Characterization and assessment of naturally mutant non-pathogenic O27 strain \textit{Escherichia coli} and their potential use as poultry probiotics

Nabila Osman\textsuperscript{1}, Shimaa A. M. Ahmed\textsuperscript{2}, Dina M. W. Shibat El-hamd\textsuperscript{2}, Ahmed I. Ahmed\textsuperscript{1}

\textsuperscript{1}Department of Poultry Diseases, Faculty of Veterinary Medicine, South Valley University, Qena, Egypt

\textsuperscript{2}Department of Poultry Diseases, Agricultural Research Center ARC, Animal Health Research Institute, Qena, Egypt

\section*{ABSTRACT}

\textbf{Objective:} The purpose of the current study was to evaluate the molecular characteristics of naturally mutant non-pathogenic O27 strain of \textit{Escherichia coli} and its efficacy as probiotic in broilers and determine the best age at which it can be administered.

\textbf{Materials and methods:} A total of 24 virulence genes using 24 sets of primers were detected using the polymerase chain reaction technique. For probiotics experiments, 60 chicks (day 1 old) were divided into three groups, 20 per group, and reared for 4 weeks. The first group was considered as a negative control. The second group was treated orally with O27 strain at first day of life for three successive days and repeated at day 21. The third group was administered orally with O27 strain at day 10 old, and repeated at day 21 old.

\textbf{Results:} The data revealed that type 1 fimbrial adhesion, salmochelin siderophore receptor, and sigma factor-binding protein were detected in O27 strain, but temperature-sensitive hemagglutinin, hemolysin secretion gene, pyelonephritis-associated Pili gene, polysaccharide capsule synthesis gene, Shiga-toxin1 gene, Shiga-toxin2 gene, Brain microvascular endothelial cell invasion, \textit{E. coli} attaching and effacing gene, heat-stable enterotoxin, heat-labile enterotoxin, \textit{eae} 1 toxin, \textit{colicin V}, \textit{verotoxin} type 2, nectrotizing cytotoxic factor type 1, colonization factor antigen I, colonization factor antigen III, \textit{coli} surface 2, \textit{coli} surface 4, serine protease zip autotransporter, vaculocytating autotransporter toxin, and serine protease EspP precursor were not detected in O27 strain. Group 2 performance parameters were significantly better ($p < 0.01$) than groups 3 and 1. Hematological and biochemical parameters did not be influenced ($p > 0.05$) by the administration of O27 strain. Antibody titers of infectious bursal disease virus and Newcastle disease virus in groups 2 and 3 were improved as compared to group 1. Group 2 had significantly higher titers than group 3. Histopathologically, all groups showed normal histopathological pictures. However, jejenum in groups 2 and 3 showed more tall, intact, and densely packed microvilli and more crypt depth than the control group.

\textbf{Conclusion:} The O27 strain of \textit{E. coli} is non-pathogenic bacteria. Its effects on growth performances and enhancement of immunity in broilers match with the same impact of probiotics, and these candidates will fit to be a good probiotic in the future. The results revealed that the effects of O27 strain at the day 1 old of life for three successive days and repeated at day 21 old are better for improving the performance and immunity of the birds. More research works about the characterized non-pathogenic \textit{E. coli} strain O27 are required for field and commercial use.

\section*{Introduction}

In many countries, the poultry industry has become an essential economic activity. In large-scale rearing facilities, where poultry is subjected to stressful conditions, disease-related problems and environmental deterioration often occur and result in significant financial losses. For many decades, antibiotics were used not only as a way of controlling infectious diseases but also for enhancing growth performance and feed efficiency [1]. However, despite extensive documentation on the evolution of
antimicrobial resistance among pathogenic bacteria, the utility of antimicrobial agents as a preventive measure has been questioned. Hence, the possibility of the antibiotic as a growth stimulant ceasing to be used for poultry and the concern about the side effects of its use as a therapeutic agent has created an environment in which consumers and manufacturers are searching for alternatives. Probiotics are considered to fill this gap, and some farmers already use them, preferably to antibiotics [2–4].

Probiotics are living microorganisms that enter actively into the gastrointestinal tract (GIT) and exert a positive influence on the host tissues [5]. Mach [6] concluded that probiotics were potentially a feasible alternative to antimicrobial growth promoters in broiler feeding, orally administered and beneficial to host health by inhibiting pathogens, improving immunity, and having a protective impact on intestinal microflora. Recent research has indicated probiotics’ possible therapeutic role in the prevention or treatment [7–10].

*Lactobacillus bulgaricus, Enterococcus faecium, L. plantarum, Escherichia coli, Bifidobacterium species, E. faecalis,* and *Streptococcus thermophilus* can be used in probiotic preparations [11]. It is believed that the microorganisms used as probiotics are non-pathogenic components of the normal microflora, such as the lactic acid bacteria. The non-pathogenic variants of pathogenic species can act in the same way as traditional probiotics do as avirulent mutants of *Clostridium difficile, E. coli,* and *Salmonella typhimurium* [12].

The naturally mutant non-pathogenic O27 strain was isolated from a liver sample of a healthy 7-day-old chick reared in commercial broiler chicken farm in Qena Governorate, Egypt, which could be an interesting candidate for a probiotic to prevent the bacterial diseases in poultry intestine and to improve growth performance and immunity of poultry [13]. The current study is intended to study the molecular genetic characteristics of naturally mutant non-pathogenic *E. coli* O27 strain by using polymerase chain reaction (PCR) and to study the pathogenicity, the age at which the naturally mutant non-pathogenic O27 strain of *E. coli* as a probiotic can be administered, and its effects on growth performance, induction of chicken antibody response, hematological and biochemical parameters, and histopathological studies.

**Materials and Methods**

**Ethical approval**

The Research Ethics Committee of the Faculty of Veterinary Medicine, University of South Valley, Egypt, has given ethical approval (approval number: Directive 2018/13/ VET-SVU). All procedures performed involving poultry during the current study were following the institutional guideline, which follows the international and national guidelines.

**Detection of virulence genes in *E. coli* O27 strain**

The PCR-technique was implemented with 24 sets of primers to detect 24 virulence genes. The genes were type 1 fimbrial adhesion (*fimH*), salmochelinsiderophore receptor (*iron*), sigma factor-binding protein (*crl*), temperature-sensitive hemagglutinin (*tsh*), hemolysin secretion gene (*hly*), pyelonephritis-associated Pili gene (*papC*), polysaccharide capsule synthesis gene (*kpsMTII*), shiga-toxin 1 (*stx1*), shigatoxin 2 (*stx2*), brain microvascular endothelial cell invasion (*ibeA*), *E. coli* attaching and effacing gene (*eaeA*), heat-stable enterotoxin (*Sta*), heat-labile enterotoxin (*Lt*), east 1 toxin (*astA*), colicin V (*cvAc*), verotoxin type 2 (*VT2e*), necrotizing cytotoxic factor type 1 (*cnf1*), colonization factor antigen I (*cfaI*), colonization factor antigen III (*cfaIII*), coli surface 2 (*CS2*), coli surface 4 (*CS4*), serine protease Pic autotransporter (*pic*), vacuolating autotransporter toxin (*vat*), and serine protease EspP precursor (*espP*) genes. PCR was applied by following QIAamp DNA Mini Kit instructions (Catalogue no. 51304) and EmeraldAmp GT PCR Master Mix (Takara) Code No. RR310A [14]. The positive PCR products for each gene were detected and visualized in single 1.5% agarose gel electrophoreses stained with ethidium bromide, viewed by the UV light.

Studying the pathogenicity, the age at which the naturally mutant non-pathogenic O27 strain of *E. coli* as a probiotic can be administered, and its effects on growth performance, induction of chicken antibody response, hematological and biochemical parameters, and histopathological studies

**Experimental birds**

Sixty-five broiler chicks (day 1 old) (Ross) were picked from a commercial hatchery. Five chicks (day 1 old) were sacrificed randomly on the first day, and samples from the liver, kidney, yolk swabs, pericardium, and air sacs were examined bacteriologically for pathogenic *E. coli*. All the results were *E. coli* negative. The remaining 60 chicks were assumed *E. coli* free, weighed individually, randomly assigned to three groups, and separately kept in a suitable environment at experimental units (Animal Health Research Institute, Qena, Egypt).

The broilers were reared for 4 weeks. The chicks of all groups were fed freely with age-appropriate commercial diets without the addition of antibacterial or anticoagulant components, before and through the experiments, and kept under daily observation of all groups with recording clinical signs and/or mortality, postmortem lesions.
The experimental birds were vaccinated with Hitchner and c所在的str. of Newcastle disease virus (NDV) on day 6 and day 18 through intraocular route, respectively, and also were immunized with an intermediate strain of infectious bursal disease virus (IBDV) through intraocular route on days 12 and 24 of age, respectively.

Experimental design

Each group containing 20 chicks was distributed in the following manner:

The first group was a negative control, fed a basal diet only. The second group was orally administered $6 \times 10^6$ Colony Forming Unit (CFU) viable organism/ml naturally mutant non-pathogenic O27 strain at the first day of life for three successive days and repeated at day 21 old. The third group was orally administered $6 \times 10^6$ CFU viable organism/ml naturally mutant non-pathogenic O27 strain of E. coli at day 10 old for three successive days and repeated at day 21 old.

Growth performance and nutrient utilization

Weights were taken on the first day and every week. Weekly feed consumption/grams were calculated every week until the fourth week. Feed conversion ratio (FCR) was obtained as the feed consumption per kg body weight (BW) gain. Performance index (PI) was obtained for each experimental diet as a ratio of average BW gain and FCR.

Hematological and biochemical analyses

At the last day of the experiment, five birds per group were slaughtered:

Whole blood was collected in a sterile ethylenediaminetetraacetic acid (EDTA) container for the estimation of differential leukocyte count (DLC) by using the standard two-slide wedge technique as in mammalian blood according to the previous studies [15–17]. Serum was analyzed for total protein by using the spectrophotometric technique (biuret method), according to Caprita et al. [18] and albumin by using the dye-binding technique, according to Gustafsson [19]. Globulins (G) were obtained through the subtraction of albumin (A) from total serum proteins, according to Ross et al. [20].

Assessment of immune response

Hemagglutination inhibition (HI) test was used to measure antibody titers to live NDV vaccine using Lasota Ag of NDV, which was prepared by reference laboratory for quality control on poultry production. In contrast, the enzyme-linked immunosorbent assay (ELISA) test was used to measure antibody titers to IBDV vaccine by using BioChek Poultry Immunoassays, an infectious bursal disease antibody test kit, Catalogue Code CK113. On the last day of the experiment, five birds per each group were selected randomly, and 5 ml of whole blood were collected from the wing vein of these birds in tubes. Whole blood was allowed to clot overnight at 4°C and then centrifuged at $3,000 \times gm$ for 10 min. The separated sera were stored at $-20^\circ$C till used in the serological tests.

Histopathological picture

Organ tissues (duodenum, jejunum, and cecum) which were collected aseptically for P/M examination were fixed in 10% formalin then washed with varying concentrations of alcohol and embedded in paraffin. Embedding cups containing organ tissue were filled with liquid paraffin and left to solidify on ice, after which the block was carefully removed to make 1-3-μm sections using a microtome. Hemotoxylin–eosin and toluidine blue staining were carried out according to the standard protocols.

Statistical analysis

The experimental data were subjected to statistical analysis using SPSS software by a completely randomized design employing a one-way analysis of variance according to Field [21] and the Chi-square test, according to Pearson [22]. The significant differences were taken at $p < 0.05$.

Results

Polymerase chain reaction

PCR results shown in Figure 1 revealed that fimH, iroN, and crl genes were detected in O27 strain, but tsh, hly, papC, kpsMTII, stx1, stx2, iberA, eaeA, STa, LT, astA, cvaC, VT2e, cnf1, caf1, cafIII, CS2, CS4, pic, vat, and espP were not detected in O27 strain.

Clinical signs and postmortem lesions

All birds of the three groups appeared healthy, full vitality, and activity with no post-mortem (PM) lesions, as shown in Figure 2.

Mortality rate

There was no mortality along the period of the experiment in all the groups.

Growth performances and nutrient utilization

Mean BW, feed consumption, and PI are shown in Table 1. Significantly ($p < 0.01$) higher performance parameters, BW, weekly feed consumption/grams, and PI were recorded in broilers of groups 2 and 3 compared to the control group (group 1). Group 2 showed significantly higher performance parameters than group 3.

Hematological findings

O27 strain of E. coli supplementation did not influence ($p > 0.05$) the DLC in two groups (groups 2 and 3), and also group 1 exhibits normal hematological parameters, as shown in Table 2.
Figure 1. PCR products of amplified of virulent genes identified in the O27 strain of E. coli visualized on agarose gel electrophoresis. The expected molecular size of amplified DNA: 508 bp for fimH gene (A), 847 bp for iroN gene (B), 250 bp for crl gene (C), 620 bp for tsh gene (D), 1177 bp for hly gene (E), 501 bp for papC gene (F), 280 bp for kpsMTII gene (G), 614 bp for stx1 gene (H), 779 bp for stx2 gene (I), 342 bp for ibelA gene (J), 248 bp for eaeA gene (K), 229 bp for StA gene (L), 606 bp for LT gene (M), 110 bp for astA gene (N), 760 bp for cvaC gene (O), 322 bp for VT2e gene (P), 620 bp for cnf1 gene (Q), 364 bp for cfaI gene (R), 437 bp for cfaII gene (S), 368 bp for CS2 gene (T), 250 bp for CS4 gene (U), 572 bp for pic gene (V), 420 bp for vat gene (W), and 547 bp for espP gene (X), Lane PC: Positive Control, Lane NC: Negative Control, Lane S: samples, and Lane (L) DNA ladder 100 bp.
Figure 2. Post-mortem (PM) results of 28-day-old chickens from group 1 (A), group 2 (B), and group 3 (C), all of them showing healthy liver and heart. (D) 28-day-old chickens from group 1 on the left, group 2 in the middle, and group 3 on the right, all of them showing normal duodenum. (E) 28-day-old chickens from group 1 on the left, group 2 in the middle, and group 3 on the right, all of them showing normal jejunum. (F) 28-day-old chickens from group 1 on the left, group 2 in the middle, and group 3 on the right, all of them showing normal cecum.

Table 1. Effect of the naturally mutant non-pathogenic O27 strain of E. coli supplementation on growth performances of broiler chickens.

| Groups | Age/week | Mean weekly BW/gm | Weekly feed consumption/gm | PI |
|--------|----------|-------------------|-----------------------------|----|
| Group 1 | First    | 143               | 800                         | 286|
| Group 2 | Second   | 152.7             | 3,725                       | 340.4|
| Group 3 | Third    | 374.5             | 6,000                       | 991.5|
| Chi-Square |        | 4.93269           | 79.9383                     | 4.49114|
| Significance |     | Non               | 0.01                        | Non|
| Group 1 | Fourth   | 669.3             | 7,000                       | 440.3|
| Group 2 |         | 842.7             | 9,000                       | 601.9|
| Group 3 |         | 750.3             | 8,000                       | 577.1|
| Chi-Square |        | 19.9648           | 250                         | 25.8383|
| Significance |     | 0.01              | 0.01                        | 0.01|
| Group 1 |         | 1,000             | 10,000                      | 769.2|
| Group 2 |         | 1,289             | 11,000                      | 991.5|
| Group 3 |         | 1,229             | 10,500                      | 945.3|
| Chi-Square |        | 39.6708           | 47.619                      | 30.5111|
| Significance |     | 0.01              | 0.01                        | 0.01|
Biochemical findings

As shown in Table 3, there were normal biochemical parameters with no significant \((p > 0.05)\) difference in all groups.

Immune response

As shown in Table 4, the supplementation of O27 strain in groups 2 and 3 increased the antibody titers against Newcastle disease (ND) and infectious bursal disease (IBD) comparing to untreated control (group 1). The birds in group 2 had significantly \((p < 0.01 \text{ and } p < 0.05)\) higher titers against ND and IBD, respectively, than those in group 3.

Histopathological finding

Normal histopathological pictures were in all birds of the three groups, but the longer, intact, and densely packed microvilli and more crypt depth in the jejunum of groups 2 and 3 compared to the control group 1 as shown in Figure 3.

Discussion

The current article studied the genetic characteristics of the O27 strain of \(E.\ coli\) and its relation to virulence because the probiotic must be avirulent. \(fimH\) gene was detected in O27 strain, and this finding agreed with Lasaro et al. [23], who mentioned that \(fimH\) was also detected in EcN (\(E.\ coli\) strain Nissle 1917) which is a probiotic of \(E.\ coli\) origin. This finding was explained by Sonnenborn and Schulze [24]. They mentioned that \(fimH\) facilitates adhesion to intestinal epithelial cells in cell culture \textit{in vitro} experiments or to the mucus layer of the intestinal wall \textit{in vivo}, facilitating colonization of the gut.

\(iroN\) gene was detected in O27 strain, and this finding agreed with Sonnenborn and Schulze [24], who found that \(iroN\) was also detected in EcN. \(iroN\) which is one of siderophores that are iron-chelating substances needed for bacterial iron uptake [25]. Iron is an essential element for \(E.\ coli\) survival. It facilitates numerous cellular activities, such as reducing peroxide, transporting electrons, and biosynthesizing nucleotides [26, 27].

\(crl\) (sigma factor-binding protein) gene was detected in O27 strain, this gene is required for normal stationary phase during stress conditions, and this result agreed with Lelong et al. [28], who mentioned that \(crl\) gene was previously believed to encode the significant curli subunit of curli (curli are surface structures that are induced by growth at stationary phase during stress conditions as low osmolarity and low temperature). It was later established that \(crl\) makes a positive contribution to stimulating the transcription of \(csgBA\), the true locus encoding for the main subunit of curli. The standard stationary-phase induction of \(csgBA\) requires RpoS as well. Based on these results, the presence of three previous genes strengthens its effects as a probiotic through mediating its colonization of the gut, increasing its ability to iron uptake needed for its cellular activities, and surviving during stationary phase under stress conditions.
hly, papC, stx1, stx2, STa, LT, cfaI, cfaIII, CS2, and CS4 virulence genes were not detected in O27 strain, and this result agreed with Sonnenborn and Schulze [24], who mentioned that these genes were not identified in EcN. The obtained data refer to the high similarity between O27 and EcN strains, which is a probiotic of E. coli origin and supports the idea of being O27 a probiotic.

kpsMTII was not detected in O27 strain, and this finding agreed with Miajlovic et al. [29] who mentioned that, in many extraintestinal pathogenic E. coli (ExPEC) strains, the existence of a capsule protects the pathogen from attacks by non-specific blood serum defense components (e.g., complement), thus making the serum resistant to bacteria. Serum resistance expanded the bloodstream’s survival time and increases a pathogen’s virulence. In light of the above, the O27 strain is serumsensitive and is rapidly destroyed in the presence of human serum or sera of other mammalian species. In the case of comparing this finding with EcN, this finding disagreed with Sonnenborn and Schulze [24], who mentioned that kpsMTII was detected in EcN, but interestingly it is serumsensitive.

tsh, ibeA, astA, cvaC, VT2e, cnf1, picesP, and vat were not detected in O27 strain, and these results agreed with Compos et al. [30], who proved that the tsh gene was found among chickens-isolated avian pathogenic Escherichia coli (APEC) strains, and also agreed with Germon et al. [31], who stated that the ibeA gene is known as the E. coli strains virulence factor and plays a significant role in some APEC strains pathogenicity. Barnes et al. [32] mentioned that the intimin produced by the eaeA gene found in enterohemorrhagic E. coli and enteropathogenic E. coli. Yamamoto and Nakazawa [33] found that astA gene has been found in enterotoxigenic E. coli. Furthermore, Janben et al. [34] mentioned that astA was described in APEC strains. Gérard et al. [35] and Skyberg et al. [36] suggested that cvaC participates in the synthesis, export, and immunity of a peptide antibiotic Colicin V, which has been discovered in APEC and associated with avian colibacillosis. Dziva and Stevens [37] found that several toxins were described in APEC strains. These involve cnf1 and cytotoxin designated VT2e. Otto et al. [38] and Henderson et al. [39] mentioned that vat and pic had been reported in APEC and human ExPEC. Hence, these genes have an essential role in APEC and other pathogenic E. coli virulence by different mechanisms, and their absence from O27 strain confirms that it is free from virulence and its high chance of being a probiotic.

Referring to the pathogenicity of O27 in vivo, all birds of the three groups appeared healthy, full vitality, and
activity with no PM lesions and did not record any mortalities along with the experiment. This result agreed with Otutumi et al. [12] who mentioned that the microorganisms used as probiotics are non-pathogenic components of the normal microflora, such as the lactic acid bacteria or non-pathogenic variants of pathogenic species, such as avirulent mutants of E. coli.

Higher performance parameters (BW, weekly feed consumption/gm, and PI) were recorded significantly ($p < 0.01$) in broilers, which administered O27 strain (second and third groups) compared to the control group (first). This result was explained by Yang et al. [40], who mentioned that probiotics improve growth performances by certain mechanisms include intestinal flora alteration, stimulation of the growth of non-pathogenic facultative anaerobic and Gram-positive lactic acid-forming bacteria and hydrogen peroxide, suppression of the growth of intestinal pathogens, and digestion and utilization of nutrients. This also comes in agreement with Otutumi et al. [12], who said that the non-pathogenic variants of pathogenic species can act in the same way as traditional probiotics do as avirulent mutants of Clostridium difficile, E. coli, and Salmonella typhimurium.

Group 2 showed significantly higher performance parameters than group 3. This result exactly comes in agreement with Baldwin et al. [41]. They explained that, at hatch, the first bacteria entering the intestine could attach to epithelial cells without competition and quickly establish, increase in number and become set the intestinal environment to best suit their own needs in terms of metabolite profiles and pH. Bacterial species diversity and distribution constitute the GIT microbiota, fluctuates post-hatch, and becomes as fast as day 3 after the establishment period, and microbiota continue to mature at a slower rate. Hence, the result which can be obtained is better to give the naturally mutant non-pathogenic O27 strain of E. coli at the first 3 days than the day 10 in broilers and repeated at 21-day old.

The O27 strain of E. coli supplementation did not influence ($p > 0.05$) the hematological and biochemical parameters in the two groups (Groups 2 and 3), and also group 1 exhibits normal hematological parameters. These results typically agreed with Alkhalf et al. [2] and Kumar et al. [42], who recorded that The hematological and biochemical parameters were not affected ($p > 0.05$) by the dietary supplementation of probiotics.

This study revealed that the supplementation of the O27 strain of E. coli in groups 2 and 3 improved the antibody titers to ND comparing to the untreated control group (First). Birds of second group had significantly ($p < 0.01$) higher titers than those in the third group, also its supplementation improved the antibody titers to IBD ($p > 0.05$) in groups 2 and 3 than in first group. Also, the second group had significantly ($p < 0.05$) higher titers than those in the third group. These results typically agreed with Talebi et al. [42] and Kumar et al. [43]. They recorded that there was a gradual increase in antibody titer against NDV and IBDV due to the supplementation of various probiotics comparing to the control group. This result was explained by Haghighi et al. [44] and Tung and Herzenberg [45], who mentioned that probiotic stimulates naturally occurring antibodies in unimmunized chickens. Functionally, B-cells participate in innate immunity by producing most of the natural serum IgM that protects against invading pathogens before adaptive immune response begins.

In this study, all birds of the three groups showed normal histopathological pictures. Still, the jejunum from birds of groups 2 and 3 showed more tall, intact, and densely packed microvilli and more crypt depth than the control group 1. These results agreed with Bai et al. [46]. They mentioned that Probiotic dietary treatment had been documented to increase the villi height and crypt depth of broilers in the small intestine compared with chickens fed with antibiotics or probiotics feed free. Increased the height of villi is advantageous for broilers because the increased surface area of the villi improved nutrient absorption [47]. Merrifield et al. [48] recorded that broilers’ gut health was characterized by intact and densely packed microvilli on administration with probiotics.

**Conclusion**

The results obtained in our study demonstrate that the O27 strain of E. coli is non-pathogenic bacteria because of the absence of the different virulence factors from it and is very close to probiotic E. coli strain Nissle 1917, which is a probiotic of E. coli origin. Our results about the naturally mutant non-pathogenic O27 strain of E. coli and its effects on growth performances and enhancement of immunity in broilers match with the same effects of probiotics, and these candidates will fit to be a good probiotic in the future. The results revealed that the effects of O27 strain at the day 1 old of life for three successive days and repeated at day 21 old are better for improving the performance and immunity of the birds. More research works about the characterized non-pathogenic E. coli strain O27 are required for field and commercial use as studying its resistance against the commonly used antibiotics in the poultry industry, studying its gastric juice resistance (low pH level) and bile acid, and studying its ability of inhibition of the growth of other common pathogens of poultry in vitro and in vivo.
Acknowledgments
The authors would like to acknowledge South Valley University, Qena, Egypt, for supports.

Conflict of interest
The authors declare that there is no conflict of interest.

Authors’ contribution
All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Abbreviations
IBDV: infectious bursal disease virus; NDV: Newcastle disease virus; ironN: salmochelin siderophore receptor; crtl: sigma factor-binding protein; Tsh: temperature-sensitive hemagglutinin; Hly: Hemolysin secretion gene; papC: pyelonephritis-associated Pili gene; kpsMTII: polysaccharide capsule synthesis gene; Stx1: Shiga-toxin1 gene; Stx2: Shiga-toxin2 gene; iberA: Brain microvascular endothelial cell invasion; eaeA: E. coli attaching and effacing gene; STa: heat-stable enterotoxin; LT: heat-labile enterotoxin; astA: east 1 toxin; cvaC: colicin V; VT2e: verotoxin type 2; Cnf1: necrotizing cytotoxic factor type 1; CFA1: colonization factor antigen I; CFAI11: colonization factor antigen III; Csa2: coli surface 2; Csf4: coli surface 4; Pic: serine protease pic autotransporter; Vat: vacuolating autotransporter toxin; espP: serine protease EspP precursor; PI: performance index.

References
[1] Gadde UD, Oh S, Lillehøj HS, Lillevold EP. Antibiotic growth promotors: perspectives on interactions between the chicken intestinal metabolome. Sci Rep 2018; 8(1):1–8; https://doi.org/10.1038/s41598-018-22004-6
[2] Alkhalif A, Alhaj M, Al-Homidam I. Influence of probiotic supplementation on immune response of broiler chickens. Egypt Poult Sci 2010; 30:271–80.
[3] Dham K, Verma V, Sawant PM, Tiwari R, Vaid RK, Chauhan RS. Applications of probiotics in poultry: enhancing immunity and beneficial effects on production performances and health - a review. J Immunol Immunopathol 2011; 13(1):1–19.
[4] Pan D, Yu Z. Intestinal microbiome of poultry and its interaction with host and diet. Gut Microbes 2014; 5(1):108–19; https://doi.org/10.4161/gmic.26945
[5] Böhm SK, Krüsk W. Probiotics: do they help to control intestinal inflammation? Ann N Y Acad Sci 2006; 1072(1):339–50; https://doi.org/10.1196/annals.1326.005
[6] Mach T. Clinical usefulness of probiotics in inflammatory bowel diseases. J Physiol Pharmacol 2006; 57(Suppl 9):23–33.
[7] Onnoord CG, Besdik SS, Ipi P, Choc M. Delivery routes for probiotics: effects on broiler performance, intestinal morphology and gut microflora. AnimNutr 2015; 1:192–202; https://doi.org/10.1016/j.aninut.2015.07.002
[8] Al-Badani HH, Abudhos AM, Al-Mufareej SI, Alzawqari M. Effects of dietary inclusion of probiotics, prebiotics and synbiotics on the intestinal histological changes in challenged broiler chickens. S Afr J Anim Sci 2016; 46:157–65; https://doi.org/10.4314/sajas.v46i2.6
[9] Gesek M, Sokol R, Lambert B, Ostroda-Komagala I. Effect of effective microorganisms on intestine morphology and morphometry in Japanese quails. Turk Vet Anim Sci 2018; 42:285–91; https://doi.org/10.3906/vet-1801-29
[10] Souza de LFA, Araujo DN, Stefani LM, Giometti IC, Cruz-Polycarpo VC, Polycarpo G, et al. Probiotics on performance, intestinal morphology and carcass characteristics of broiler chickens raised with lower or higher environmental challenge. Austral J Vet Sci 2018; 50:35–41; https://doi.org/10.4067/S0719-81322018000100107
[11] Fuller R. Probiotics in man and animals. J Appl Bact 1989; 66:365–78; https://doi.org/10.1111/j.1365-2672.1989.tb05105.x
[12] Otutumi LIK, De Moraes García ER, Góis MM, Loddi MM. Variations on the efficacy of probiotics in poultry. In: Rigobelo EC (ed.) (Rijeka: InTech), Probiotics in animals, London, EC3R 6AE, UK, 2012, pp 203–230.
[13] Ahmed SAM, Ahmed IA, Osman N, Shabat El-Hand DMW. Problem of Escherichia coli infection in broilers in qena province. Assiut Vet J 2018; 50:35–41; https://doi.org/10.1007/s41025-018-0287-8
[14] van der Velden J, Pfitsch EF, Montias T. Molecular biology. In: Molecular cloning. Laboratory manual. 2nd edition, Cold Spring Harbor Laboratory press, New York, NY, p 268, 1989.
[15] Schalm OW, Jain NC, Carroll EJ. Veterinary hematology. 3rd edition, Lea and Febiger, Philadelphia, PA, p 21, 1975.
[16] Cokes EH. Veterinary clinical pathology. 4th edition, WB Saunders Co, Philadelphia, PA, pp 53–54, 1986.
[17] Campbell TW. Avian hematology and cytology. Iowa State University Press, Ames, IA, 1988.
[18] Caprira R. Principii si tehnici in biochimie. Mirton, Timisoara, UK, pp 125–9, 2001.
[19] Gustafsson JE. Automated serum albumin determination by use of the immediate reaction with bromcresol green reagent. Clin Chem 1978; 24(2):369–73; https://doi.org/10.1093/clinchem/24.2.369
[20] Ross GJ, Christie G, Halliday WG, Jones RM. Determination of haematocrit by the microcentrifuge method. J Clin Pathol 1976; 29(6):499–502; https://doi.org/10.1136/jcp.29.6.499
[21] Pearson K. On the criterion that a given system of deviations from the probable in the case of a correlated system of variables is such that it can be reasonably supposed to have arisen from random sampling. Philos Mag 1900; 50:157–75; https://doi.org/10.1080/14787440009463897
[22] Lasaro MA, Salinger N, Zhang J, Wang Y, Zhong Z, et al. F1C fimbriae play an important role in biofilm formation and intestinal colonization by the Escherichia coli commensal strain Nissle 1917. Appl Environ Microbiol 2009; 75(4):273–81; https://doi.org/10.1128/AEM.01144-08
[23] Pearson K. On the criterion that a given system of deviations from the probable in the case of a correlated system of variables is such that it can be reasonably supposed to have arisen from random sampling. Philos Mag 1900; 50:157–75; https://doi.org/10.1080/14787440009463897
[24] Sonnenborn U, Schulze J. The non-pathogenic Escherichia coli. In: Köhn EM, Glaeser A (eds) The influence of dietary inclusion of probiotics, prebiotics and synbiotics on the immediate reaction with bromcresol green reagent. Clin Chem 1978; 24(2):369–73; https://doi.org/10.1093/clinchem/24.2.369
[25] Lasaro MA, Salinger N, Zhang J, Wang Y, Zhong Z, et al. F1C fimbriae play an important role in biofilm formation and intestinal colonization by the Escherichia coli commensal strain Nissle 1917. Appl Environ Microbiol 2009; 75(4):273–81; https://doi.org/10.1128/AEM.01144-08
Escherichia coli: salmonchelin and aerobactin contribute more to virulence than heme in a chicken infection model. BMC Microbiol 2012; 12:143; https://doi.org/10.1186/1471-2180-12-143

[28] Lelong C, Aguiluz K, Luche S, Kuhn L, Garin J, Raballoud T, et al. The CrI-RpoS regulon of Escherichia coli. Mol Cell Proteom 2007; 6(4):648–59; https://doi.org/10.1074/mcp.M600191-MCP200

[29] Mijalovic H, Cooke NM, Moran GP, Rogers TR, Smith SG. Response of extraintestinal pathogenic Escherichia coli to human serum reveals a protective role for RcS-regulated exopolysaccharide colanic acid. Infect Immun 2014; 82(1):298–305; https://doi.org/10.1128/JI.00800-13

[30] Compos TA, Stehling EG, Ferreira A, De Castro AFP, Brocchi M, DaSilveira WD. Adhesion properties, fimbrial expression and PCR detection of adhesin-related genes of avian Escherichia coli strains. Vet Microbiol 2005; 106(3):275–85; https://doi.org/10.1016/j.vetmic.2004.12.025

[31] Germon P, Chen YH, He L, Blanco JE, Bree A, Schouler C, et al. ibeA, a virulence factor of avian pathogenic Escherichia coli. Microbiol 2005; 151(4):1179–96; https://doi.org/10.1099/mik.0.27809-0

[32] Barnes HJ, Nolan lk, Vaillancourt JA. Colibacillosis in Saif YM, Benson HJ. Microbiological applications. 8th edition, McGraw Hill, New York, NY, p 95, 2008. Bacterial Population Counts. ICBN# 0-07-231889-9.

[33] Yamamoto T, Nakazawa M. Detection and sequences of the enteroaggregative Escherichia coli heat-stable enterotoxin 1 gene in strains isolated from piglets and calves with diarrhea. J Clin Microbiol 1997; 35(1):223–7; https://doi.org/10.1128/JCIM.35.1.223-227.1997

[34] Janben T, Schwarz C, Preikschat F, Voss M, Philipp HC, Wieler LH. Virulence associated genes in avian pathogenic Escherichia coli (APEC) isolated from internal organs of poultry having died from colibacillosis. Int J Med Microbiol 2001; 291(5):371–8; https://doi.org/10.1016/S0307-9456(00)00143

[35] Gérard F, Pradel N, Wu LF. Bacterial activity of colicin V is mediated by an intermembrane protein, Sdac, of Escherichia coli. J Bacteriol 2005; 187(6):19–50; https://doi.org/10.1128/JB.187.6.1945-1950.2005

[36] Skyberg JA, Johnson TJ, Johnson JR, Clabots C, Logue CM, Nolan LK. Acquisition of avian pathogenic Escherichia coli plasmids by a commensal E. coli isolate enhances its abilities to kill chicken embryos, grow in human urine, and colonize the murine kidney. Infect Immun 2006; 74(11):6287–92; https://doi.org/10.1128/IAI.00363-06

[37] Dziva F, Stevens MP. Colibacillosis in poultry: unravelling the molecular basis of virulence of avian pathogenic Escherichia coli in their natural hosts. Avian Pathol 2008; 37(4):355–66; https://doi.org/10.1080/03079450802216652

[38] Otto BR, Van Dooren SJ, Nuijens JH, Lurink J, Oudega B. Characterization of a hemoglobin protease secreted by the pathogenic Escherichia coli strain EBL. J Exp Med 1998; 180(6):1091–103; https://doi.org/10.1084/jem.180.6.1091

[39] Henderson JR, Czecculín J, Eslava C, Noriega F, Nataro JP. Characterization of Pic, a secreted protease of Shigella flexneri and enteroaggregative Escherichia coli. Infect Immun 1999; 67(11):5587–96; https://doi.org/10.1128/IAI.67.11.5587-5596.1999

[40] Yang Y, Li PA, Chocht M. Dietary modulation of gut microbiota in broiler chickens: a review of the role of six kinds of alternatives to in-feed antibiotics. Worlds Poult Sci J 2009; 65(1):97–114; https://doi.org/10.1016/S00439339(09)000087

[41] Badwin S, Hughes RJ, Van TTH, Moore RJ, Stanley D. At-hatch administration of probiotic to chickens can introduce beneficial changes in gut microbiota. PLoS One 2018; 13(3):e0194825; https://doi.org/10.1371/journal.pone.0194825

[42] Kumar L, Singh PK, Kumar M. Effect of dietary supplementation of combination of probiotics on the growth performance and immune response of broiler chickens. Anim Nutr Feed Technol 2013; 13(1):15–25; https://doi.org/10.1080/03079450802356995

[43] Talebi A, Amirzadeh B, Mokhtari B, Gahri H. Effects of a multi-strain probiotic (PrimaLac) on performance and antibody responses to Newcastle disease virus and infectious bursal disease virus vaccination in broiler chickens. Avian Pathol 2008; 37(5):509–12; https://doi.org/10.1080/03079450802356995

[44] Haghighi HR, Gong J, Cyles CL, Hayes MA, Zhou H, Sanei B, et al. Probiotics stimulate production of natural antibodies in chickens. Clin Vaccine Immunol 2006; 13:975–80; https://doi.org/10.1128/CVI.00161-06

[45] Tung JW, Herzenberg LA. Unraveling B-1 progenitors. Curr Opin Immunol 2007; 19(2):150–5; https://doi.org/10.1016/j.coi.2007.02.012

[46] Bai SP, Wu AM, Ding XM, Lei Y, Bai J, Zhang KY, et al. Effect of probiotic-supplemented diets on growth performance and intestinal immune characteristics of broiler chickens. Poult Sci 2013; 92(3):663–70; https://doi.org/10.3382/ps.2012-02813

[47] Awad WA, Ghareeb K, Abdel-Raheem S, Böhm J. Effects of dietary inclusion of probiotic and symbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. Poult Sci 2009; 88(1):49–56; https://doi.org/10.3382/ps.2008-00244

[48] Merrifield DL, Dimitroglou A, Bradley G, Baker TM, Davies SJ. Soybean meal alters autochthonous microbial populations, microvilli morphology and compromises intestinal enterocyte integrity of rainbow trout, Oncorhynchus mykiss (Walbaum). J Fish Dis 2009; 32(9):755–66; https://doi.org/10.1111/j.1365-2761.2009.01052.x