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The beneficial effects of a multistrain potential probiotic, formic, and lactic acids with different vaccination regimens on broiler chickens challenged with multidrug-resistant *Escherichia coli* and *Salmonella*

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ABSTRACT

The effects of a multistrain potential probiotic (Protexin[®]), acids, and a bacterin from multidrug-resistant *E. coli* O26, O78, *S. Enteritidis* (1,9,12 g.m1,7), and *S. Typhimurium* (1,4,5,12.i.1,2) on the immune response, haematological parameters, cytokines, and growth parameters of broiler chickens challenged with bacterin live serotypes were investigated. Two experiments were designed using 300 one-day-old chicks (Arbor Acres) randomly assigned to 15 groups. The first experiment comprised 9 groups, including positive and negative control groups and other groups received Protexin[®], acids, and the bacterin (0.2 ml/SC), either alone or in combination, on the 1st day. The second experiment contained 6 groups, including positive and negative control groups and other groups received a combination of Protexin[®], acids, and the bacterin (0.5 ml/SC) on the 8th day. All the groups except the negative control groups were challenged on the 8th and 16th days in both experiments, respectively, with mixed live bacterin serotypes. The groups that received Protexin[®], acids, and the bacterin either alone or in combination revealed significant improvements in the immune response to the bacterin ($p \leq 0.05$). The groups in the 1st experiment and most the 2nd experiment groups showed a reduced mortality rate and decreased levels IFN- γ , IL-4, and IL-12 cytokines ($p \leq 0.05$). Moreover, these groups demonstrated increases in haematological parameters and reduced rates of infection-caused anaemia. These groups showed significant increases in growth performance parameters, such as body weight, weight gain, and the feed conversion ratio (FCR) ($p \leq 0.05$). There was a beneficial effect on 1-day-old chickens produced by combining Protexin[®], acids, and the bacterin (0.2 ml/SC).

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1. Introduction

Constant human population growth is strongly linked to animal-based food products of high quality. The uncontrolled use

of antimicrobials has led to the emergence of drug-resistant microorganisms that represent a threat to public health (Rousham et al., 2018). Consequently, alternative substances, including probiotics and acidifiers have been used to produce similar effects (Cameron and McAllister, 2019). Protexin[®] is a commercially available multistrain potential probiotic which considered as a complementary feed for animals. The Protexin[®] contained bacteria showed antimicrobial activity against *Salmonella* Typhimurium LT2, *Escherichia coli* NCFB 1989, *Staphylococcus aureus* NCTC 8532, *Enterococcus faecalis* NCTC 775, and *Clostridium difficile* ATCC 43,594 (Tejero-SariNena et al., 2012). Many studies have confirmed the safety and efficacy of the Protexin[®] on immune response, blood chemistry, and growth performance. The Protexin[®]

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supplementation improved the immune response to Newcastle disease virus and lowered the counts of cecal *E. coli* (Khan et al., 2013). Nutrilac® contained lactic and formic acids that significantly reduced the shedding of intestinal pathogens and lesions in broiler chickens (Abd El-Ghany et al., 2015). *E. coli* O26, O78, *Salmonella* Typhimurium, and *Salmonella* Enteritidis infect chicken farms in Egypt with significant economic losses and are implicated in human infections. Multidrug-resistant (MDR) *E. coli* and *Salmonella* serotypes interfere with treatment and can transfer resistance to various pathogens (Tawyabur et al., 2020). Killed vaccines, probiotics, and acids have been used to control MDR bacterial agents through enhanced immune response and competitive exclusion (Kabir, 2009; Salehi et al., 2012; Nhung et al., 2017; Abdelhamid et al., 2018). Probiotics enhance the activities of macrophages and natural killer cells, modulate cytokine and immunoglobulin secretion, and promote intestinal epithelial barrier integrity (La Fata et al., 2018). The probiotics activate B lymphocytes that transform into antibody-secreting plasma cells; that will enter the gut-draining lymphoid tissue (Bibas Bonet et al., 1999; Cetin et al., 2005; Park et al., 2016). Probiotic supplementation results in a significant increase in the total leukocyte counts and causes lymphocyte proliferation that enhances the immune response against pathogens (Cetin et al., 2005; Khaksefidi and Ghoorchi, 2006). Pathogenic bacteria induce inflammation that increases the secretion of gamma interferon (IFN)- γ , tumor necrosis factor (TNF), interleukin (IL)-1 β , IL-6, and IL-12 cytokines (Tohidi et al., 2012; Hu et al., 2015). IL-4 stimulates the proliferation of B cells and T cells and differentiates CD4⁺ T cells into Th2 cells (Annamalai and Selvaraj, 2012). The supplementation of chickens with *Lactobacillus* species can significantly mitigate alterations in pro-inflammatory cytokine gene expression profiles (Mead, 2000; Hu et al., 2015).

Hence, this study aimed to evaluate the effects of a multistrain potential probiotic (Protexin®), Nutrilac® containing (lactic and formic acids) either alone or in combination with different vaccination regimens of a bacterin containing MDR *Escherichia coli* O26, O78, *Salmonella* Enteritidis (1,9,12 g.m 1,7), and *Salmonella* Typhimurium (1,4,5,12.i.1,2) serotypes on the immune response against these live pathogens. Additionally, mortality rates, cytokine production, and growth performance parameters were also investigated.

2. Materials and methods

2.1. The potential probiotic and acids

The used potential probiotic was Protexin® (ADM Protexin Ltd, Lopen Head, Somerset, United Kingdom), a commercial potential probiotic available in Egypt, it is white dry powder containing 2×10^9 cfu/g of *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subspecies *bulgaricus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Bifidobacterium bifidum*, *Enterococcus faecium*, *Streptococcus thermophiles*, *Aspergillus oryzae*, and *Candida pintolepesii*. It was used at a dose of 1 g/ litre at the first 5–7 days of age. The Nutrilac® (I.G.A.Ega vet, England), is composed of 90% lactic acid and 10% formic acid, was administered alone or in combination with the Protexin®, and bacterin at a dose of 1 ml/ litre.

2.2. Vaccine preparation

A multistrain killed vaccine was prepared from the following multi-drug resistant (MDR) poultry pathogens: *E. coli* O26, O78, *S. Enteritidis* (1,9,12.g.m 1,7), and *S. Typhimurium* (1,4,5,12.i.1,2). These serotypes were selected due to their serious infections in broiler farms in Egypt (Abd El-Mongy et al., 2018; Sedeik et al.,

2019). The selected serotypes were tested against 20 antimicrobials: amikacin, ampicillin/sulbactam, amoxicillin, amoxicillin/clavulanic acid, cefotaxime, ciprofloxacin, cloxacillin, doxycycline, erythromycin, gentamycin, imipenem, levofloxacin, nalidixic, norfloxacin, ofloxacin, penicillin, rifamycin, streptomycin, tobramycin, and vancomycin. All the serotypes were resistant to all the applied antimicrobials except imipenem, with a multiple antimicrobial resistance index of 0.95, which was calculated as the ratio between the number of ineffective antimicrobials and the total number of used types. The serotypes were cultivated on nutrient broth and incubated for 18 h at 37 °C. The broth cultures were washed twice by centrifugation (11,000x g for 20 min) and resuspended in sterile PBS (pH 7.2). The bacterial density was adjusted to a final concentration of 8×10^{11} / 1 ml using a spectrophotometer at 600 nm. The bacteria were killed with 0.5% formalin, and inactivation was confirmed by plating on MacConkey agar medium and incubation for 24 h at 37 °C. Then, the culture was washed with saline, and Montanide ISA 206VG (Seppic, France), was used as an adjuvant and mixed with the culture at a ratio of 1:1. The stability of the vaccine was conferred by adding 2.5% of skimmed milk (Barbour et al., 2002). There were two types of formulated vaccines; the first one contained a bacterial concentration of 2×10^{11} / 0.2 ml and was administered to the groups that were vaccinated on the first day of life in the first experiment, and the second type contained a bacterial concentration of 2×10^{11} /0.5 ml and was administered to the groups that were vaccinated on the 8th day of life in the second experiment (Charles et al., 1994; Muir et al., 1998). Vaccine safety was detected using 45 specific pathogen-free chicks that were purchased from the Qum Oshim specific pathogen-free (SPF) farm at Fayoum Governorate, Egypt. The chicks were randomly grouped and injected with a double field dose as follows: 15 chicks were injected with 0.4 ml/ SC vaccine on the first day of age, 15 chicks were injected with 1 ml/ SC vaccine on the 8th day of age, and 15 chicks were kept as controls. The chicks were observed for 2 weeks to monitor local reactions, disease symptoms, and death (OIE, 2018).

2.3. Experimental design and bird groupings

A total of 300 chicks (Arbor Acres) were used for 35-day experiments; the 1st experiment was based on the utilization of the Protexin®, acids, and vaccination with 0.2 ml/ SC of the multistrain vaccine administered on the first day of age. The second experiment relied on the administration of the Protexin® and acids on the first day of age with vaccination of the chicks using the vaccine at a dose of 0.5 ml/ SC on the 8th day of age. The chicks were randomly divided into 15 groups (20 chicks per group), and the groups are listed in Table 1. The groups G1a, G2, G4, G5, G6, G7, G9, G11, and G13 were assigned to the first experiment. The G1b, G3, G8, G10, G12, and G14 were assigned to the second experiment. All the chicks were vaccinated against Newcastle disease (ND) on day 7 and 20, avian influenza subtype H5N1 on day 9, infectious bronchitis (IB) on day 12 and infectious bursal disease (IBD) on day 18. The institutional animal care and use committee (IACUC) at the Faculty of Veterinary Medicine, University of Sadat City, approved the protocol with an allotted approval number of 230/2018.

2.4. Challenge with different serotypes

The bacterial challenge was performed with a mixed culture of the same vaccine serotypes. The challenge dose was adjusted using a spectrophotometer to 1×10^8 cfu/ ml, this dose was determined according to Trabees et al. (2018). The chicks were challenged orally with 1 ml at the age of 8 days in the first experiment groups and the age of 16 days in the second experiment groups. The chal-

Table 1
Group number, treatment, and challenge.

Group no.	Treatment/age	Time of challenge (day)
G1a	Negative control received a basal diet (for the 1st experiment)	–
G1b	Negative control received a basal diet (for the 2nd experiment)	–
G2	Positive control group	8th
G3	Positive control group	16th
G4	Probiotic administration on the 1st day	8th
G5	Probiotic product administration on the 1st day	8th
G6	Probiotic product + probiotic administration on the 1st day	8th
G7	Mixed vaccine (0.2 ml/SC) on the 1st day	8th
G8	Mixed vaccine (0.5 ml/ SC) on the 8th day	16th
G9	Probiotic + mixed vaccine (0.2 ml/SC) on the 1st day	8th
G10	Probiotic + mixed vaccine (0.5 ml/SC) on the 8th day	16th
G11	Probiotic product + mixed vaccine (0.2 ml/SC) on the 1st day	8th
G12	Probiotic product + mixed vaccine (0.5 ml/SC) on the 8th day	16th
G13	Probiotic + probiotic product + mixed vaccine (0.2 ml/SC) on the 1st day	8th
G14	Probiotic + probiotic product + mixed vaccine (0.5 ml/SC) on the 8th day	16th

challenge was implemented one week after vaccination (Frommer et al., 1994). The mortality rates of each group were recorded before and after the challenge.

2.5. Collection of blood samples

In the first experiment, blood samples were collected from the jugular vein on the 7th day and by puncturing the wing vein on days 12 and 15. In the second experiment, blood samples were collected on days 15, 20, and 25 by puncturing the wing vein. The blood was divided into two samples; the first sample was kept in disodium ethylenediaminetetraacetic acid (EDTA) for haematological assays. The second sample was placed in plain centrifuge tubes, left to clot, and then centrifuged to separate the serum samples which were stored at -20°C until the implementation of ELISA assays.

2.6. Evaluation of the immune response of each group within the two experiments

The response of chicks to each bacterial serotype was evaluated. Briefly, 96-well microtiter plates were coated with $100\ \mu\text{l}$ of well of antigen containing 1×10^8 killed MDR *E. coli* O26, O78, *S. Enteritidis* (1,9,12.g.m 1,7), and *S. Typhimurium* (1,4,5,12.i.1,2) cells were prepared in a carbonate/ bicarbonate coating buffer (pH 9.6). After blocking and addition of serum under test the rabbit anti-chicken IgG-horseradish peroxidase-conjugate (AP162P-Sigma-Aldrich-Germany) was used and the automated ELISA plate reader at an absorbance of 450 nm was implemented. The cut-off values were calculated according to the formula of Penha Filho et al. (2016): cut-off value = OD sample-OD negative control/OD positive control-OD negative control.

2.7. Evaluation of the inflammatory cytokines in both experiments

The serum IL4, IL12 and IFN- γ levels were evaluated using the MyBioSource (USA) ELISA kits according to the manufacturer's instructions. These cytokines were evaluated at the age of the 12th day in the first experiment and the 20th day in the second experiment.

2.8. Evaluation of the haematological parameters in both experiments

The packed cell volume (PCV), haemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were measured according to the protocols described by Feldman et al. (2000). Erythrocyte (RBC) count was performed according to the protocol reported by Natt and Herrick (1952).

2.9. Broiler performance parameters

Body weight (the recorded weight of the birds at the end of each week), body weight gain (the difference between two successive weights), and the feed conversion ratio (determining the ratio between feed intake and body weight gain) were evaluated after random selection of 5 birds from each group. The feed intake per day was calculated as the difference between the amount of supplemented feed and the remaining feed, and the weekly feed intake was calculated based on these data.

2.10. Statistical analysis

All the data of the immune response, cytokine levels, haematological parameters, and growth parameters were expressed as the standard error of means. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by a multiple comparison Duncan test after Snedecor and Cochran (1980) to detect differences among treated groups. The statistical difference was regarded as significant when $p < 0.05$.

3. Results

3.1. The results of the immune response in the first experiment

The immune responses to *S. Enteritidis* (1,9,12.g.m 1,7), and *S. Typhimurium* (1,4,5,12.i.1,2) elucidated significant differences between all groups of all ages with ($p \leq 0.05$). Groups G6, G7, and G9 showed higher responses to *S. Typhimurium* at the age of 7 days. Additionally, groups G5, G6, G7, G9, and G13 had the highest responses to *S. Enteritidis*. At the age of 12 days, groups G4, G11, and G13 produced the highest responses to *S. Typhimurium*, while groups G4, G5, G6, G7, G11, and G13 produced the highest responses to *S. Enteritidis*. Moreover, groups G4, G5, G6, G7, G9, G11, and G13 had the highest results at the age of 15 days, contrasting with the results at the younger ages for *S. Typhimurium* and *S. Enteritidis*. Concerning the immune responses to *E. coli* O26 and O78, there were significant differences between all groups and the control group ($p \leq 0.05$). At the age of 7 days, groups G5, G7, and G9 had the highest responses to both serotypes. While the response in most groups at the age of 12 days was lower than that at the younger age, groups G7 and G13 had the highest responses to *E. coli* O26. However, for *E. coli* O78, groups G4, G5, G11, and G13 produced the highest responses and exceeded the corresponding responses at the younger age, while groups G6, G7, and G9 had lower responses than the previous younger age. Besides, the response to *E. coli* O26 and O78 at the age of 15 days indicated that groups G4, G5, G6, G7, G11, and G13 had the highest results, surpassing the results recorded at the previous younger ages (Table 2).

3.2. The results for the immune response in the second experiment

Focusing on the immune response to *S. Typhimurium* at the age of 15 days, there were significant differences among all groups ($p \leq 0.05$) except G10 and G12, while for the immune response

Table 2
Results of immune response of the first experiment.

Group	G 1a	G 2	G 4	G 5	G 6	G 7	G 9	G 11	G 13
7	S. Typhimurium S. Enteritidis E. coli O26 E. coli O78	0.59 ± 0.1 ^e 0.586 ± 0.1 ^f 0.511 ± 0.03 ^d 0.678 ± 0.1 ^{ae}	0.493 ± 0.06 ^b 0.578 ± 0.05 ^g 0.459 ± 0.02 ^e 0.630 ± 0.03 ^g	0.605 ± 0.16 ^b 0.668 ± 0.2 ^d 0.59 ± 0.3 ^b 0.713 ± 0.04 ^d	1.40 ± 0.08 ^a 0.898 ± 0.06 ^b 0.471 ± 0.04 ^a 0.898 ± 0.06 ^b	1.3 ± 0.03 ^b 0.9302 ± 0.07 ^a 0.6526 ± 0.06 ^a 0.9302 ± 0.07 ^a	1.071 ± 0.05 ^c 0.807 ± 0.07 ^c 0.521 ± 0.08 ^c 0.807 ± 0.07 ^c	0.594 ± 0.05 ^e 0.589 ± 0.01 ^f 0.433 ± 0.03 ^f 0.576 ± 0.01 ^a	0.635 ± 0.01 ^d 0.608 ± 0.01 ^e 0.506 ± 0.02 ^{ab} 0.653 ± 0.07 ^f
12	S. Typhimurium S. Enteritidis E. coli O26 E. coli O78	0.69 ± 0.2 ^f 0.44 ± 0.01 ^g 0.48 ± 0.2 ^f 0.4 ± 0.2 ^f	1.052 ± 0.06 ^d 0.762 ± 0.05 ^{ab} 0.522 ± 0.03 ^d 0.762 ± 0.05 ^d	1.292 ± 0.11 ^b 0.928 ± 0.09 ^b 0.473 ± 0.008 ^e 0.928 ± 0.09 ^b	0.969 ± 0.001 ^e 0.7325 ± 0.04 ^d 0.438 ± 0.006 ^c 0.75 ± 0.04 ^{ad}	0.962 ± 0.04 ^e 0.77 ± 0.046 ^d 0.703 ± 0.097 ^a 0.77 ± 0.046 ^d	0.612 ± 0.01 ^{ab} 0.618 ± 0.05 ^e 0.478 ± 0.06 ^{ab} 0.644 ± 0.02 ^f	1.153 ± 0.08 ^c 0.896 ± 0.04 ^c 0.542 ± 0.01 ^d 0.896 ± 0.04 ^c	1.319 ± 0.17 ^h 1.036 ± 0.1 ^a 0.643 ± 0.08 ^{ab} 1.036 ± 0.1 ^a
15	S. Typhimurium S. Enteritidis E. coli O26 E. coli O78	0.58 ± 0.1 ^c 0.73 ± 0.2 ^e 0.78 ± 0.1 ^g 0.52 ± 0.1 ^e	1.297 ± 0.03 ^e 0.982 ± 0.04 ^d 0.605 ± 0.04 ^c 0.982 ± 0.04 ^c	1.591 ± 0.15 ^c 1.105 ± 0.015 ^c 0.640 ± 0.06 ^b 1.105 ± 0.015 ^b	1.705 ± 0.18 ^b 1.011 ± 0.05 ^c 0.658 ± 0.06 ^b 1.011 ± 0.05 ^b	1.224 ± 0.04 ^e 0.953 ± 0.06 ^d 0.61325 ± 0.034 ^c 0.95325 ± 0.05 ^c	1.123 ± 0.33 ^f 0.887 ± 0.13 ^{ab} 0.505 ± 0.04 ^a 0.899 ± 0.12 ^{ab}	1.543 ± 0.04 ^d 1.209 ± 0.04 ^b 1 ± 0.1 ^a 1.209 ± 0.04 ^a	1.768 ± 0.06 ^a 1.221 ± 0.04 ^a 0.617 ± 0.07 ^{ab} 1.221 ± 0.04 ^a

Each value represents the mean of 5 replicates, the superscript letters within the same row elucidate that there are significant differences between values (P < 0.05).

to *S. Enteritidis* significant differences were present among all groups and the control group ($p \leq 0.05$). Groups G8, G10, G12, and G14 produced the highest responses to both *S. Typhimurium* and *S. Enteritidis*. Furthermore, there were significant differences among the responses of all groups at the ages of 20 and 25 days to both *S. Typhimurium* and *S. Enteritidis* ($p \leq 0.05$). For *S. Typhimurium*, groups G8, G10, G12, and G14 produced the highest responses, but these responses were lower than those measured at the younger age, which could be due to the challenge. The responses of all groups at the age of 25 days confirmed that groups G8, G10, G12, and G14 produced the highest responses to both *S. Typhimurium* and *S. Enteritidis* and the responses of these groups were higher than that observed at the younger age for *S. Enteritidis*. For the response to *E. coli* O26 at the age of 15 days, there were significant differences among all groups ($p \leq 0.05$) except G10 and G12. Groups G8, G10, G12, and G14 produced the highest responses to both *E. coli* O26 and O78. The immune response to *E. coli* O26 at the age of 20 days evidenced significant differences between all groups except G10 and G12. All the responses were lower than the G1b response, and the responses were weaker than the corresponding responses at the younger age. For the responses to *E. coli* O78 at the age of 20 days, all the groups showed significant differences ($p \leq 0.05$). The G8, G10, G12, and G14 produced the highest results, and their responses were lower than the corresponding responses at the younger age. Moreover, the responses to both serotypes at the age of 25 days were significantly different ($p \leq 0.05$) from the response of the control group. Groups G8, G10, G12, and G14 produced the highest results, and their responses were higher than those observed at the younger ages for both *E. coli* O26 and O78 (Table 3).

3.3. Results of mortalities from all tested groups

There were significant differences in the mortality rates among all groups before challenge ($p \leq 0.05$) except for the following groups: G1a, G1b, and G2; G4 and G5; G6, G9, and G10; and G7, G12, G13, and G14. The groups G1a, G1b, G2, G3, and G8 exhibited high mortality rates. After the challenge, all the groups showed significant differences ($p \leq 0.05$) in mortality except the groups G4, G5, and G10 and G9, G12, G13, and G14, while G2 and G3 exhibited high mortality rates. Calculating the total mortalities, groups G1a, G1b, G2, G3, and G8 exhibited higher mortality than the other groups, while G11 showed no mortality (Table 4).

3.4. Results of cytokines in both experiments

Focusing on the levels of cytokines produced by all treated groups in the first and second experiments, there were significant differences among all groups with respect to the levels of IFN- γ , IL-4, and IL-12 ($p \leq 0.05$). Also, the levels of these cytokines in the treated groups were lower than those in the positive control groups, indicating lower inflammation in response to the pathogenic *E. coli* and *Salmonella* serotypes used for infection and challenge and proving the protective effects of the potential probiotic (Protexin[®]), acids, and vaccine (Table 5 and 6).

3.5. Results of haematological parameters in both experiments

3.5.1. Erythrogram of the first experiment

On the 7th day of age, there were no significant changes in erythrogram parameters in the treated groups compared with the control group, although the RBC values were increased in G7. On day 12, there were significant reductions in the PCV, Hb, and RBC values in all groups except G13 compared with the control group. The most significant reductions in these values were recorded in G2. However, compared with the infected group G2, all challenged

Table 3
Results of immune response of the second experiment.

Group		G1b	G 3	G 8	G 10	G 12	G 14
15	S. Typhimurium	0.65 ± 0.1 ^c	0.69 ± 0.1 ^b	1.83 ± 0.12 ^a	1.8 ± 0.04 ^a	1.8 ± 0.11 ^a	1.81 ± 0.1 ^a
	S. Entritidis	0.656 ± 0.1 ^c	0.686 ± 0.1 ^c	1.303 ± 0.1 ^b	1.2 ± 0.02 ^b	1.4 ± 0.1 ^a	1.5 ± 0.1 ^a
	E. coli O26	0.59 ± 0.03 ^b	0.611 ± 0.01 ^b	0.8985 ± 0.07 ^{ab}	0.93 ± 0.03 ^a	0.93 ± 0.02 ^a	0.95 ± 0.03 ^a
20	E. coli O78	0.76 ± 0.06 ^c	0.773 ± 0.03 ^c	1.303 ± 0.1 ^b	1.4 ± 0.01 ^b	1.6 ± 0.1 ^a	1.7 ± 0.1 ^a
	S. Typhimurium	0.95 ± 0.01 ^c	0.39 ± 0.2 ^d	1.499 ± 0.11 ^b	1.356 ± 0.09 ^b	1.714 ± 0.02 ^a	1.384 ± 0.03 ^b
	S. Entritidis	0.89 ± 0.1 ^b	0.486 ± 0.2 ^c	1.137 ± 0.11 ^a	1.031 ± 0.03 ^a	1.152 ± 0.02 ^a	1.146 ± 0.004 ^a
25	E. coli O26	0.95 ± 0.3 ^b	0.511 ± 0.02 ^d	0.85 ± 0.04 ^c	0.9 ± 0.05 ^a	0.9 ± 0.1 ^a	0.92 ± 0.02 ^a
	E. coli O78	0.84 ± 0.01 ^c	0.673 ± 0.04 ^d	0.994 ± 0.2 ^b	1.031 ± 0.03 ^a	1.152 ± 0.02 ^a	1.146 ± 0.004 ^a
	S. Typhimurium	1.1 ± 0.1 ^c	0.49 ± 0.1 ^d	1.486 ± 0.03 ^b	1.624 ± 0.16 ^a	1.707 ± 0.1 ^a	1.622 ± 0.2 ^a
	S. Entritidis	0.91 ± 0.04 ^b	0.586 ± 0.1 ^c	1.265 ± 0.03 ^a	1.134 ± 0.1 ^a	1.235 ± 0.1 ^a	1.156 ± 0.1 ^a
	E. coli O26	0.75 ± 0.2 ^d	0.5 ± 0.03 ^e	0.99 ± 0.03 ^c	1 ± 0.14 ^b	1.01 ± 0.1 ^b	1.2 ± 0.02 ^a
	E. coli O78	0.95 ± 0.1 ^c	0.7 ± 0.05 ^d	1.265 ± 0.03 ^a	1.3 ± 0.1 ^a	1.235 ± 0.1 ^a	1.126 ± 0.1 ^b

Each value represents the mean of 5 replicates, the superscript letters within the same row elucidate that there are significant differences between values ($P < 0.05$).

Table 4
Results of mortalities from all tested 14 groups.

Group	G 1a	G 1b	G 2	G 3	G 4	G 5	G 6	G 7	G 8	G 9	G 10	G 11	G 12	G 13	G 14
Before challenge	8	9	5	6	4	4	2	3	8	2	2	0	3	3	3
After challenge	No	No	7	6	2	2	3	0	0	1	2	0	1	1	1
Total	8/20 (40%)	9/20 (45%)	12/20 (60%)	12/20 (60%)	6/20 (30%)	6/20 (30%)	5/20 (25%)	3/20 (15%)	8/20 (40%)	3/20 (15%)	4/20 (20%)	0/20 (0.0%)	4/20 (20%)	4/20 (20%)	4/20 (20%)

Table 5
Results of cytokines of the first experiment.

Cytokines	G 1a	G 2	G 4	G 5	G 6	G 7	G 9	G 11	G 13
IFN- γ	70.5 ± 0.04 ^b	111.3 ± 0.01 ^a	67.1 ± 0.1 ^c	59.93 ± 0.1 ^f	70.3 ± .001 ^b	47.6 ± 0.04 ^h	58.6 ± 0.01 ^e	63.1 ± .08 ^d	57.1 ± 0.17 ^g
IL-12	62.2 ± 0.01 ^b	98.7 ± 0.02 ^a	56.4 ± 0.1 ^d	49.6 ± 0.1 ^f	61.3 ± 0.04 ^c	39.8 ± 0.046 ⁱ	47.6 ± 0.1 ^g	52.4 ± 0.04 ^e	46.1 ± 0.1 ^h
IL-4	50.4 ± 0.02 ^b	79.2 ± 0.1 ^a	45.2 ± 0.2 ^c	38.3 ± 0.01 ^e	50.1 ± 0.03 ^b	31.2 ± 0.02 ^g	36.9 ± 0.04 ^f	42.1 ± 0.13 ^d	36.8 ± 0.03 ^f

IFN- γ , Interferon gamma

IL, Interleukin

Each value represents the mean of 5 replicates, the superscript letters within the same row elucidate that there are significant differences between values ($P < 0.05$).

Table 6
Results of cytokines of the second experiment.

Group	G 1b	G 3	G 8	G 10	G 12	G 14
IFN- γ	45.6 ± 0.01 ^{ab}	121 ± 0.01 ^b	45.3 ± 0.02 ^{ab}	43.1 ± 0.09 ^{ab}	38.6 ± 0.02 ^{ab}	37.1 ± 0.03 ^a
IL-12	37.2 ± 0.02 ^{ab}	108 ± 0.02 ^b	34.2 ± 0.03 ^{ab}	36.1 ± 0.03 ^{ab}	22.4 ± 0.02 ^a	31.3 ± 0.004 ^{ab}
IL-4	30.2 ± 0.1 ^{ab}	95 ± 0.1 ^b	26.4 ± 0.05 ^{ab}	23.4 ± 0.05 ^a	29.8 ± 0.1 ^{ab}	27.1 ± 0.02 ^{ab}

Each value represents the mean of 5 replicates, the superscript letters within the same row elucidate that there are significant differences between values ($P < 0.05$).

groups showed significant increases in the PCV, Hb, and RBC values, and the most significant increase was recorded in G13. Additionally, significant reductions in the MCH and MCHC values were observed in G2, while the MCHC was reduced in G4 compared with the control group. On day 15, the PCV and Hb values were significantly reduced in all groups except G13 compared with the control group. In addition, the RBC values were significantly decreased in G2 and G5. The MCH values were reduced in all groups except G7 and G13; however, significant reductions in MCHC were also observed in all groups except G7, G9, G11, and G13. In comparison with the infected group G2, all the challenged groups showed significant increases in the PCV, Hb, RBC, MCH, and MCHC values, with the most significant increase reported in G13 (Supplementary Data Table 1).

3.5.2. Erythrogram of the second experiment

On the 15th day of age, none of the groups showed any significant changes in erythrogram parameters. On the 20th day, the PCV, Hb, and RBC values were decreased in all groups, with the most significant decrease observed in G3, compared with the control group; however, the RBC value in G14 showed no significant

change. Furthermore, the MCVs were reduced in G10 and G12, MCH values were reduced in all groups except G14, and the MCHC values were reduced in all groups except G12 and G14 compared with the control group. There were significant decreases in the MCH and MCHC values in G3. In comparison to the infected group G3, all challenged groups showed significant increases in the PCV, Hb, RBC, MCH, and MCHC values, except the PCV value in G8, which showed no significant change, and the MCH values in G8 and G10, which showed no significant changes. However, the MCV values were decreased in all challenged groups. The challenged groups G12 and G14 showed the most significant increases in the PCV, Hb, RBCs, MCH, and MCHC values. On the 25th day, all the erythrogram parameters were significantly reduced in all groups compared with the control group, with the most significant reduction reported in the infected group G3; only the MCV values in G3 and G8 and MCHC values in G12 and G14 showed no significant changes. In comparison with the G3 or infected group, all challenged groups showed significant increases in the PCV, Hb, RBC, MCH and MCHC values, except the MCH value in G8, which showed no significant change. However, the MCV values were clearly decreased in all challenged groups except G8. The chal-

lenged group G14 showed the most significant increases in the PCV, Hb, RBC, MCH, and MCHC values (Supplementary Data Table 2).

3.6. Results of growth parameters

The body weights in G1a, G1b, G2, and G3 in the first week were significantly different from that in the other groups (G4, G5, G6, G7, G8, G9, G10, G11, G12, G13, and G14), which showed higher body weights than G1, G2, and G3 ($p < 0.05$). The body weight gains in the first week in G1a, G1b, G2, and G3 were significantly different from that in the other groups ($p \leq 0.05$). The FCRs at the age of 7 days showed significant differences among all groups except for the FCR among the following groups: G1, G2, and G3; G5 and G7; G9, G10, and G13; and G11, G12, and G14 ($p \leq 0.05$), although groups G1a, G1b, G2, G3, and G8 produced the lowest results. The other groups showed high FCRs. The body weights at the age of 14 days in G1 and G3 were significantly different from that in the other groups (G2, G4, G5, G6, G7, G8, G9, G10, G11, G12, G13, and G14) ($p \leq 0.05$). The body weight in G2 was higher than that in the other groups, as weight decreased due to the challenge. The body weight gains at the age of 14 days in G1 and G2 were significantly different from that in the other groups, as the remaining groups exhibited higher body weight gain ($p \leq 0.05$). The FCR at the age of 14 days was confirmed to be significantly different among all groups ($p \leq 0.05$) except for the FCR between G6 and G7 and among G9, G10, and G13; the groups G9–G14 gave the highest results. Considering body weight at the age of 21 days, G2 chicks had the lowest body weight, and G7 chicks had the highest body weight with a significant difference between their body weights and other groups ($p \leq 0.05$). Regarding body weight gain at the age of 21 days, G3 exhibited the lowest weight gain, while G7 showed the highest weight gain, and there was a significant difference between each of these two groups and the other groups ($p \leq 0.05$). Interpreting the results for the FCR at the age of 21 days, there were significant differences among all groups except for the FCR between G5 and G7, G9 and G10, and G13 and G14 ($p \leq 0.05$); the groups G5–G7 and G9–G14 had the highest ratios. The body weights at the age of 28 days showed significant differences among all groups ($p \leq 0.05$) except for the body weights among the groups G9, G10, and G14, and the groups G11, G12, and G13. Although G2 had the lowest body weight, the groups G4–G14 had high results, and group G7 had the highest result. Regarding body weight gain at the age of 28 days, G3 exhibited the lowest gain, while G7 showed the highest gain, with significant differences among all groups ($p \leq 0.05$). The FCR data at the age of 28 days showed that all groups exhibited significant differences ($p \leq 0.05$), although there were no significant differences among G4, G9, G10, G12, and G14 and between G11 and G13; the groups G4–G6 and G9–G14 showed the highest results. The body weights at the age of 35 days proved that the G2 was the lowest, while G7 had the highest body weight. There was a significant difference between these two groups and significant differences between these two groups and the other groups ($p \leq 0.05$); there were no significant differences among G9, G10, G11, G12, G13, and G14. Concerning the weight gain at the age of 35 days, both G2 and G3 showed the lowest gains, while G7 had the highest gain, and there were significant differences between these groups and the other groups ($p < 0.05$). Moreover, there were no significant differences among G6, G9, G10, G11, G12, G13, and G14. The FCR data at the age of 35 days showed significant differences among all groups ($p < 0.05$) except for the FCR between G1 and G8, among G9, G10, and G13 and

between G11 and 14; the groups G9–G14 showed the highest results (Supplementary Data Table 3).

4. Discussion

Avian colibacillosis and salmonellosis are notorious infectious diseases that cause significant economic losses in the global poultry industry (Kabir, 2010). The beneficial roles of Protexin[®], acids, and vaccines in the protection of poultry and large animals from MDR bacteria were confirmed (Asai et al., 2011; Belanger et al., 2011). Concerning the immune responses to *E. coli* O26, O78, *S. Enteritidis* (1,9,12.g.m 1,7), and *S. Typhimurium* (1,4,5,12.i.1,2) serotypes in various groups in the first and second experiments, there were significant differences among all groups of all ages ($p \leq 0.05$). These enhanced responses were due to the immunomodulatory effects of the Protexin[®] and acids, as they increase the activity of macrophages, NK cells, GALT, B-1 cells, and enhance the secretion of immunoglobulins, and promote gut epithelial barrier integrity (Haghighi et al., 2006; Kabir, 2009). Moreover, probiotics containing *Lactobacillus acidophilus*, and *Bifidobacterium bifidum* lead to significant improvement of systemic antibody response (Haghighi et al., 2005). This effect, in turn, is reflected in increased chicken resistance to pathogens, and improved growth performance (Bibas Bonet et al., 1999; Cetin et al., 2005; Park et al., 2016). The addition of the Protexin[®] or acids, either alone or in combination with different vaccination regimens, lowered mortality at the beginning of rearing age and after challenge with mixed live bacterin serotypes. Simultaneous vaccination with 0.2 ml of mixed vaccine in 1-day-old chicks and daily supplementation with acids in the G11 showed no mortality in response to MDR bacteria. The decreased mortality rates induced by the Protexin[®], acids, and bacterin are in agreement with Tarabees et al. (2018), and Khedr et al. (2015). Protexin[®] supplementation established a balance in the intestinal flora, competed for nutrients, produced volatile fatty acids that imparted a bacteriostatic effect, and decreased the intestinal pH to limit the colonization of pathogenic bacteria without any side effects on the kidneys or liver (Mookiah et al., 2014). Moreover, the lowered mortality could be regarded to probiotic stimulation of T cells in the caecal tonsils and enhancement of NK cell activities (Yurong et al., 2005). The measured levels of IFN- γ , IL-12, and IL-4 at the ages of 12 and 20 days in the two experiments were lower in the treated groups than in the positive control groups, with significant differences of $p \leq 0.05$. The increased cytokine and inflammatory marker levels in the positive control group infected with *E. coli* and *Salmonella* serotypes agreed with the results of Brisbin et al. (2010), who confirmed increased expression of IL-12p40, IFN- γ and other Th1-type cytokines in caecal tonsil mononuclear cells treated with *S. Typhimurium*. The decreased cytokine levels indicated reduced inflammatory changes due to the beneficial effect of the Protexin[®], acids, and vaccine (Chen et al., 2012; Hu et al., 2015; Wang et al., 2017).

For the erythrogram parameters, the chicks infected with the mixed live bacterin serotypes revealed microcytic hypochromic anaemia evidenced by significant reductions in the values of PCV, Hb, RBCs, MCH, and MCHC (Brown et al., 2010). Infection-caused anaemia is characterized by a reduced erythropoietin hormone (EPO) response, reduced erythroid precursor proliferation, and decreased RBC survival. A major contributor to infection-caused anaemia is hepcidin, a hormone that inhibits the release of stored cellular iron and iron transfer from enterocytes into the systemic circulation. In addition, both iron deficiency and sepsis can enhance RBC apoptosis, exacerbating anaemia, if not compensated

for by erythropoiesis (Lang and Qadri, 2012). Iron is essential for the survival and pathogenesis of *Salmonella*; thus, iron withdrawal from the circulation mediated by hepcidin is a host-protective mechanism (Wessling-Resnick, 2015). Other groups challenged with the mixed live bacterin serotypes, particularly G13 and G14 showed values near to that of the negative control group, they showed mild microcytic hypochromic anaemia, confirming the beneficial effects of the Protexin[®], acids, and vaccine on chicken health (Manafi, 2015). Probiotics, formic, and lactic acids enhance the concentration of Hb, the haematocrit level, and the RBC count in broiler chickens (Wang et al., 2010; Panwar et al., 2017). The body weight, body weight gain, and the FCR were improved by the Protexin[®], acids, and vaccine with different regimens, which agrees with the findings of Tarabees et al. (2018) and Ezema et al. (2011). The improvement in growth parameters was probably due to increased feed consumption and improved nutrient digestibility (Gao et al., 2008). Furthermore, the supplementation with a mixture of yeasts and other microbes increases the digestibility of dry matter, crude protein, energy, Ca, P, and some amino acids in broiler chickens (Li et al., 2008). Enhanced broiler chicken performance after probiotic supplementation is related to increased levels of amylase and carbohydrase enzymes and other beneficial products in the intestinal milieu (Aziz Mousavi et al., 2018). Moreover, dietary supplementation with probiotics and prebiotics enhance the growth performance, intestinal morphology, and nutrient absorption of broiler chickens (Awad et al., 2008). The enhanced chicken immune response reduces the colonization of their alimentary tract by poultry pathogens of zoonotic importance which in turn reduces the potential contamination of chicken carcasses, thus improving the quality of consumed chicken meat.

5. Conclusions

Taken together, the results elucidated the beneficial effects of Protexin[®], lactic and formic acids, and a multistrain bacterin formulated from avian pathogenic MDR *E. coli* O26, O78, S. Enteritidis (1,9,12 g.m 1,7), and S. Typhimurium (1,4,5,12.i.1,2) serotypes. The single or combined utilization of these products produced an immunomodulatory effect, decreased mortality, improved erythrogram parameters, lowered pro-inflammatory cytokine levels, and increased growth performance parameters, especially when administered together on the first day of age. Future molecular studies are required to unravel the signalling mechanisms that lead to these vital alterations.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.sjbs.2021.02.017>.

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