



## Individual and combined effects of dietary supplementation of probiotic and essential oil on the growth performance, immunity status, gut health and jejunal histomorphology of broiler chickens

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Received: 10 April 2022; Accepted: 10 July 2022

### ABSTRACT

The present study was conducted to evaluate the individual and combined effects of probiotic and essential oils on the performance of broilers. Day-old broiler chicks (240) were allocated to 5 groups in completely randomized design to give 6 replications per treatment group with 8 birds in each replicate. The birds of different groups were supplemented with antibiotic growth promoter (CTC 15%) at the level of 335 mg/kg ( $T_1$ ), essential oil formula (TCLVC 1.5%) ( $T_2$ ), *L. fermentum* NKN51 ( $10^7$  cfu/g) along with MOS (0.3%) and sodium butyrate (0.03%) ( $T_3$ ), and combination of *L. fermentum* NKN51 ( $10^7$  cfu/g) and essential oil formula (TCLVC 1.5%) in presence of MOS (0.3%) and sodium butyrate (0.03%) ( $T_4$ ). The control group was provided basal diet only with no other supplementation. The birds were reared for a period of 6 weeks and examined for growth performance including weekly body weight, average daily gain, weekly FCR, average feed intake, cell mediated and humoral immunity, gut microbiology, jejunal histology and stress parameters including heterophil-lymphocyte ratio and serum corticosterone levels. The group supplemented with combination of probiotic and essential oil formula (TCLVC 1.5%) had significantly better growth performance, cell mediated and humoral immunity and improved jejunal histology in terms of villus height, villus width, VH: CD ratio and intestinal absorptive surface area. No significant difference was observed in stress levels of different groups in terms of heterophil-lymphocyte ratio and serum corticosterone levels. The study depicted that combined supplementation of probiotic and essential oil had affirmative response on performance parameters of broiler chickens.

**Keywords:** Broiler, Essential oil, *L. fermentum*, Probiotic

The validation of antibiotics was a revolution in ruling pathogenic infections and augmenting the efficiency of nutrient absorption (Engberg *et al.* 2000). Antibiotics are being used routinely to cure and prevent infections in humans and animals. Further, their use for non-therapeutic purposes such as growth promotion in intensive farming of food producing animals including poultry is now a well-known practice. However, in the last few years, enormous use of these compounds has resulted in intense complication of antibiotic resistance (Forgetta *et al.* 2012). The existence of antibiotic residues in feed and environment (Gonzalez Ronquillo and Angeles Hernandez 2017) adversely affects human and animal health (Ghosh *et al.* 2019). Further, emergence and dissemination of antibiotic resistance is a universal affair and the scenario is so threatening that there are no novel classes of antibiotics introduced since late 1980s.

Increased consumer's awareness and continuous concern of society and researchers against adverse outcome

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of antibiotic use has augmented the exploration for such alternatives (Diarra and Malouin 2014), that can be used in place of growth promoting antibiotics in food animals including poultry. Currently, the application of probiotics, GRAS (Generally Recognized As Safe) chemicals and essential oils has got the attention of researchers with the possibility of exhibiting similar beneficial effects through their natural possible ways thereby presenting themselves as promising alternatives. However, their weak antibacterial activity leads to the incorporation of higher doses in order to achieve an antibacterial effect, which renders their usage limited to specific conditions only (Silva and Domingues 2017). Hence, the objective of the present study was to evaluate the performance of broilers with the incorporation of probiotic and essential oil at accurate level in their basal diet.

### MATERIALS AND METHODS

Day-old broiler chicks (240; Caribro Vishal), procured from broiler farm, ICAR-Central Avian Research Institute, Uttar Pradesh, India were reared for a period of 6 weeks at divisional experimental farm, ICAR-CARI, Bareilly. The

birds were randomly allotted to five experimental groups in a completely randomized design (CRD). Each group consisted of 6 replicates of 8 birds, housed under deep litter system using rice husk as the bedding material provided with the floor space allocation of 1 square ft per bird. The study was conducted with the approval of Institutional Animal Ethics Committee (IAEA) (No. 452/01/ab/CPCSEA). A total of five treatment groups (i.e. T<sub>1</sub>, T<sub>2</sub>, T<sub>3</sub>, T<sub>4</sub> and control group) were made, where the birds of different treatment groups were supplemented with the pre-decided levels of probiotic and essential oils (Table 1). The basal diet offered was common for all the five groups as recommended by ICAR (2013). The used probiotic, *Lactobacillus fermentum* NKN51 and essential oil formula were procured from

Table 1. Formulation of the different treatment groups with varied levels of *L. fermentum* NKN51 and essential oils formula alone or in combination

| Treatment group | Diet formula                                                                                           |
|-----------------|--------------------------------------------------------------------------------------------------------|
| Control         | BD only                                                                                                |
| T <sub>1</sub>  | BD + AGP @ 335 mg/kg (CTC, 15%)                                                                        |
| T <sub>2</sub>  | BD + TCLVC (1.5×)                                                                                      |
| T <sub>3</sub>  | BD + <i>L. fermentum</i> @ 10 <sup>7</sup> cfu/g + MOS @ 0.3% + Sodium butyrate @ 0.03%                |
| T <sub>4</sub>  | BD + TCLVC (1.5×) + <i>L. fermentum</i> @ 10 <sup>7</sup> cfu/g + MOS @ 0.3% + Sodium butyrate @ 0.03% |

BD, Basal diet; AGP, Antibiotic growth promoter; CTC, chlortetracycline; TCLVC(1.5×), combination of thymol, carvacrol, linalool, vitamin C and copper sulphate in 1.5× concentration-pre-standardized by Indian Institute of Technology, Roorkee; MOS, mannan oligosaccharide.

Indian Institute of Technology, Roorkee. The essential oil formula's (TCLVC) 1.5× concentration was developed by combining the 5 ingredients including thymol, linalool, carvacrol, vitamin C and copper sulphate in a constant ratio, where 1.5× strength of the formula (Thymol - 15 mg/L, Carvacrol - 37.5 mg/L, Linalool - 60 mg/L, vitamin C - 225 mg/L, copper sulphate - 1.5 µM) was used for the present experiment.

The birds from each group were evaluated for growth performance in terms of weekly body weight, weekly feed conversion ratio, average feed intake and average daily gain. For immune performance, *in vivo* cell mediated immune response (CMI) to phyto-haemagglutinin type P (PHA-P) was studied as described by Cheng and Lamont (1988) on 28<sup>th</sup> day post-hatch (n=12). Further, the humoral immune response against 1% sheep RBC was studied by injecting the birds (n=12) with 1 ml of 1% sheep RBC suspension intravenously on 35<sup>th</sup> day post-hatch in broilers which was followed by collection of blood, 5 days post-immunization (Siegel and Gross 1980) and the HA test. Further, the gut health was examined through gut microbiology with the counting of total plate counts, *E. coli* counts and *Lactobacillus* counts. The colonies were counted for each of the respective plates and were expressed as log<sub>10</sub> value for colony forming units per gram

of sample (cfu/g) (APHA 2001). At the end of the trial, jejunal histomorphology was also done. The histological sections were observed under low power of microscope (10×) for measuring villus height, villus width, crypt depth and crypt width and villus height-crypt depth ratio with the help of Zeiss Primo star Software. Further, the mean absorptive surface area was also calculated with the help of the following formula (Kisielinski *et al.* 2002).

$$M = (\text{villus } W \times \text{villus } L) + (\text{villus } W/2 + \text{crypt } W/2)^2 - (\text{villus } W/2)^2 / (\text{villus } W/2 + \text{crypt } W/2)^2$$

where, M, intestinal absorptive surface area; villus W, mean width of villi; villus L, mean length of villi and crypt W, mean width of crypts.

Further, the birds were also examined for stress parameters by evaluating heterophil: lymphocyte ratio (Gross and Siegel 1983) and serum corticosterone level estimation with the help of ready to use ELISA kit from LDN<sup>R</sup> immunoassays and services. The data obtained in this study were analyzed with the use of SPSS software (Version 21; IBM SPSS 2012), where one-way analysis of variance was performed for all the parameters. The effects were determined significant at the level of P<0.05 and the separation of mean was done by Duncan multiple range test (Duncan 1955).

## RESULTS AND DISCUSSION

The groups fed with combination of essential oil formula (1.5×) and *L. fermentum* NKN51 (i.e. 10<sup>7</sup>cfu/gm) in presence of prebiotics and acidifier performed better in terms of body weight gain, feed conversion ratio, average daily gain and reduced feed intake up to six weeks of age (Table 2). Various previous studies have also documented that supplementation of essential oils in broiler feed resulted in improvement in the growth performance of the broilers in terms of body weight, feed conversion ratio, average daily gain, etc (Yang *et al.* 2018), which is attributable to the increase in the release of endogenous enzymes in response to the EOs supplementation and thereby leading to enhanced digestibility of nutrients and more gut passage of chickens (Lee *et al.* 2004). Further, probiotics are also well known for their positive effects on the growth performance parameters (Li *et al.* 2019). Hence, the synergistic effects of essential oils and probiotic bacteria would have contributed to the improved growth performance of the broiler birds as the essential oils have higher MIC against probiotics, thus it would have performed synergistically in combination when the essential oils, i.e. thymol, carvacrol and linalool were provided in combination at a very low concentration. The probiotics are well known to perform better in presence of prebiotics like MOS, imparting a substrate to the probiotic for fermentation resulting in their better survival and acidifiers which favours the probiotic growth by reducing the pH and thus under a positive environment, the combined effect of *L. fermentum* and essential oils formula was possibly found to be significantly better as compared to when, essential oils formula and *L. fermentum* were used alone.

Table 2. Individual and combined effects of probiotic and essential oil supplementation on weekly body weight, average feed intake and feed conversion ratio in broilers (Mean±SE)

| Group          | Body weight (g)             |                         |                         |
|----------------|-----------------------------|-------------------------|-------------------------|
|                | 0-2 week                    | 0-4 week                | 0-6 week                |
| T <sub>1</sub> | 261.6±3.8 <sup>c</sup>      | 882.2±3.5 <sup>d</sup>  | 1698.5±2.4 <sup>d</sup> |
| T <sub>2</sub> | 249.4±3.1 <sup>ab</sup>     | 847.0±3.6 <sup>b</sup>  | 1641.0±3.9 <sup>b</sup> |
| T <sub>3</sub> | 255.9±4.9 <sup>bc</sup>     | 868.6±3.4 <sup>c</sup>  | 1646.1±2.2 <sup>b</sup> |
| T <sub>4</sub> | 278.8±4.0 <sup>d</sup>      | 883.5±2.8 <sup>d</sup>  | 1687.7±2.4 <sup>c</sup> |
| Control        | 240.4±4.6 <sup>a</sup>      | 832.4±4.9 <sup>a</sup>  | 1629.9±5.8 <sup>a</sup> |
| P value        | 0.001                       | 0.001                   | 0.001                   |
| Group          | Average feed intake (g)     |                         |                         |
|                | 0-2 week                    | 0-4 week                | 0-6 week                |
| T <sub>1</sub> | 227.1±5.6 <sup>a</sup>      | 518.6±2.6 <sup>a</sup>  | 831.2±7.7 <sup>b</sup>  |
| T <sub>2</sub> | 219.1±2.6 <sup>a</sup>      | 520.2±13.3 <sup>a</sup> | 814.9±5.1 <sup>a</sup>  |
| T <sub>3</sub> | 222.3±6.3 <sup>a</sup>      | 518.6±8.6 <sup>a</sup>  | 812.6±3.5 <sup>a</sup>  |
| T <sub>4</sub> | 248.5±7.5 <sup>b</sup>      | 513.8±6.9 <sup>a</sup>  | 816.8±1.4 <sup>a</sup>  |
| Control        | 219.8±2.6 <sup>a</sup>      | 525.9±7.0 <sup>a</sup>  | 846.9±3.1 <sup>c</sup>  |
| P value        | 0.007                       | 0.893                   | 0.001                   |
| Group          | Feed conversion ratio (FCR) |                         |                         |
|                | 0-2 week                    | 0-4 week                | 0-6 week                |
| T <sub>1</sub> | 1.38±0.00 <sup>a</sup>      | 1.68±0.01 <sup>a</sup>  | 1.90±0.02 <sup>a</sup>  |
| T <sub>2</sub> | 1.41±0.02 <sup>a</sup>      | 1.73±0.02 <sup>ab</sup> | 1.97±0.01 <sup>ab</sup> |
| T <sub>3</sub> | 1.41±0.03 <sup>a</sup>      | 1.71±0.04 <sup>ab</sup> | 1.97±0.02 <sup>b</sup>  |
| T <sub>4</sub> | 1.37±0.03 <sup>a</sup>      | 1.69±0.02 <sup>a</sup>  | 1.90±0.02 <sup>a</sup>  |
| Control        | 1.42±0.02 <sup>a</sup>      | 1.78±0.02 <sup>b</sup>  | 2.05±0.03 <sup>c</sup>  |
| P value        | 0.510                       | 0.082                   | 0.002                   |

T<sub>1</sub>, Basal diet + AGP (CTC, 15%) @ 335 mg/kg; T<sub>2</sub>, Basal diet + EOs formula (1.5×); T<sub>3</sub>, Basal diet + *L. fermentum* @ 10<sup>7</sup> cfu/g + MOS @ 0.3% + Butyrate @ 0.03%; T<sub>4</sub>, Combination of T<sub>2</sub> + T<sub>3</sub>; Control, basal diet only. Mean±SE given with different superscripts column-wise differ significantly (P<0.05).

Further, the current study revealed the improvement in the immune status of the broiler chicks supplemented with the probiotic and the essential oils alone or in combination as compared to the control group, which was fed the basal diet (Table 3). The obtained results are in agreement with the previous studies stating that the immune response of chickens can be enhanced with the use of probiotic and prebiotics together (Al-Khalaifah 2018). The essential oils have been documented to have role in improving immune status of the broiler birds as per the earlier findings (Adaszyńska-Skwirzyńska and Szczerbińska

2017); however, their effect on immune status of broilers was found significantly better in presence of the probiotic bacteria in the present study. The finding can also be supported by a study conducted by Rewatkar *et al.* (2019) demonstrating that the immune status of broilers was improved with the supplementation of encapsulated *Saccharomyces cerevisiae* as a probiotic and Oregano (*Origanum vulgare*) oil, which is in agreement with our findings too.

The antimicrobial effects of probiotics has been discussed by various researchers in earlier studies showing that their supplementation in diet of broilers can reduce the pathogen level including total viable count and Enterobacteriaceae group of bacteria in gut (de Souza *et al.* 2019), which may be a result of lowered pH (Halimi and Mirsalehian 2016) due to production of organic acid and presence of sodium butyrate also reduces the pH level thus reducing the microbe level in the gut and presence of prebiotic provides substrate for fermentation leading to the survival of probiotic bacteria. Due to this possible reason, the *Lactobacillus* count was significantly higher for groups supplemented with *L. fermentum* (i.e. 10<sup>7</sup> cfu/gm), MOS (i.e. 0.3%) and sodium butyrate (0.03%) (Table 4). Further, the antimicrobial activity of essential oils is thought to be the result of their lipophilic nature, which helps them to permeate the microbes through their cell membrane and mitochondria and thus hamper their energy metabolism (Gopi *et al.* 2014). Due to the higher MIC of EOs against probiotics as mentioned in previous studies, *Lactobacillus* was able to survive at the lower concentration of essential oils in EO formula (1.5×) supplemented to them.

The present study showed improved villus height, villus width and VH: CD ratio along with increased intestinal absorptive surface area in the broiler groups supplemented with combination of essential oil formula and probiotic in presence of MOS (i.e. 0.3%) and sodium butyrate (0.03%) (Table 5). Various previous studies have proclaimed the positive effects of probiotic on intestinal histomorphology documenting that supplementation of probiotic in feed of birds can improve the villus height and villus area (Hutsko *et al.* 2016). As far as the effect of essential oil on intestinal histology is concerned, a study conducted by Yang *et al.*

Table 3. Individual and combined effects of probiotic and essential oil supplementation on cell mediated and humoral immunity, serum corticosterone level and Heterophil-Lymphocyte ratio in broilers (Mean±SE)

| Treatment group | Cell mediated immunity (mm)<br>(PHA-P Method) | Humoral immunity<br>(Log <sub>2</sub> value) | Serum corticosterone<br>(nmol/lit) | H L ratio               |
|-----------------|-----------------------------------------------|----------------------------------------------|------------------------------------|-------------------------|
| T <sub>1</sub>  | 0.74±0.11 <sup>a</sup>                        | 3.01±0.04 <sup>b</sup>                       | 71.96±10.93 <sup>a</sup>           | 0.26±0.021 <sup>a</sup> |
| T <sub>2</sub>  | 0.76±0.08 <sup>ab</sup>                       | 3.01±0.05 <sup>b</sup>                       | 87.16±9.55 <sup>a</sup>            | 0.24±0.032 <sup>a</sup> |
| T <sub>3</sub>  | 1.00±0.08 <sup>bc</sup>                       | 3.07±0.06 <sup>bc</sup>                      | 81.83±8.97 <sup>a</sup>            | 0.25±0.016 <sup>a</sup> |
| T <sub>4</sub>  | 1.20±0.03 <sup>c</sup>                        | 3.19±0.07 <sup>c</sup>                       | 78.38±11.15 <sup>a</sup>           | 0.27±0.017 <sup>a</sup> |
| Control         | 0.64±0.07 <sup>a</sup>                        | 2.82±0.04 <sup>a</sup>                       | 78.44±8.24 <sup>a</sup>            | 0.24±0.015 <sup>a</sup> |
| P value         | 0.001                                         | 0.001                                        | 0.864                              | 0.756                   |

T<sub>1</sub>, Basal diet + AGP (CTC, 15%) @ 335 mg/kg; T<sub>2</sub>, Basal diet + EOs formula (1.5×); T<sub>3</sub>, Basal diet + *L. fermentum* @ 10<sup>7</sup> cfu/g + MOS @ 0.3% + Butyrate @ 0.03%; T<sub>4</sub>, Combination of T<sub>2</sub> + T<sub>3</sub>; Control, basal diet only. Mean±SE given with different superscripts column-wise differ significantly (P<0.05).

Table 4. Individual and combined effects of probiotic and essential oil supplementation on gut microbiology in broilers (Mean±SE)

| Treatment group | Total plate count (Log <sub>10</sub> value) | <i>Lactobacillus</i> count (Log <sub>10</sub> value) | <i>E. coli</i> count (Log <sub>10</sub> value) |
|-----------------|---------------------------------------------|------------------------------------------------------|------------------------------------------------|
| T <sub>1</sub>  | 7.52±0.32 <sup>a</sup>                      | 7.26±0.09 <sup>a</sup>                               | 5.60±0.08 <sup>a</sup>                         |
| T <sub>2</sub>  | 7.49±0.07 <sup>a</sup>                      | 7.53±0.04 <sup>b</sup>                               | 5.56±0.05 <sup>a</sup>                         |
| T <sub>3</sub>  | 7.55±0.11 <sup>a</sup>                      | 9.19±0.03 <sup>d</sup>                               | 5.56±0.05 <sup>a</sup>                         |
| T <sub>4</sub>  | 7.43±0.04 <sup>a</sup>                      | 8.13±0.02 <sup>c</sup>                               | 5.46±0.06 <sup>a</sup>                         |
| Control         | 8.05±0.05 <sup>b</sup>                      | 7.46±0.06 <sup>b</sup>                               | 6.03±0.07 <sup>b</sup>                         |
| Total±SE        | 7.61±0.08                                   | 7.92±0.16                                            | 5.64±0.05                                      |
| P value         | 0.080                                       | 0.001                                                | 0.001                                          |

T<sub>1</sub>, Basal diet + AGP (CTC, 15%) @ 335 mg/kg; T<sub>2</sub>, Basal diet + EOs formula (1.5×); T<sub>3</sub>, Basal diet + *L. fermentum* @ 10<sup>7</sup> cfu/g + MOS @ 0.3% + Butyrate @ 0.03%; T<sub>4</sub>, Combination of T<sub>2</sub> + T<sub>3</sub>; Control, basal diet only. Mean±SE given with different superscripts column-wise differ significantly (P<0.05).

(2018) demonstrated that the supplementation of essential oils in broiler's diet enhanced the villus height and VH: CD ratio in jejunum resulting in augmented digestion and efficiency of absorption with increased intestinal absorptive surface area. Thus, essential oil supplementation in broiler diet can improve the morphology of the gut. The present study revealed that there is the possibility of using the essential oils and probiotic bacteria together for their affirmative response on the intestinal histology in a synergistic way as probiotic can easily survive at the lower concentration of essential oils (Pakbin 2017, Ambrosio *et al.* 2019). Few studies proclaim that sometimes, the phytochemical essential oils can lead to the intestinal tissue irritation resulting in reduction of intestinal surface, which might be due to many factors such as the nature of essential oil used, their concentration and combination used.

No significant difference was observed in the HL ratio and serum corticosterone levels of any of the groups (Table 3). On the contrary, some of the previous studies proclaimed the affirmative response of probiotics (Simmering and Blaut 2001) and essential oils (Mohiti and Ghanaatparast

2017) in reduction of the stress level in broilers.

Hence, the data obtained from the present study depicts that combined supplementation of *L. fermentum* NKN51 (10<sup>7</sup> cfu/g) and essential oil formula (TCLVC 1.5×) developed by IIT Roorkee has an affirmative response on various performance parameters of broiler chickens.

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Table 5. Individual and combined effects of probiotic and essential oil supplementation on intestinal (Jejunal) histomorphology in broilers (Mean±SE)

| Treatment group | Villus height (VH) (µm)    | Villus width (VW) (µm)    | Crypt depth (CD) (µm)     | Crypt width (CW) (µm)   | VH: CD ratio            | Intestinal absorptive surface area (µm) <sup>2</sup> |
|-----------------|----------------------------|---------------------------|---------------------------|-------------------------|-------------------------|------------------------------------------------------|
| T <sub>1</sub>  | 1166.60±20.87 <sup>c</sup> | 109.17±2.78 <sup>a</sup>  | 136.85±2.51 <sup>ab</sup> | 53.28±1.76 <sup>a</sup> | 8.57±0.22 <sup>ab</sup> | 18.85±0.54 <sup>ab</sup>                             |
| T <sub>2</sub>  | 1106.73±14.14 <sup>b</sup> | 120.03±4.14 <sup>b</sup>  | 121.97±6.62 <sup>a</sup>  | 50.05±1.04 <sup>a</sup> | 9.64±0.61 <sup>b</sup>  | 17.95±0.43 <sup>a</sup>                              |
| T <sub>3</sub>  | 1310.41±12.31 <sup>d</sup> | 134.14±2.50 <sup>c</sup>  | 138.71±1.44 <sup>b</sup>  | 51.57±1.85 <sup>a</sup> | 9.49±0.14 <sup>b</sup>  | 20.10±0.56 <sup>b</sup>                              |
| T <sub>4</sub>  | 1384.45±19.92 <sup>c</sup> | 127.87±2.37 <sup>bc</sup> | 131.75±6.99 <sup>ab</sup> | 51.52±1.56 <sup>a</sup> | 10.92±0.46 <sup>c</sup> | 21.64±0.47 <sup>c</sup>                              |
| Control         | 1040.59±19.48 <sup>a</sup> | 102.60±4.30 <sup>a</sup>  | 134.60±5.70 <sup>ab</sup> | 50.01±1.32 <sup>a</sup> | 7.97±0.36 <sup>a</sup>  | 17.89±0.61 <sup>a</sup>                              |
| Total±SE        | 1201.76±14.98              | 118.76±1.86               | 132.78±2.34               | 51.29±0.68              | 9.32±0.20               | 19.29±0.27                                           |
| P value         | 0.001                      | 0.001                     | 0.177                     | 0.549                   | 0.001                   | 0.001                                                |

T<sub>1</sub>, Basal diet + AGP (CTC, 15%) @ 335 mg/kg; T<sub>2</sub>, Basal diet + EOs formula (1.5×); T<sub>3</sub>, Basal diet + *L. fermentum* @ 10<sup>7</sup> cfu/g + MOS @ 0.3% + Butyrate @ 0.03%; T<sub>4</sub>, Combination of T<sub>2</sub> + T<sub>3</sub>; Control, basal diet only. Mean±SE given with different superscripts column-wise differ significantly (P<0.05).

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