Evaluation of Enterococcus faecium NCIMB 11181 and Clostridium butyricum probiotic supplements in post-weaning rabbits reared under thermal stress conditions

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ABSTRACT

An 8-week feeding experiment was designed to examine the influences of single or/and double strains of probiotic compared to antibiotic colistin on growth, hematological variables, blood serum metabolites and caecal fermentation in post-weaning rabbits exposed to heat stress conditions. A total of one hundred (35-day-old; average initial body weights 694 ± 7.33) New Zealand White rabbits were distributed into five groups. The experimental groups were the basal diet (control), or basal diet fortified with 120 mg colistin (COL); 5 \times 10^6 cfu Clostridium butyricum (CB); 2 \times 10^8 cfu Enterococcus faecium NCIMB 11181 (EF); or 2.5 \times 10^6 cfu C. butyricum + 1 \times 10^8 cfu E. faecium/kg diet (CB + EF). The results demonstrated that both the EF and CB + EF treatments enhanced (p < .05) final body weight and overall feed conversion ratio compared to control and COL treatment. The carcase yield percentage and relative organs weight were insignificantly affected in all treated groups. The COL treatment increased (p < .05) mean corpuscular volume and albumin/globulin ratio compared to the control group. Compared to the control, all probiotic and COL groups decreased (p < .05) serum total triglycerides and very low-density lipoprotein. The concentrations of Serum total protein, globulin, high-density lipoprotein, complement component 3, and lysozyme activity were higher (p < .05) in probiotic groups than those in the control group. Probiotic application improved the caecal total volatile fatty acids production and the intestinal histomorphometry parameters. In conclusion, EF and CB + EF probiotic supplements could be used as alternatives to antibiotic colistin to enhance the growth and health of weaned rabbits under heat stress conditions.

HIGHLIGHTS

- Two strains of probiotics as alternatives to antibiotics were studied in rabbits under heat stress conditions.
- Supplemental EF and CB + EF probiotic enhanced rabbit growth and feed utilisation.
- Probiotic application improved biochemical and immunological indicators.
- The application of the tested probiotics improved the caecal volatile fatty acids production and the intestinal histomorphometry parameters.
- EF and CB + EF probiotic supplements could be used as an alternative to antibiotics to promote the growth of rabbits under heat stress conditions.

Introduction

Numerous antibiotics, including chlorotetracycline, tyiamulin, bacitracin, monensin, and colistin, have been commonly used in European commercial rabbit farms in prophylactic dosages to act as growth promoters or to control pathogenic bacteria (Mateos et al. 2010; Attia et al. 2019). However, repeated use of antibiotics leads to increasing their residue in animal products together with antibiotic resistance in bacteria in both animal and human (Romero et al. 2012). Due to these restrictions, in addition to the few antibiotic numbers authorised by the European Union, there was an imperative need to search for viable alternatives to antibiotics in rabbit feeding. These alternatives should
be able to promote rabbit growth and maintain the sanitary status of the digestive system (Attia, Hamed, Abd El-Hamid, Al-Harthi, et al. 2015; Attia, Hamed, Abd El-Hamid, Shahba, et al. 2015; Attia et al). In tropical and subtropical regions, rabbits suffer from hypersensitivity to high temperatures due to their dense fur and few sweat glands (Daader et al. 2018). Under hot climate, especially during summer, rabbits suffer from slow growth, loss of appetite, and poor feed utilisation which leads to increased mortality rates and serious economic losses (Hassan et al. 2021). Therefore, it is essential to enhance the heat stress tolerance of growing rabbits using practical approaches such as nutritional strategies. Probiotic products are a very appropriate choice as possible growth promoters in the rabbit industry as they positively boost the immune system, reduce pathogens, and improve intestinal barrier function (Attia et al. 2018). Additionally, the probiotic combats the problem of bacterial resistance resulted from antibiotics residues.

*Enterococcus faecalis* is a Gram-positive facultative anaerobic coccus, which belonging to lactic acid-producing bacterial species (Domann et al. 2007). Currently, the probiotic strain *E. faecium* 11181 (EF) was approved by the European Food Safety Authority (EFSA) as a feed additive to improve animal growth performance (EFSA 2012; Pajarillo et al. 2015). This strain has been reported to efficiently enhance feed utilisation, daily weight gain, and gut health by hindering the gut pathogens proliferation and promoting the growth of beneficial bacteria (Vrotniakien _ et al. 2013; Pajarillo et al. 2015).

*Clostridium butyricum* (CB), a Gram-positive anaerobe bacterium, produces butyrate in the gastrointestinal of healthy animals (Yang et al. 2012; Wang et al. 2019). Furthermore, it has stronger tolerance ability in high temperature environment, low pH, bile salt, compared with other probiotics. Consequently, CB has been recognised as effective and safe feed supplement (Kong et al. 2011). In an earlier experiment in broilers, CB has been demonstrated to boost the immune response, enhance growth performance indices, and modify the microflora structure (Yang et al. 2012). Furthermore, CB can increase the content of caecal volatile fatty acid (Han et al. 2018), maintain the intestinal flora balance, and stimulate the nutrients absorption (Mallo et al. 2010).

There are fewer studies deals with CB and EF probiotics in rabbits than in other monogastric farm species. Furthermore, knowledge on the effects of tested strains on the growth, intestinal morphology, and heat stress tolerance in post-weaning rabbits is limited. Based on the earlier reported beneficial impacts of both probiotic strains, we hypothesise that could be used as alternatives to antibiotic to stimulate the rabbit growth under summer conditions. Hence, the existing study was carried out to investigate the effects of EF and CB as a substitute for antibiotic colistin on growth, blood metabolites, immune function, intestinal morphology, and volatile fatty acids production in weaned rabbits reared under heat stress conditions.

**Materials and methods**

**Animals, design, and management**

This study was performed at the Rabbit Research Farm, Faculty of Agriculture, Zagazig University, Zagazig, Egypt during the hot climatic summer season.

The rabbits were reared under similar sanitary, management, and environmental conditions. All animals were acclimatised for one week before starting the experiment and fed on the control diet without additives until the start of the trial. The rabbits received the experimental diets formulated to cover their nutrient requirements according to De Blas and Mateos (2010) recommendations. All animals were raised jointly in galvanised wire cages (Dimensions of each cage were 35 x 40 x 60 cm) in a naturally ventilated room. The cages had manual feeders and automatic nipple drinkers to continuously supply clean fresh water. The experimental pelleted diet and water were offered *ad libitum*. All care and experimental procedures including test animals in the current study were conducted in line with the scientific and ethical regulations recommended by the European Parliament Directive (2010/63/EU).

For the experimental trial, a total of 100 (35-day-old; average initial body weights 694 ± 7.33 g) New Zealand White male rabbits were selected and randomly distributed into five groups. Each group contained ten replicates, with two rabbits per replicate (cage). The experimental groups were: (1) basal diet without probiotic and antibiotic supplement (control), (2) basal diet + colistin at 120 mg/kg diet (3) basal diet + CB at 5 x 10^8 cfu/kg diet, (4) basal diet + EF at 2 x 10^8 cfu/kg diet, or (5) basal diet + CB and EF complex at 2.5 x 10^6 cfu and 1 x 10^6 cfu/kg diet, respectively. The dose of probiotic supplements used in this experiment has followed the recommendation of the producing company. The colistin level was selected based on Romero et al. (2012) recommendation. CB (Clostrimix®) was obtained from Cheil Bio Company.
growth rate (RGR) = \left(\frac{W_{1} - W_{0}}{W_{0}}\right) \times 100$, where $W_{1}$ refers to the final weight, and $W_{0}$ denotes the initial weight. Feed conversion ratio (FCR) was estimated as the ratio of feed intake (g) to body weight gain (g).

**Carcass traits**

At the feeding trial end, five rabbits per treatment were weighed and sacrificed to assess the carcass characteristics. When complete bleeding was reached, skin, and all entails were removed, and the carcass weight and some carcass organs were weighed (Blasco et al. 1993). The weight of the heart, liver, kidneys, spleen, and lungs were weighed and recorded as g/kg of pre-slaughter weight (SW). Carcase yield, as percentage, was measured by dividing the carcase weight on SW.

**Blood sampling**

At day-91 of age, five rabbits from each group were subjected to collections of blood sample. Two separate blood samples of each rabbit were collected from the lateral ear vein without harming the rabbits. The first sample (2 ml) was collected into an EDTA (Ethylendiaminetetraacetic acid) tube to estimate hematological evaluations. The second aliquot was used to separate the serum after centrifugation at 3000 rpm for 20 min. The collected sera was kept at $-20^\circ$C until measuring the biochemical and immunological parameters.

**Hematological and biochemical parameters**

In the aliquot contained EDTA (1 mg/ml), total erythrocytes (RBCs), total leukocytes (WBCs), and haemoglobin (Hb) were determined following the methods of Jain (1986). Consequently, the mean cell haemoglobin [MCH = (Hb × 10)/RBCs], mean corpuscular volume [MCV = (PCV × 10)/RBCs], and mean corpuscular haemoglobin concentration [MCHC = (Hb × 10)/PCV] were calculated. The sera samples were used to estimate the total protein, albumin, total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL) contents spectrophotometrically by commercial kits (Diamond Diagnostic, Dokki, Giza, Egypt) according to the manufacturer’s guidelines. Serum globulin content of the tested animals in each group was computed by subtracting the albumin from total protein levels. Using commercial ELISA kits, serum concentrations of lysozyme activity and

**Temperature-humidity index calculation**

Throughout the experiment, relative humidity and ambient temperature were recorded by an automatic thermo-hygrometer (TFA Dostmann GmbH and Company, KG, Wertheim, Germany). Temperature-humidity index (THI) was used to assess the degree of exposure to natural thermal stress. The THI value was calculated based on Marai et al. (2001) equation as follows: $\text{THI} = \text{Tem} - [(0.31 - 0.31 \times \text{RH}) (T - 14.4)]$, where RH refers to relative humidity percentage/100 and ambient temperature in Celsius. The values obtained are categorised as follows, THI $<27.8$ used as a thermal comfort zone, 27.8 to $<28.9$ indicates mild to moderate thermal stress, 28.9 to $<30.0$ indicates severe thermal stress, and $>30.0$ shows very severe thermal stress condition.

**Performance measurements**

The growth performance trial was continued for eight consecutive weeks (from 35 to 91-day of age). Feed intake was recorded for each cage by measuring the remaining quantities of diet then subtracting them from the supplied before giving the next quantities. All rabbits were weighed separately at the start of the feeding trial (35-d), 63, and 91 day of age. The relative

**Table 1. Ingredients and composition of the basal diet.**

| Components               | [g/kg feed] | Nutrient levels                | [g/kg feed] |
|--------------------------|-------------|--------------------------------|-------------|
| Lucerne meal             | 340         | Dry matter                     | 910.5       |
| Soybean meal             | 140         | Crude protein (N $\times$ 6.25) | 170.9       |
| Barely grain             | 170         | Ether extract                  | 29.8        |
| Wheat bran               | 220         | Ash                             | 86.3        |
| Yellow corn              | 80          | Neutral detergent fibre         | 344.9       |
| Cane molasses            | 30          | Acid detergent fibre           | 176.5       |
| Dicalcium phosphate      | 10          |                                 |             |
| Sodium chloride          | 5           |                                 |             |
| DL-methionine            | 1           |                                 |             |
| Premix*                  | 4           |                                 |             |
| Total                    | 1000        |                                 |             |

*Premix contains per kg (minerals and vitamins mixture): vitamin A, 20,000 U; vitamin D$_{3}$, 15,000 U; vitamin E, 8.33 g; vitamin K, 0.33 g; vitamin B$_{1}$, 0.33; vitamin B$_{2}$, 1.0 g; vitamin B$_{6}$, 0.33 g; vitamin B$_{12}$, 1.7 mg; panthenic acid, 3.33 g; biotine, 33 mg; folic acid, 0.83 g; choline chloride, 200; manganese 80 g; zinc 60 g; iron 30 g; copper 4 g; iodine 0.5 g; selenium 0.1 g; and cobalt 0.1 g.
complement component 3 was determined following the manufacturer directives.

**Determination of caecal volatile fatty acid profile**

Samples from caecum contents were immediately collected from five rabbits per group after slaughtering. The caecal contents were squeezed into a beaker through four layers of cheesecloth. A caecal content of 1 ml was added to 200 μl meta-phosphoric acid 25% (w/v) in microcentrifuge tubes and centrifuged for 20 min at 30,000×g (15,000 rpm, JA–17 rotor) and. The supernatant was refrigerated at −20°C for future analysis. The supernatant was removed to GC vials for VFA analysis according to Palmquist and Conrad (1971).

**Histopathological and histomorphometric measurements of the duodenum**

At the end of the trial, rabbits were sacrificed, the abdomen was opened through a midline incision and the small intestine was gently removed. The segments of the duodenum (2 cm posterior to stomach) were collected from each rabbit and were first rinsed with saline (0.85% NaCl). Then cross-sectional lengths of 1 cm were cut from the duodenum. The collected tissue samples were fixed in 10% formalin solution and consequently dehydrated, cleared in xylene, embedded and blocked in paraffin by an automatic tissue processor. Then, four μm thickness sections were cut via a rotary microtome and lastly stained with Haematoxylin and Eosin (H&E) according to Suvarna et al. (2013). Five sections per animal were inspected in a blinded manner by the light microscope (Olympus, Tokyo, Japan) at diverse magnifications to qualitatively and quantitatively detect histological changes resulted from different experimental materials.

From each animal, duodenal sections that represented the best view of villus and crypts were selected to be analysed using stereological image software, with its micrometric ruler. The following parameters were evaluated in five randomly-selected sections stained with H&E according to Seyyedin and Nazem (2017) and Stamilla et al. (2020). (1) Villous height (VH) was defined from their tip to the villus–crypt junction. (2) Villous width (VW) estimated by measuring the average of the base and tip of the villus. (3) Crypt depth (CD) was measured as the distance from top of crypt to the invagination between two villi. (4) Submucosal layer thickness (SMT). (5) Muscular layer thickness (MT). (6) Tunica serosa thickness (TST).

**Statistical analysis**

For the body weight and weight gain data, the experimental unit was the individual rabbit (20 rabbits per treatment), and, for the feed intake and feed conversion data, the experimental unit was the cage (10 cages per treatment). For the carcase traits and hemato-biochemical parameters, five rabbits per treatment were used. The SPSS.21® software was used to analyse the obtained data. (IBM Cooperation, Armonk, NY, USA). The data were statistically analysed via one-way ANOVA, according to the following model: $Y_{ij} = \mu + D_i + e_{ij}$, where $\mu =$ the overall mean, $D_i =$ dietary supplement effect, and $e_{ij} =$ random error. Differences among means were analysed by Tukey’s test. Probability values of < .05 were considered significant.

**Results**

**Meteorological data**

Throughout the experiment, the average daily relative humidity and the ambient temperature inside the farm were recorded between 50% and 68% (60.19% ± 1.20%) and 27.80°C to 36.10°C (31.78°C ± 0.58°C), respectively. Accordingly, the calculated THI values were ranged from 26.35 to 33.28. The overall mean of THI was 29.62 ± 0.49 throughout the experiment, which indicates that the rabbits were subjected to severe thermal stress following Marai et al. (2001) equation.

**Growth performance and carcase traits**

Final body weight was higher ($p = .042$) and weight gain and RGR (d35–d91) tended to be greater ($p = .054$) in the groups (Table 2). No significant differences were recorded on daily feed intake among groups during all experimental intervals. Dietary EF or EF+CB supplementation in rabbits diets had significantly ($p = .028$) enhanced FCR (35 and 91 d) compared with those fed the control diet (Table 2). On the other hand, the carcase yield percentage and relative organs (heart, liver, kidneys, spleen, and lungs) weight were insignificantly affected in the treated groups (Table 3).

**Hemato-biochemical and immunological parameters**

The data shown in Table 4 indicates that dietary supplemental EF or EF+CB significantly ($p = .011$) increased blood haemoglobin levels as compared with
Table 2. Effect of dietary supplemental probiotics and colistin as growth promoters on growth performance of broiler rabbit exposed to heat stress conditions.

| Parameter                        | Experimental groups | Pooled SEM | p-Value |
|----------------------------------|---------------------|------------|---------|
|                                  | Control             | Colistin   | EF      | CB      | EF + CB |         |
| Body weight, g                   |                     |            |         |         |         |         |
| d 35 (Initial)                   | 688                 | 712        | 685     | 698     | 689     | 7.33     | .807    |
| d 63                             | 1246                | 1339       | 1371    | 1307    | 1380    | 24.21    | .418    |
| d 91 (Final)                     | 1697\(^b\)          | 1782\(^b\) | 1898\(^a\) | 1821\(^b\) | 1925\(^a\) | 27.02    | .042    |
| Weight gain, g                   |                     |            |         |         |         |         |
| d 35–d 63                        | 558                 | 628        | 686     | 609     | 692     | 25.24    | .438    |
| d 63–d 91                        | 452                 | 443        | 528     | 515     | 546     | 16.63    | .182    |
| d 35–d 91 (Overall)              | 1010                | 1071       | 1214    | 1123    | 1237    | 28.92    | .054    |
| Relative growth rate, %          |                     |            |         |         |         |         |
| d 35–d 63                        | 57.12               | 60.50      | 66.32   | 60.52   | 66.67   | 1.95     | .485    |
| d 63–d 91                        | 30.99               | 28.69      | 32.54   | 32.87   | 33.06   | 1.11     | .729    |
| d 35–d 91 (Overall)              | 84.42               | 85.55      | 93.86   | 88.94   | 94.47   | 1.54     | .117    |
| Daily feed intake, g             |                     |            |         |         |         |         |
| d 35–d 63                        | 73.65               | 68.14      | 73.36   | 71.00   | 67.57   | 1.22     | .380    |
| d 63–d 91                        | 117.79              | 108.07     | 110.78  | 117.37  | 115.40  | 2.03     | .491    |
| d 35–d 91 (Overall)              | 95.72               | 88.10      | 92.07   | 94.19   | 91.49   | 1.30     | .426    |
| Feed: Gain                       |                     |            |         |         |         |         |
| d 35–d 63                        | 3.90                | 3.19       | 3.16    | 3.35    | 2.85    | 0.16     | .333    |
| d 63–d 91                        | 7.33                | 6.94       | 5.98    | 6.93    | 6.05    | 0.26     | .404    |
| d 35–d 91 (Overall)              | 5.36\(^a\)         | 4.67\(^b\) | 4.26\(^b\) | 4.76\(^b\) | 4.23\(^a\) | 0.13     | .028    |

SEM stands for standard error of the mean. \(^a\)\(^b\)Means in a row with different superscripts differ significantly.

Ef: control diet + Enterococcus faecium NCIMB 11181; CB: control diet + Clostridium butyricum; EF + CB: control diet + Enterococcus faecium NCIMB 11181 + Clostridium butyricum.

Table 3. Effect of dietary supplemental probiotics and colistin as growth promoters on carcase traits (as % of pre-slaughter weight, SW) of broiler rabbit exposed to heat stress conditions.

| Parameter                  | Experimental groups | Pooled SEM | p-Value |
|----------------------------|---------------------|------------|---------|
|                            | Control             | Colistin   | EF      | CB      | EF + CB |         |
| Carcase yield, %           | 57.87               | 57.47      | 57.84   | 57.19   | 58.23   | 0.35     | .931    |
| Liver, g/kg SW             | 27.62               | 30.91      | 25.99   | 29.46   | 27.90   | 0.78     | .355    |
| Kidneys, g/kg SW           | 6.89                | 6.79       | 6.05    | 6.72    | 7.45    | 0.32     | .792    |
| Heart, g/kg SW             | 2.58                | 2.55       | 2.92    | 2.48    | 3.73    | 0.18     | .160    |
| Lungs, g/kg SW             | 6.65                | 7.62       | 6.65    | 6.95    | 7.63    | 0.33     | .821    |
| Spleen, g/kg SW            | 0.71                | 0.59       | 0.44    | 0.86    | 0.42    | 0.07     | .242    |

SEM stands for standard error of the mean.

Ef: control diet + Enterococcus faecium NCIMB 11181; CB: control diet + Clostridium butyricum; EF + CB: control diet + Enterococcus faecium NCIMB 11181 + Clostridium butyricum.

Table 4. Effect of dietary supplemental probiotics and colistin as growth promoters on hematological parameters of broiler rabbit exposed to heat stress conditions.

| Parameter                  | Experimental groups | Pooled SEM | p-Value |
|----------------------------|---------------------|------------|---------|
|                            | Control             | Colistin   | EF      | CB      | EF + CB |         |
| Erythrogram                |                     |            |         |         |         |         |
| RBCs, 10^6/\muL             | 4.73                | 4.60       | 5.33    | 4.78    | 4.99    | 0.09     | .063    |
| Hb, g/dL                   | 10.93\(^b\)        | 11.30\(^b\) | 12.43\(^a\) | 11.33\(^b\) | 12.30\(^a\) | 0.18     | .011    |
| Hct, %                     | 34.47               | 37.50      | 39.10   | 36.73   | 36.23   | 0.60     | .156    |
| MCV, fL                     | 72.82\(^b\)        | 81.72\(^a\) | 74.16\(^b\) | 76.85\(^a\) | 76.52\(^b\) | 1.02     | .038    |
| MCH, pg/dL                 | 23.17               | 24.60      | 23.65   | 23.70   | 24.89   | 0.22     | .062    |
| MCHC, g/dL                 | 31.91               | 31.07      | 31.90   | 30.84   | 32.55   | 0.33     | .147    |
| Platelets, 10^3/\muL        | 363.25              | 290.75     | 311.75  | 347.75  | 324.75  | 19.41    | .819    |

Leukogram

| WBCs, 10^3/\muL             | 5.75                | 4.94       | 5.81    | 5.78    | 4.63    | 0.23     | .367    |
| Lymphocyte, %              | 64.75               | 60.69      | 64.38   | 65.58   | 61.99   | 1.39     | .819    |
| Monocytes, %               | 2.00                | 2.00       | 2.25    | 2.25    | 2.25    | 0.08     | .736    |
| Neutrophils, %             | 25.58               | 35.98      | 31.29   | 29.42   | 33.35   | 1.57     | .293    |
| Eosinophils, %             | 2.25                | 1.25       | 2.00    | 2.00    | 2.00    | 0.14     | .234    |

SEM stands for standard error of the mean. \(^a\)\(^b\)Means in a row with different superscripts differ significantly.

Ef: control diet + Enterococcus faecium NCIMB 11181; CB: control diet + Clostridium butyricum; EF + CB: control diet + Enterococcus faecium NCIMB 11181 + Clostridium butyricum; RBCs: red blood cells; Hct: the haematocrit; Hb: haemoglobin; MCV: mean corpuscular volume; MCHC: mean corpuscular haemoglobin concentration; WBCs: white blood cells.
control. Also, dietary administration with colistin led to a significant \( p = .038 \) increase in the level of MCV compared to the non-supplemented group. Regarding the leukograms, there was no significant change in WBCs count, lymphocytes, monocytes, neutrophils, and eosinophils percentages among all experimental groups (Table 4).

As shown in Table 5, the serum concentrations of total protein, globulin, and HDL were increased \( (p < .001) \) in the probiotic-supplemented groups. In contrast, lower total glycerides \( (p = .002) \) and VLDL \( (p = .003) \) values were obtained in rabbits groups fed on colistin or probiotics. Also, total cholesterol levels of EF and EF + CB groups were decreased \( (p = .039) \) compared with the control. Colistin-supplemented rabbits had a higher \( (p < .001) \) A/G ratio than the other groups. Lysozyme activity in the probiotic-supplemented groups was significantly \( (p = .004) \) higher than that in the non-supplemented group. The levels of serum C3 of EF and EF + CB groups were significantly increased \( (p = .005) \) compared with those of the control rabbits (Table 5).

**Caecal fermentation**

Both the EF and EF + CB treatments increased \( (p = .018) \) the caecal total VFA and butyrate concentration compared to the non-supplemented group (Table 6). The propionate amount in all probiotic-supplemented groups was significantly \( (p < .001) \) increased compared with control group. In the same line, the EF + CB treatment resulted in the highest \( (p = .017) \) proportions of propionate (Table 6).

**Histological evaluation of tissues of duodenum**

Histological examination of the duodenum of control heat stressed group showed marked injury (Figure Table 5. Effect of dietary supplemental probiotics and colistin as growth promoters on protein and lipid profiles and immunological indices of broiler rabbit exposed to heat stress conditions.

| Parameter               | Experimental groups | Control | Colistin | EF | CB | EF + CB | Pooled SEM | p-Value |
|-------------------------|---------------------|---------|----------|----|----|---------|------------|---------|
| **Protein profile**     |                      |         |          |    |    |         |            |         |
| TP, g/dL                |                     | 6.00b   | 5.27b    | 8.04a | 7.53a | 7.91a   | 0.28       | <.001   |
| ALB, g/dL               |                     | 2.22    | 2.91     | 2.55 | 2.36 | 3.00    | 0.12       | .136    |
| GLOB, g/dL              |                     | 3.78b   | 2.36b    | 5.49a | 5.18a | 4.91a   | 0.29       | <.001   |
| A/G, %                  |                     | 0.61b   | 1.89a    | 0.46b | 0.47b | 0.61b   | 0.13       | <.001   |
| **Lipid profile**       |                      |         |          |    |    |         |            |         |
| TC, mg/dL               |                     | 238.00a | 195.25ab | 166.00b | 195.25ab | 178.00b | 5.42       | .039    |
| TG, mg/dL               |                     | 283.25a | 200.25b  | 188.00ac | 174.75bc | 122.75  | 14.60      | .002    |
| HDL, mg/dL              |                     | 35.25a  | 39.25bc  | 46.75a | 44.25h | 55.00a  | 1.81       | <.001   |
| LDL, mg/dL              |                     | 147.35  | 91.20    | 109.80 | 97.95 | 115.85  | 8.84       | .318    |
| VLDL, mg/dL             |                     | 54.28a  | 40.08b   | 37.60bc | 34.93bc | 24.53c  | 2.78       | .003    |
| **Immunological indices** |                 |         |          |    |    |         |            |         |
| C3, mg/dL               |                     | 118.25b | 130.00ab | 141.25a | 130.00ab | 139.75a | 2.43       | .005    |
| Lysozyme, U/ml          |                     | 10.75c  | 14.25bc  | 17.75ab | 17.75ab | 20.75a  | 1.00       | .004    |

SEM stands for standard error of the mean. a,bMeans in a row with different superscripts differ significantly.

**Table 6. Effect of dietary supplemental probiotics and colistin as growth promoters on caecal volatile fatty acids (VFA) of broiler rabbit exposed to heat stress conditions.**

| Parameter               | Experimental groups | Control | Colistin | EF | CB | EF + CB | Pooled SEM | p-Value |
|-------------------------|---------------------|---------|----------|----|----|---------|------------|---------|
| **Caecal VFA concentration (mmol/L)** |                     |         |          |    |    |         |            |         |
| Total                   |                     | 102.88b | 113.44ab | 126.73a | 120.96ab | 128.85a | 2.98       | .018    |
| Acetate                 |                     | 80.01   | 87.97    | 98.42 | 91.62 | 94.96   | 2.28       | .083    |
| Propionate              |                     | 6.64b   | 7.22b    | 9.15a | 8.97a | 10.42a  | 0.37       | <.001   |
| Butyrate                |                     | 15.21b  | 16.87b   | 18.00b | 19.09ab | 22.39a  | 0.48       | .029    |
| Valerate                |                     | 1.02    | 1.38     | 1.16  | 1.28  | 1.08    | 0.06       | .233    |
| **Caecal VFA proportions (%)** |                   |         |          |    |    |         |            |         |
| Acetate                 |                     | 77.77   | 77.55    | 77.66 | 75.74 | 73.70   | 0.78       | .774    |
| Propionate              |                     | 6.45b   | 6.36b    | 7.22b | 7.42ab | 8.09a   | 0.25       | .017    |
| Butyrate                |                     | 14.78   | 14.87    | 14.20 | 15.78 | 17.38   | 0.68       | .708    |
| Valerate                |                     | 0.99    | 1.22     | 0.92  | 1.06  | 0.84    | 0.05       | .054    |

SEM stands for standard error of the mean. a,bMeans in a row with different superscripts differ significantly.

**Ef:** control diet + Enterococcus faecium NCIMB 11181; **CB:** control diet + Clostridium butyricum; **EF + CB:** control diet + Enterococcus faecium NCIMB 11181 + Clostridium butyricum; **TP:** total protein; **ALB:** albumin; **GLOB:** globulin; **A/G:** albumin/globulin ratio; **TC:** total cholesterol; **TG:** total triglycerides; **HDL:** high-density lipoprotein; **LDL:** low-density lipoprotein; **VLDL:** very low-density lipoprotein; **C3:** complement component 3.
Histomorphometric measurements were performed to histopathological alterations of the duodenum. As mentioned in Table 7, the villus height in the duodenum was increased in the treatment groups compared with heat stressed group. However, the villus width decreased in treated groups except control group. It was noticeable that the groups treated with probiotics and colistin showed increasing in Crypt depth compared with the heat stressed group. Also, the submucosal layer thickness was lesser in probiotics and colistin groups than control group. However, muscular layer thickness in the duodenum was greater compared to the control group. It should also be noted that a significant decreasing in tunica serosa thickness compared to heat stressed group (Table 7).

## Discussion

Throughout the current experiment, based on the calculated THI value (29.62), growing rabbits were severely heat stressed during the feeding trial. Nevertheless, distinctive improvement in growth performance, blood constituents, and immune response were evident because of EF and/or CB dietary supplementation.

Initially, a growth-enhancing activity represented by a significant improvement in final body weight and FCR as well as a rising tendency of weight gain and FCR were apparent in growing rabbit fed diet supplemented with EF and EF + CB in the summer season. In weaning piglets, this EF strain has been displayed to efficiently augment daily weight gain and enhance feed conversion (Herzig et al. 2003; Vrotniakien et al. 2013) and broiler (Wu et al. 2019). In this study, the growth improvement in EF group could be probably owed to increases in the lactic acid bacteria count or improve the structure of intestinal mucosal thus augmenting the absorption of nutrients (Samli et al. 2007). Moreover, EF probiotic has also been shown to increases growth performances in monogastric animals like pigs via augmenting jejunal mucosa absorptive and secretory ability, enhancing intestinal barrier

### Table 7. Effect of dietary supplemental probiotics and colistin on histomorphometric measurements in the intestine of broiler rabbit exposed to heat stress conditions.

| Parameter                        | Control | Colistin | EF | CB | EF + CB | Pooled SEM | p-Value |
|----------------------------------|---------|----------|----|----|---------|------------|---------|
| Histomorphometry measurements    |         |          |    |    |         |            |         |
| Villus length (µm)               | 222.20± | 304.80±  | 332.60± | 367.20± | 589.80± | 26.30      | <.001   |
| Villus width (µm)                | 191.00± | 160.80±  | 143.60± | 135.00± | 107.80± | 6.60       | <.001   |
| Crypt depth (µm)                 | 88.00±  | 68.40±   | 48.60±  | 49.00±  | 50.00±  | 4.49       | <.001   |
| Submucosa (µm)                   | 153.00± | 99.00±   | 86.60±  | 88.40±  | 69.40±  | 6.93       | <.001   |
| Muscle (µm)                      | 69.40±  | 79.40±   | 90.40±  | 85.40±  | 106.20± | 2.92       | <.001   |
| Serosa (µm)                      | 66.60±  | 77.00±   | 45.60±  | 35.00±  | 32.00±  | 4.78       | .001    |

SEM stands for standard error of the mean. a,bMeans in a row with different superscripts differ significantly. The EF: control diet + Enterococcus faecium NCIMB 11181; CB: control diet + Clostridium butyricum; EF + CB: control diet + Enterococcus faecium NCIMB 11181 + Clostridium butyricum.
function, boosting disease resistance to pathogenic infection and preventing diarrhoea (Kreuzer-Redmer et al. 2016). On the other hand, the growing rabbit fed diet fortified with CB showed a tendency for enhanced growth but not significant. This could be linked to increased production of SCFAs in the caecum especially butyric acid that has high potency to enhance feed utilisation via balancing gut micro-ecological environments (Nakanishi et al. 2003).

In the existing study, it was evident that EF or EF + CB dietary supplementation considerably improved blood haemoglobin content in growing rabbits reared under thermal stress circumstances. In the intestinal tracts, lactic acid bacteria such as EF

Figure 1. Representative photomicrographs of duodenum of growing rabbit reared under summer conditions of different experimental groups. (A–C) control group fed non-supplemented diets, (D–F) colistin group, (G–I) Enterococcus faecium (EF) NCIMB 11181 group, (J–L) Clostridium butyricum (CB) group, (M–O) EF + CB group. Villi: V; Necrotic masses: N; Intestinal glands: G; Muscular layer: M; Serosa: red arrow; Crypt: C; Inflammatory cell infiltrations: I; Epithelium: E; Erosions: S; Stroma: ST; Intraepithelial lymphocytosis: L; Hyperplasia: HV; Congestion: black arrow; Goblet cell: green arrow (1st, 2nd, 3rd column of photos stained with H&E at 40×, 100×, and 400×, respectively).
produces lactic acid that increases the bioavailability of iron, the main component of haemoglobin (Marciano et al. 2015; Vonderheid et al. 2019). Also, this type of probiotic has been reported to produce bacteriocins and enzymes that facilitate the food digestion, decrease the intestinal pH, and subsequently increase the minerals absorption (Huang et al. 2019).

In the present study, the probiotic-supplemented growing rabbits showed marked enhancement in lysozyme activity than that of the non-supplemented group. In addition, the serum C3 levels of EF and EF + CB groups were significantly increased with an improvement tendency in CB group; suggesting that the EF + CB high efficacy in enhancing non-specific immune function in rabbits. Generally, the probiotic strains ability to initiate specific immune responses has been owed to the ability of theses bacterial types to retain their mucus adhesion potency following inactivation (Ouwehand et al. 2000). Consequently, immune cells detect individual structural components of bacterial cells and elicit specific immune responses (Taverniti and Guglielmetti 2011). In poultry, Wu et al. (2019) suggested that a main mechanism underlying the immunomodulatory effects of EF could be the modulation of the expression of proinflammatory and anti-inflammatory cytokines and other immune mediators. From another perspective, numerous researchers have demonstrated that the immune function and volatile fatty acids are closely related (Singh et al. 2014; Kelly et al. 2015). In this regard, in weaned piglets, Wang et al. (2019) confirmed that CB and EF stimulate the VAFs production by controlling the intestinal flora structure, thus boosting the immune function. This link between increased VFA content and enhanced immune function is apparent in our study. Nevertheless, the mechanisms that underlie the immunomodulatory elicited by EF and/or CB in relation to rabbit health need to be more investigated.

Herein, the serum levels of total protein and globulin were increased in the EF and/or CB-supplemented groups. Such increment in serum total protein in the rabbit by the inclusion of probiotic in their diets indicated that these probiotic strain has affected protein metabolism (El-Shafei et al. 2019), which is harmonious with the enhancement observed in the body weights of treated groups. Additionally, the increased globulin content is a significant indicator of the enhanced immune function.

In the present study, the caecal total VFA content was significantly increased in EF and EF + CB treated growing rabbits with an increasing tendency in CB treated ones. Moreover, the propionate concentration was significantly in all probiotic-supplemented groups. Similarly, earlier studies confirmed the increased VFA amounts in the caecal digesta of broilers (Han et al. 2018) and pigs (Wang et al. 2019) fed diet supplemented with other strains of EF and CB. The incremented caecal VFA has been reported to be associated with several favourable effects including repair and regeneration of intestinal epithelial cells, reducing the pH of the intestine, and prohibiting the growth of harmful microorganisms (Pryde et al. 2002; Józefiak et al. 2008).

In the current experiment, EF and CB dietary supplementation to growing rabbits reared under summer conditions showed a notable hypcholesterolemic effect. Several mechanisms have been hypothesised for the probiotics associated hypocholestermic activity including bile acids enzymatic deconjugation via probiotics bile-salt hydrolase (Lambert et al. 2008), cholesterol assimilation by probiotics (Pereira and Gibson 2002), cholesterol co-precipitation with deconjugated bile (Liong and Shah 2006), cholesterol attachment to probiotics cell walls (Liong and Shah 2005), cholesterol integration into the probiotics cell membranes throughout growth (Lye, Rahmat-Ali, et al. 2010), cholesterol conversion into coprostanol (Lye, Rusul, et al. 2010) and short-chain fatty acids production upon probiotics fermentation (De Preter et al. 2007).

In the current study, growing rabbits reared under thermal conditions and fed non-supplemented diets showed various pathological perturbations in their intestinal sections. Of note, the earlier group showed the smallest duodenal villus length and width but the larger crypt depth. On the other hand, the single or combined EF and CB addition to the rabbit diet significantly increased duodenal villus length and width but reduced crypt depth. The increased width and length of villus suggests that the intestinal absorption area is expanded, mature enterocytes are accumulated, and the absorption and digestion capacity is increased (Kim et al. 2012). Moreover, the diminishing crypt size refers to the recovery of intestinal villus by speeding up the regenerative enterocyte rate (Salim et al. 2013). Consequently, the gut can fight distressing damage by toxins or pathogens. Consistently, Wang et al. (2019) indicated that dietary CB or EF improved the intestinal health development and absorption of nutrients in weaned piglets by reducing the crypt depth and incrementing the villus height. Also, in weaned piglets, EF enhanced the intestinal villi length (Galeano et al. 2016). Additionally, Chen et al. (2018) reported that the duodenal, jejunal, and ileal villus
height was increased in lipopolysaccharide-challenged weaned piglets fed CB supplemented diet. Meanwhile, EF increased the villus length in the jejunum of suckling piglets (Liu et al. 2017). The increased VFA content could be partly shared to the improvement of intestinal health as earlier evidence suggested that microbiota-derived butyrate is vital for the preservation of the integrity of intestinal epithelium (Singh et al. 2014; Kelly et al. 2015).

Based on the estimated hematological, biochemical, and histological analysis, we could propose that the growth enhancing activity of the tested probiotics could be the outcome of the enhanced immune function, improved intestinal health, and augmented beneficial metabolites like caecal VFA. In this regard, several earlier studies confirmed the positive association of growth performance, the immune function, and the intestinal integrity in rabbits with diverse feed supplements (Alagawany et al. 2018; Al-Sagheer et al. 2019, 2020; Ayyat et al. 2021).

Of note, the combined EF and CB dietary supplementation resulted in a significant improvement of HDL, villus length, villus width, and intestinal muscle thickness rather than the single probiotic effect. Moreover, a maximal enhancement in other parameters including FBW, FCR, TG, HDL, VLDL, lysozyme activity, propionate, and crypt depth was evident in the EF + CB group. Such improvement denotes the favourable synergistic effect resulted from using these two probiotics. Similarly, Zhang et al. (2012) demonstrated that addition of the Lactobacillus salivarius and Bacillus subtilis probiotics combination to the diet of layer hens had a greater effect on growth and immune function than single strains.

Conclusion

Our results showed that EF or/and CB supplementation resulted in a significant improvement in final body weight, feed conversion ratio, immune function, and blood biochemistry in post weaned rabbits as compared with the control and colistin treatment under thermal stress. Furthermore, the application of the tested probiotics improved the caecal total volatile fatty acids and propionate concentrations along with the intestinal histomorphometry parameters. The results suggested that supplementation of growing rabbits diets with $2 \times 10^8$ cfu EF or $2.5 \times 10^6$ cfu CB + $1 \times 10^8$ cfu EF/kg feed could be used as an alternative to antibiotics to promote the growth under heat stress conditions.

Ethical approval

All care and experimental procedures including test animals in the current study were conducted in line with the scientific and ethical regulations recommended by the European Parliament Directive (2010/63/EU).

Disclosure statement

No potential conflict of interest was reported by the author(s).

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