which served as control group

Ingredients	Pre-starter (1 - 7 days)	Starter (8 - 21 days)	Finisher (22 - 42 days)
Yellow maize	53.10	56.00	59.50
Soya bean meal	40.50	36.70	32.50
Vegetable oil	2.50	3.45	4.30
Dicalcium phosphate	1.50	1.50	1.50
Common salt	0.35	0.30	0.30
Mineral mixture*	1.30	1.30	1.30
Vitamin premix	0.20	0.20	0.15
B complex	0.10	0.10	0.10
Trace minerals	0.05	0.05	0.05
DL-Methionine	0.30	0.30	0.30
Toxin binder	0.10	0.10	0.10
Total	100.0	100.0	100.0
Nutrient composition			
ME (Kcal/kg) [a]	3023.00	3091.00	3163.00
Crude protein (%) [b]	22.45	21.52	19.76
Calcium (%) [a]	1.01	1.01	1.01
Phosphorous (%) [a]	0.47	0.47	0.47
Lysine (%) [a]	1.21	1.24	1.09
Methionine (%) [a]	0.55	0.50	0.53

* Mineral mixture: Each 100 g contains Magnesium oxide - 1.48 g, Ferrous sulphate - 6.0 g, copper sulphate - 0.05 g, Manganese Sulphate - 0.04 g, Potassium Iodide - 0.001 g, Potassium Chloride - 17.09 g and Sodium selenite - 0.001 g.
[a] calculated value; [b] analyzed values

Vaccination regime: The chicks on 7th day were vaccinated with ND with B_1 strain followed by booster dose on 21st day through ocular or nasal route. On 14th day chicks were vaccinated against IBD intermediate vaccine followed by booster dose on 28th day through ocular route.

Gut microbial count: At the end of the experiment, two birds from each replicate in T_1 to T_4 treatment groups were slaughtered. Microbial counts of gut were assessed for *Escherichia coli* and *Lactobacillus spp.* count. The intestinal contents from caecum were collected aseptically in sterile container and immediately subjected to enumeration of gut microbes as per spread plate method (Postgate, 1969) [20]. Specific media such as MacConkey agar was used for *Escherichia coli* count, whereas *Lactobacillus* was assessed on MRS (de Man, Rogosa and Sharpe) agar by pour plate method (Mackie and Mccartney, 1996) [16]. Here ten folds serial dilution of intestinal contents was used to assess the bacterial count. The bacterial counts were expressed as $\log_{10}$ colony forming units (CFU)/gram of the sample from intestinal content (Weir, 1990) [30].

The data pertaining to various parameters of the trial were subjected to statistical analysis by one way analysis of variance (ANOVA) using SPSS 16 statistical software (Snedecor and Cochran, 1994) [28]. The statistical analysis was done at 95 % level of significance ($P \leq 0.05$). The significant mean differences among the treatment groups were determined using Tukey's multiple comparison test.

Result and Discussion

The findings of the groups supplemented with the combination of natural thiosulfinate, cinnamaldehyde and naphthoquinones at 250 mg/kg (T_3) and 500 mg/kg (T_4) in diets revealed significantly ($P \leq 0.05$) decreased *Escherichia coli* count compared with control group (T_1) whereas microbial counts were similar with antibiotic supplemented group (T_2). Supplementing combinations at 500 mg/kg (T_4) revealed significantly ($P \leq 0.05$) increased *Lactobacillus spp.* count compared to control group (T_1), whereas similar with groups T_2 and T_3. While higher L*actobacillus spp*. counts were recorded in group supplemented with herbal extracts combination at 500 mg/kg diet (T_4) presented in Table 1 and depicted in figure 1.

The findings of gut microbial count of experimental birds were in accordance with the Peinado *et al*. (2012) [19] who supplemented derived propyl propane thiosulfinates in broilers at 45 and 90 mg/kg in diets revealed significantly ($P \leq 0.01$) lower *Escherichia coli* count in the cecal contents. Similarly, Zweil *et al*. (2016) [32] studied effect of lycopene and allicin on broilers each fed at 100, 200 and 300 mg/kg diets found significantly ($P \leq 0.05$) decreased total aerobic, anaerobic bacterial and coliform count in allicin supplemented group compared with control, lycopene and antibiotic fed group and here the greatest reduction of bacterial count was observed in the intestinal chicks fed 300 mg/kg allicin in diet. Garlic derivatives allicin (thiosulfinate) exhibited a wide spectrum of antibacterial activity against pathogenic bacteria's *viz*. *Escherichia* spp., *Salmonella* species and *Clostridium* spp. due to their chemical reaction with thiol groups of various enzymes such as the acetyl-CoA-forming system consisting of acetate kinase and phosphotransacetylase-CoA synthetase (Focke *et al*., 1990; Ross *et al*., 2001) [7, 22].

The findings of gut microbial count of experimental birds were in agreement with Saied *et al*. (2022) [24] who supplemented dietary cinnamon oil at 500, 1000 and 1500 mg/kg (cinnamaldehyde at 60-75%) in broiler diets revealed significantly ($P \leq 0.05$) decreased *Escherichia coli* count and significantly ($P \leq 0.05$) increased *Lactobacilli spp.* count in the caecum compared to control group but similar to antibiotic-treated group. Similarly, Yang *et al*. (2019) [31] studied effect of feeding cinnamon essential oil in broilers diet at 100 mg/kg showed significantly ($P \leq 0.05$) increased the *Lactobacillus* species count and significantly ($P \leq 0.001$) decreased *Escherichia coli* count in caecum. Cinnamaldehyde reduced the *Escherichia coli* counts in the caecum, possibly due to the capacity of cinnamaldehyde to disrupt the bacterial cell membranes. Additionally, the

cinnamaldehyde stimulates mucus release in the intestinal tract, which in turn reduces the adhesion of pathogens to the epithelial cells in the gut (Jamroz *et al*., 2006) [14]. Dietary cinnamon oil increased the growth of *Lactobacillus spp.* while inhibiting *Campylobacter spp.* and *Escherichia coli* in the ileum and cecum of poultry here the production of SCFAs is attributed towards the better *Lactobacillus spp.* fermentation which are responsible for the maintenance of gut ecosystem and preventing the pH-sensitive pathogenic bacteria (Tabak *et al*., 1999) [29].

The findings of gut microbial count of experimental birds were in corroboration with the Kiavandani *et al*. (2021) [15] who studied effect of *Lawsonia inermis* (henna) on broilers fed at 0.15%, 0.20% and 0.25% diets found significantly ($P \leq 0.05$) lower total aerobic count and *Escherichia coli* whereas significantly ($P \leq 0.05$) higher *Lactobacilli spp.* count than broilers fed with control diet and flavophospholipol (AGP) supplemented groups. The reason could be *Lawsonia inermis* (henna) extract in *in- vitro* study reported antimicrobial activity against gram negative such as *Escherichia coli* and *Klebsiella species* indicates great significance as substitute antimicrobial agent in therapeutics (Gull *et al*., 2013) [12]. Similar results on better microbial count reported by Dieumou *et al*. (2009) [6]; Shams *et al*. (2012) [12]; Ruiz *et al*. (2015) [23] and Manafi *et al*. (2016) [17].

The results of above experiment on gut microbial count were in contrary with studies conducted by Pathak *et al*. (2017) [18] who supplemented combination of cinnamaldehyde and calcium formate at 500 mg/kg in broiler diets reported no effect against *Escherichia coli* and *Lactobacillus spp*. count. Similarly, Chowdhury *et al*. (2018) [5] supplemented cinnamon oil at 300 mg/kg in broiler diets reported no significant ($P>0.05$) effect against *Lactobacillus spp*. count.

Conclusion

Based on results of the experiment, it was concluded that the inclusion of natural thiosulfinate, cinnamaldehyde and naphthoquinones combination at 500 mg/kg diet had positive effect on caecal microbial composition by lowering pathogenic bacteria such as *Escherichia coli* spp. and increasing the beneficial bacteria *Lactobacilli spp*. count compared to control group.

Table 1: Effect of supplementing combination of natural thiosulfinate, cinnamaldehyde and naphthoquinones at 250 and 500 mg/kg diets on gut microbial count (log10 CFU/g) (Mean ± SE) in commercial broilers.

Experimental group	Description of the treatment	*E. coli* count	*Lactobacillus* count
T_1	Control group	6.907 ^{a} ± 0.16	8.105 ^{b} ±0.16
T_2	Antibiotics (250mg/kg diet)	6.191 ^{b} ± 0.15	8.350 ^{ab} ± 0.20
T_3	Herbal extracts combination (250mg/kg diet)	6.351 ^{b} ± 0.13	8.314 ^{ab} ± 0.09
T_4	Herbal extracts combination (500mg/kg diet)	6.307 ^{b} ± 0.10	8.728 ^{a} ± 0.14

$^{a, b}$ Means in the same column with no common superscript differ significantly ($P \leq 0.05$)

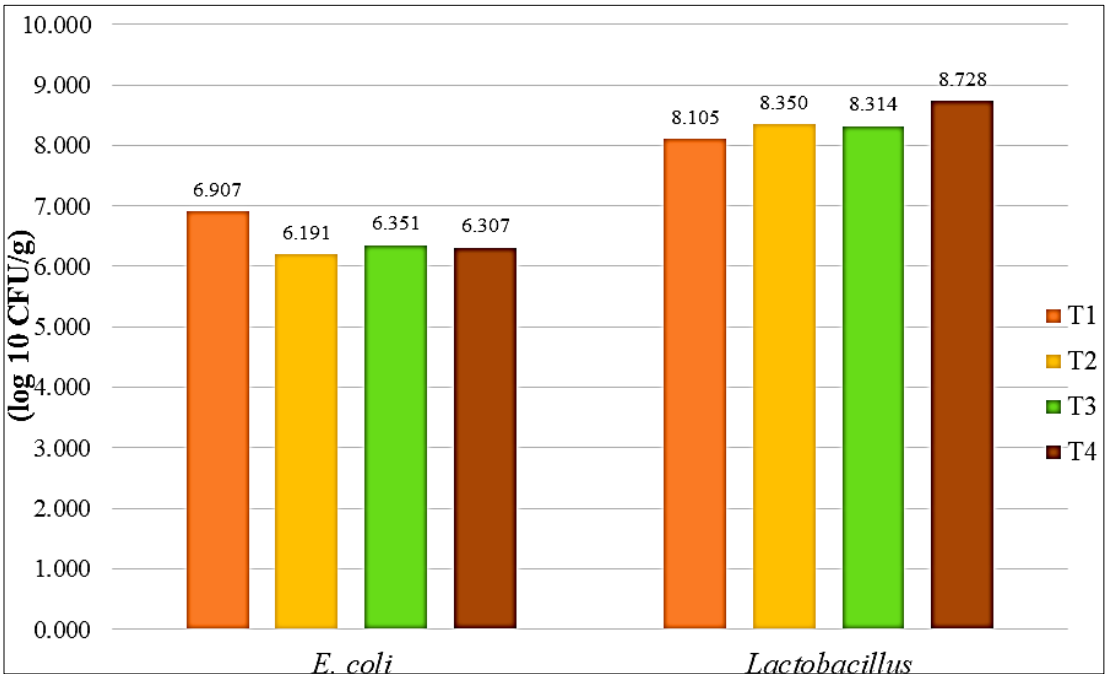

Fig 1: Effect of supplementing combination of natural thiosulfinate, cinnamaldehyde and naphthoquinones at 250 and 500 mg /kg diets on gut microbial count (log10 CFU/g) in commercial broilers

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