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Antibacterial and Immuno-Modulatory Effects of Probiotics and Phytobiotics in Spent Rhode Island Red Layers

Muhammad Mushtaq[1], Muhammad Shuaib[1]*, Aamir Iqbal[2], Abubakar Sufyan[3], Muqader Shah[6], Noor Ul Ain Nawaz[4], Muhammad Ismail Khan[5], Hamayun Khan[6], Muhammad Shahkar Uzair[1], Qudrat Ullah[6] and Usman Zeb[7]

[1]Department of Poultry Science, Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture, Peshawar, Pakistan.
[2]Department of Animal and Poultry Production, Faculty of Veterinary and Animal Sciences, Ponch University, Rawalkot, Azad Kashmir
[3]Department of Livestock and Poultry Production, Bahauddin Zakariya University, Multan, Pakistan
[4]Department of Pharmacy, City University of Science and Information Technology, Dalazak Road, Peshawar
[5]Department of Zoology, Islamia College, Peshawar
[6]College of Veterinary Sciences, Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture, Peshawar, Pakistan
[7]Institute of Biotechnology and Genetic Engineering, The University of Agriculture, Peshawar, Pakistan

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***Bacillus clausii*, Egg-type birds, Immunology, Microflora count, *In-vitro*, Poultry production, Rhode island red**

ABSTRACT

This research study was designed to evaluate the synergistic effects of phytobiotics and probiotics during the induced mix (*Escherichia coli* and *Salmonella*) infection in egg-type birds. Six different phytobiotic extracts with a constant dose level of probiotic were first evaluated through *in-vitro* screening by disk diffusion method against the resisted *E. coli* and *Salmonella paratyphi*. Based on *in-vitro* screening, phytobiotics were further evaluated by *in-vivo* trial. The potential of phytobiotics with probiotics was evaluated by evaluating the mortality, morbidity, intestinal microbiota count, and serum immunoglobulin (IgA, IgM, and IgG) during the induced mixed infection. Birds were inoculated with *E. coli* (O157:H7) and *S. paratyphi* at the rate of 1:1x10^9 cfu/ml each. Birds were divided into 12 groups having three replicates. Phytobiotics extracts at concentration of 1, 100, and 1000mg/L were used for all the three selected medicinal plants with probiotics @ 0.03ml/L (12×10^6 spores). The *in-vitro* evaluation of phytobiotics (Fenugreek, *B. Lycium*, and *T. arjuna*) extracts with probiotic (*Bacillus clausii*) showed more potential by inhibiting the growth of selected bacterial strains. It was noticed that phytobiotics and probiotics at 1000mg/L+0.03ml/L dose level showed improved microbial count, IgA, IgM, IgG, mortality, and morbidity against the infection as compared to the control and standard group. It was concluded from the present experiment that phytobiotics with probiotics possess antimicrobial and immune-modulatory properties.

INTRODUCTION

The poultry industry of Pakistan is one of the vibrant sub-sectors of the Pakistan livestock industry and plays a vital role in fulfilling the demand for animal protein among the consumer of Pakistan. Advancements in poultry husbandry practices in feeding management lead to the incorporation of different feed additives during ration formulation. These feed additives include antibiotics, pro-pre-biotics, phytobiotics, exogenous enzymes, and a balanced diet, which act as growth promoters in

* Corresponding author: shoaibwzr@gmail.com
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poultry production (Mehdi *et al*., 2018). Several poultry husbandry practices in feeding management are reported by previous studies (Roess *et al*., 2013) that become the risk factors for transmitting of drug resisted infectious zoonotic-infection. Microbes are disease-causing agents in all living organisms. Structurally they are tiny in size but cause profound damage to the living body. Antimicrobial/antibiotic is the class of drugs that can kill or arrest the multiplication of these microbes. With the advancement of antimicrobial drugs against the different categories of microbes, the microbes developed resistance against these antimicrobial drugs shortly or passively to stabilize their survival in the environment. *Escherichia coli* infections are widely distributed among poultry of all ages and categories. Salpingitis (inflammation of the oviduct) due to *E. coli* infections could be also observed in growing birds. Extra intestinal pathogenic *Escherichia coli* (ExPEC) constitutes ongoing health concerns for women, newborns, elderly, and immunocompromised individuals due to increased numbers of urinary tract infections (UTIs), newborn meningitis, abdominal sepsis, and septicemia (Manges and Johnson, 2012). Recognizing and treating the zoonotic risk posed by ExPEC would greatly enhance food safety and positively impact human health (Mellata, 2013). *Salmonella* is a group of bacteria that causes typhoid fever, food poisoning, gastroenteritis, enteric fever, and other illnesses. Meat and eggs are known to be a source of human pathogens such as *Campylobacter, Listeria*, and *Salmonella*, which frequently leads to a food recall of the suspected contaminated products (Olugbenga *et al*., 2021). In humans, the main source of infection is the consumption of contaminated poultry meat and eggs. Because of its significant risk to public health, *Salmonella virchow* is one of five serovars that has been given priority by the European Union (EU) for investigations on poultry farms to control its entry into the food chain (Snow *et al*., 2007: Arnold *et al*., 2010). Resistance produced by microbes against the synthetic antimicrobials lead to human health hazard with low immunity, therefore, the photochemical (natural antimicrobials) from the plants' kingdom got attention (Kazi *et al*., 2022). Biomolecules of plant origin and probiotics appear to be one of the alternatives for the control of these antibiotic-resistant human pathogens in producing animals/birds. Plant materials are used widely in traditional systems of medicine (Aziz *et al*., 2018). Plant extracts, also known as phytobiotics, have been exploited in animal nutrition, particularly for their antimicrobial, anti-inflammatory, antioxidant, and antiparasitic activities (Motoi, 2021). Biologically active components of plants are mostly secondary metabolites, such as terpenoids, phenolics, glycosides, and alkaloids (Baan and Hasan, 2022). These secondary metabolites may have a protective function in vegetal tissues. These compounds are assumed to be involved in plant defense and most of them may possess antimicrobial properties (Khameneh *et al*., 2019). Probiotics are beneficial live micro-organisms, which administration in the host confers one or more specified health benefits (Roess *et al*., 2013). Application of probiotics can result in structural and compositional alteration in intestinal architecture and microflora by improving the absorptive sites and reduction in pathogenic microbiota. It can help in the production of intestinal-lactic acid and hydrogen peroxide (H_2O_2), which further causes the inhibition/reduction in oxidation processes. This phenomenon of probiotic in the host intestinal site, resulting in the inhibition of aerobic pathogens (toxin amines and ammonia), promote the production of intestinal essential digestive enzymes, and vitamin-B complex and also stimulate the host appetite (Singh *et al*., 2004). As per previous studies by Guo *et al*. (2004) and Jamroz *et al*. (2003) that bioactive compounds of phytobiotics are considered potential agents by promoting beneficial intestinal-microbiota (probiotics) without influencing the growth of pathogenic microflora spp. Considering this beneficial aspect of phytobiotics compounds on the promoting gut-probiotics, it can provide an optimal precondition for effective protection against zoonotic infectious pathogens and involve in the host gut-immune/defense system (Wenk, 2003). Therefore, the present research study was designed to evaluate the synergistic effects of phytobiotics and probiotics during the induced mixed (*E. coli* and *salmonella*) infection in egg-type birds.

MATERIALS AND METHODS

Biochemical materials and phytobiotics extracts preparation

The phytobiotics (*Berberis lycium* bark, Fenugreek seeds, *Terminalia arjuna* seeds, *Nigella sativa* seeds, *Withania coagulans* seeds, *Peganum harmala* seeds) and probiotics (*Bacillus clausii*) materials were obtained from Forest Institute Peshawar and Veterinary Teaching Hospital (VTH) of College of Veterinary Sciences, Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture Peshawar, Pakistan. Methanolic extracts of medicinal plants were prepared according to the standard protocol of Dabur *et al*. (2004). Clinical isolates of the poultry microorganisms [*Salmonella paratyphi* and *Escherichia coli* (O157:H7)] were used as challenged microbiota. The strains of the microorganisms were collected from the Microbiology Lab of the College of Veterinary Sciences, The University of Agriculture Peshawar, Pakistan.

In-vitro *antibacterial activities of methanolic extract of phytobiotics and probiotic*

The methanolic extracts of 6 phytobiotics with the combination of *Bacillus clausii* were screened against 2-bacterial strains, *Escherichia coli* and *Salmonella*. These challenged microbiotas were obtained from the Microbiology Lab of the College of Veterinary Sciences. Active cultures of microbiota for experimental purposes were prepared by transferring a loop-full of cells from previously stored stock cultures (maintained at 4°C on slopes of nutrient agar) to Mueller-Hinton broth (MHB) containing test tubes. The test tubes were incubated for 24 h at 37°C and 25°C, respectively, without agitation in the incubator. For achieving optical densities (OD) corresponding to $2x10^6$ CFU/ml, the cultures were diluted with fresh MHB.

According to the standard protocol developed by Bauer *et al*. (1966), the antibacterial activities of extracts and probiotics were performed. The Mueller Hinton agar (MHA) plates were prepared by pouring 15ml of molten media into sterile petri-plates and allowed to dry for 5 min. The drying process was followed by swabbing 0.1% of inoculum suspension uniformly and allowed to dry for 5 min. The different concentrations of phytobiotics extracts with the probiotic and antibiotic discs were loaded at a distance of 24 mm from each other and 12mm from the plate edge. The plates were properly labeled, sealed with Parra-film, and inoculated at 37 °C for 24-48 h. To avoid any sort of contamination biosafety cabinet level II (ESCO, USA) was used. The zone of inhibition against the tested pathogens was recorded in millimeters (mm) after 48 h. About 10% DMSO-containing disc was taken as a negative control to compare the tested plates against the tested pathogens. The process was performed in triplicate and the triplicate average was analyzed as the mean.

In-vivo *antibacterial activities of methanolic extract of phytobiotics and probiotic*

The inoculum was prepared from a stock culture of *E. coli* and *Salmonella typhimurium* through a developed technique by Eman and Hoda (2008). A high concentration of the inocula ($1.0x10^9$) was prepared to increase the probability of establishing the disease condition in the experimental birds. One hundred and eighty (180) healthy laying birds of age 75 weeks were used for this experiment. The birds were obtained from the laying house of the Department of Poultry Science, The University of Agriculture Peshawar, Pakistan. The birds were assigned randomly to isolated cages in the house on a 16/08 light-dark cycle. The birds were allowed to acclimatize to their new environment for a week before inoculation and were tested to ensure that they will negative for *E. coli* and *Salmonella*. Feed and water were provided from the day the birds procure until the completion of the experiment.

Seventy-five weeks-old Rhode Island Red (RIR) birds were divided into 12 groups of 3 replicates (n=5). The phytobiotics extracts and probiotics incorporated in drinking water to the different treatment groups at the rate of 1mg/L+0.03ml/L (12×10^6 spores), 100mg/L+0.03ml/L (12×10^6 spores) and 1000mg/L+0.03ml/L (12×10^6 spores). The supplemented water was offered throughout the entire five weeks period of the experiment.

On the 4th day of the experiment, the volume of the inoculum was introduced into each bird as prescribed by Eman and Hoda (2008). All the groups were orally challenged with 1.0ml of *E. coli* and *Salmonella paratyphi* inoculum at a dose of $1:1 \times 10^9$cfu/ml except the negative control group.

For determination of performance indicators the birds were routinely observed for mortality if any due to microbe infections. The post-mortem was performed to identify the possible cause of mortality. The birds were physically observed for any signs of illness during the entire period of the experiment. The gut-microbiota count was performed according to the standard protocol developed by Barrow and Feltharn (1993) by using the standard plate count technique. The preparation of media for the count was performed according to the manufacturer's specifications. About 1 ml ileum digesta was used for the count. The ileo-digesta was processed through serial dilution processing (10-3 dilution level) in sterile 15 ml test tubes (each tube consisting of 9 ml of 0.1% of sterile peptone water) and vortexed. Approximately 1 ml of the diluted digesta was pipetted/inoculated on plate count agar and MacConkey agar, following the incubation at 37°C for 24 h. The required/discrete colonies of microbiota on the plate were examined and counted by using a colony counter and expressed as log10 CFU/ml as results.

Immunoglobulin's (Ig) determination

Immunoglobulin (Ig) was determined in egg-type birds according to the developed protocol of Delhanty and Solomon (1966), Yamamoto and Glick (1982), Martin *et al*. (1989), and Qureshi and Havenstein (1994). Sheep red blood cell (SRBCs) suspension was used for the determination of Ig-antibodies in egg-type birds. About 15 ml of blood was collected from healthy sheep reared at University Dairy Farm in an EDTA tube and washed with equal V/V with phosphate buffer saline (PBS). The packed cells at 2.5/0.25 V/V were taken in PBS followed by a washing process. Birds were immunized with 0.1-ml (0.25%) of sheep RBCs suspension. The blood was collected about 14-d of post-immunization and serum was collected by centrifugation at 4000 rpm for 10 mins for determination of serum antibodies against SRBCs.

Statistical analysis

The data were analyzed through a statistical package scientific analysis system (SAS) by using a complete randomized design (CRD) as described by Steel and Torrie (1981).

Table I. *In-vitro* antibacterial activities of different concentrations of methanolic extracts of phytobiotics probiotic *B. clausii* and Ciprofloxacin.

Phytobiotics	Methanolic extract+ *B. clausii*	Mean±SE (mm in radius)	
		E. coli	*Salmonella*
Ciprofloxacin	40 µl	20.56±0.86	22.65±0.58
B. lycium	0.02 µl + 5 µl	8.00 ±0.28^{e}	9.00±0.28^{e}
	0.04 µl + 5 µl	8.70 ±0.11^{ed}	9.40±0.11^{ed}
	0.06 µl + 5 µl	9.46±0.12^{d}	9.96±0.12^{d}
T. foenum-graecum	0.02 µl + 5 µl	9.00±0.57^{d}	12.00±0.28^{c}
	0.04 µl + 5 µl	11.00±0.57^{c}	13.00±0.57^{b}
	0.06 µl + 5 µl	13.00±0.28^{a}	17.00±0.57^{a}
T. arjuna	0.02 µl + 5 µl	7.00±0.57^{f}	7.00±0.28^{g}
	0.04 µl + 5 µl	9.00±0.57^{d}	8.00±0.28^{f}
	0.06 µl + 5 µl	12.00±0.57^{b}	9.00±0.57^{e}
N. sativa	0.02 µl + 5 µl	1.50±0.11^{h}	0.00±0.00^{h}
	0.04 µl + 5 µl	1.60±0.05^{h}	0.00±0.00^{h}
	0.06 µl + 5 µl	1.60±0.15^{h}	0.00±0.00^{h}
W. coagulans	0.02 µl + 5 µl	0.00±0.00^{i}	0.00±0.00^{h}
	0.04 µl + 5 µl	0.00±0.00^{i}	0.00±0.00^{h}
	0.06 µl + 5 µl	0.00±0.00^{i}	0.00±0.00^{h}
P. harmala	0.02 µl + 5 µl	2.00±0.28^{h}	0.00±0.00^{h}
	0.04 µl + 5 µl	2.43±0.08^{h}	0.00±0.00^{h}
	0.06 µl + 5 µl	3.63±0.08^{g}	0.00±0.00^{h}
P-value		0.0212	0.0121

B. clausii, Bacillus clausii; B. lycium, Berberis lycium; T. arjuna, Terminalia arjuna; N. sativa, Nigella sativa; W. coagulans, Withania coagulans; P. harmala, Peganum harmala

Means in column with different superscript are significantly different at α=0.05

RESULTS

Methanolic extracts of different phytobiotics and probiotics showed variable antibacterial activity against selected bacterial pathogenic strains as compared to the standard group of ciprofloxacin (broad spectrum antibiotic) and control (DMSO). Based on the zone of inhibition, the phytobiotic extracts and probiotic against the challenged pathogenic strains, the direct-proportional trend was observed that the increased sensitivity against these tested bacteria (*E. coli*) with increasing the extract level in all treatments except the *Withania coagulans*. Similar findings were recorded for *Salmonella* in the group of *B. lycium*, Fenugreek and *T. arjuna* extracts with the probiotic. The other phytobiotics failed to show any response against the challenged bacterial strains. The highest sensitivity (zone of inhibition) of phytoprobiotics was noticed in fenugreek + probiotic at a level of 60+5 µl (13.00 mm), followed by *T. arjuna* + probiotic @ 60 + 5 µl (12.00 mm) and fenugreek + probiotic @ 40 +5 µl (11.00 mm) against the *E. coli*. In the case of *Salmonella* as challenged bacteria, methanolic extract of fenugreek with probiotic @ 60, 40, and 20 + 5 µl showed the highest (17.00, 13.00, and 12.00 mm) sensitivity as compared to other phytoprobiotics (Table I). Based on the *in-vitro* antibacterial screening of phytoprobiotics extracts against the multi drugs resistance (MDR) *E. coli* and *Salmonella*, the selected plant extracts with probiotics were further evaluated for antibacterial activities *in-vivo* tests. Phytoprobiotics combination was further evaluated as an antibacterial agent by observing the mortality and morbidity in the challenged experiment. The highest mortality was observed in positive control as compared to the standard group of quanlone group of antibiotics (Ciprofloxacin). Indirect correlation/proportion was observed in phytoprobiotics combination groups regarding mortality. The lowest mortality was observed in the standard group of ciprofloxacin, followed by the phyto-pro-biotics combo-groups at the highest concentration (3.33 %). Less morbidity was observed in the negative control group while the highest morbidity was observed in the positive control. Ciprofloxacin-treated group morbidity was very low (+), while in phytoprobiotics combination treated groups high morbidity was observed for Ph(Fg) E+Bc at the rate of 1mg/L+0.03 ml/L and 100mg/ L+0.03 ml/L. Low morbidity was observed in *B. lycium* + probiotic (Ph(Bl)E+Bc) treated group at the rate of 1000mg/L+0.03 ml/L was (+) compared to the positive control group (Table II). It reflects that medicinal plant extracts with probiotic supplementation have antibacterial properties. Intestinal microflora count was found significant ($P<0.05$) variable data (Table III). Significantly the highest count was recorded in positive control and as well as in treatment groups with a low concentration of extract combination with probiotics for each *E. coli* and *Salmonella* inoculation. We observed the indirect trends of phytoprobiotics (increasing level) with intestinal microflora count (decreasing). The count for selected microflora was recorded lowest in the antibiotics treatment group, followed by phytoprobiotics groups at the highest concentration (1000mg/L+0.03ml/L), 4.15, 3.41, 5.12, 5.42, and 5.81 (CFU/g) for *E. coli* and 4.35, 5.35 and 5.81 (CFU/g) for *Salmonella*, respectively. Serum humoral immunity response was estimated through

Table II. *In-vivo* synergistic effects of different concentrations of methanolic extracts of phytoprobiotics on zootechnical indicators of egg type birds during induced infection.

Groups	Treatment (Extract+Probiotic)	Mixed infection (*E. coli* + *Salmonella*)	Means±SE		Morbidity (Severity index)
			Feed intake (%)	Mortality (%)	
Negative control	No infection, No treatment	--	94.69±0.39^{a}	0.00±0.00^{b}	+
Positive control	Infection without treatment	1.0x10^{9}	64.54±1.89^{i}	10±0.00^{a}	++++
Standard	Ciprofloxacin (2mg/kg)	1.0x10^{9}	90.60±0.80^{b}	0.00±0.00^{b}	+
($MP_{(Tf)}$E+Bc)	1mg/L+0.03ml/L	1.0x10^{9}	70.59±0.95^{gf}	6.66±3.33^{ba}	+++
	100mg/L+0.03ml/L	1.0x10^{9}	77.16±0.61^{d}	6.66±3.33^{ba}	+++
	1000mg/L+0.03ml/L	1.0x10^{9}	81.76±0.52^{c}	3.33±3.33^{ba}	++
($MP_{(Bl)}$E+Bc)	1mg/L+0.03ml/L	1.0x10^{9}	68.78±1.11^{gh}	6.66±3.33^{ba}	++
	100mg/L +0.03ml/L	1.0x10^{9}	72.25±0.51^{ef}	3.33±3.33^{ba}	++
	1000mg/L+0.03ml/L	1.0x10^{9}	80.39±0.54^{c}	3.33±3.33^{ba}	+
($MP_{(Ta)}$E+Bc)	1mg/L+0.03ml/L	1.0x10^{9}	66.83±0.56^{ih}	10±0.00^{a}	+++
	100mg/L+0.03ml/L	1.0x10^{9}	73.32±0.53^{e}	6.66±3.33^{ba}	+++
	1000mg/L+0.03ml/L	1.0x10^{9}	74.80±0.87^{ed}	3.33±3.33^{ba}	++
P-value			0.0120	0.0214	--

$MP_{(Fg)}$E, methanolic extracts of phytoprobiotics (*Trigonella foenum-graecum*)
$MP_{(B1)}$E, methanolic extracts of phytoprobiotics (*Berberis lycium*)
$MP_{(Fg)}$E, methanolic extracts of phytoprobiotics (*Terminalia arjuna*)
Means in column with different superscript are significantly different at α=0.05. Severity Index: +, very low morbidity; ++, low morbidity; +++, milled morbidity; ++++, sevier morbidity.

Table III. Synergistic effects of different concentrations of methanolic extracts of phytoprobiotics on gut microflora count during induced infection.

Groups	Treatment (Extract+Probiotic)	Mix infection (*E. coli* +*Salmonella*)	Means (Log^{10} CFU/g)	
			E. coli	*Salmonella*
Negative control	No infection, No treatment	--	5.21^{b}	4.51^{bc}
Positive control	Infection without treatment	1.0x10^{9}	8.4^{a}	7.48^{a}
Standard	Ciprofloxacin (2mg/L)	1.0x10^{9}	4.15^{c}	3.41^{c}
($MP_{(Tf)}$E+Bc)	1mg/L+0.03ml/L	1.0x10^{9}	7.54^{a}	7.25^{a}
	100mg/L+0.03ml/L	1.0x10^{9}	6.89^{ab}	6.74^{ab}
	1000mg/L+0.03ml/L	1.0x10^{9}	4.35^{c}	5.12^{b}
($MP_{(Bl)}$E+Bc)	1mg/L+0.03ml/L	1.0x10^{9}	7.87^{a}	7.09^{a}
	100mg/L +0.03ml/L	1.0x10^{9}	6.45^{ab}	6.48^{ab}
	1000mg/L+0.03ml/L	1.0x10^{9}	5.35^{b}	5.42^{b}
($MP_{(Ta)}$E+Bc)	1mg/L+0.03ml/L	1.0x10^{9}	7.42^{a}	7.52^{a}
	100mg/L+0.03ml/L	1.0x10^{9}	6.75^{ab}	6.27^{ab}
	1000mg/L+0.03ml/L	1.0x10^{9}	5.81^{b}	5.81^{b}
P-value	--	--	0.0001	0.0001

Bc, *Bacillus clausii*, For other abbreviations, see Table II.
Means in column with different superscript are significantly different at α=0.05

the detection/measured the titer of IgM, IgA, and IgG in egg-type birds (Table IV). Significantly increased level of serum immunoglobulin titer was recorded in the positive control group as compared to negative control and treatment groups (post infection). Significant serum humoral immunity response was recorded in the antibiotic standard group (1.95 IgM, 1.85 IgA, and 2.15 IgG), followed by the highest concentration of phytobiotics extract with probiotic (1000mg/L+0.03ml/L) treatment groups.

Table IV. Synergistic effects of different concentrations of methanolic extracts of phytoprobiotics on immune status during induced infection.

Groups	Treatment (Extract+Probiotic)	Mixed infection (*E. coli* +*Salmonella*)	Means (g/L)		
			IgM	IgA	IgG
Negative control	No infection, No treatment	--	1.43^{c}	1.51^{c}	1.62^{c}
Positive control	Infection without treatment	1.0x10^{9}	2.98^{a}	3.15^{a}	2.74^{a}
Standard	Ciprofloxacin (2mg/L)	1.0x10^{9}	1.95^{b}	1.85^{c}	2.15^{b}
($MP_{(Tf)}$E+Bc)	1mg/L+0.03ml/L	1.0x10^{9}	2.52^{a}	2.4^{b}	2.65^{a}
	100mg/L+0.03ml/L	1.0x10^{9}	2.05^{b}	1.94^{b}	2.14^{b}
	1000mg/L+0.03ml/L	1.0x10^{9}	1.75^{b}	1.84^{b}	1.92^{bc}
($MP_{(Bl)}$E+Bc)	1mg/L+0.03ml/L	1.0x10^{9}	2.41^{a}	2.55^{b}	2.32^{ab}
	100mg/L +0.03ml/L	1.0x10^{9}	2.04^{ab}	2.14^{b}	2.05^{b}
	1000mg/L+0.03ml/L	1.0x10^{9}	1.98^{b}	2.00^{c}	1.95^{bc}
($MP_{(Ta)}$E+Bc)	1mg/L+0.03ml/L	1.0x10^{9}	2.56^{a}	2.41^{b}	2.43^{ab}
	100mg/L+0.03ml/L	1.0x10^{9}	2.15^{ab}	2.21^{b}	2.14^{b}
	1000mg/L+0.03ml/L	1.0x10^{9}	2.02^{b}	1.98^{c}	2.00^{bc}
P-value			0.0425	0.0415	0.0471

For details of groups and treatments, see Table III.
Means in column with different superscript are significantly different at α=0.05

DISCUSSION

The present research work mainly focused on the potential of medicinal plants to overcome microbial resistance and the prevention of *E. coli* infection without any immunosuppression. The highest sensitivity (zone of inhibition) of phytoprobiotics was noticed in fenugreek + probiotic at a level of 60+5 µl (13.00 mm), followed by *T. arjuna* + probiotic @ 60 + 5 µl (12.00 mm) and fenugreek + probiotic @ 40 +5 µl (11.00 mm) against the *E. coli* and *Salmonella*. This inhibition might be due to polyphenols, tannins, flavonoids, and saponins' activities. Polyphenols, flavonoids, and saponins cause bacterial cell wall membrane disruption and leakage of the cell wall/cell membrane (Negi, 2012; Sung and Lee, 2008; Cushnie and Lamb, 2005; Francis *et al*., 2002; Ikigai *et al*., 1993) while tannins affect the microbial cellular metabolism by inhibiting the enzyme, phosphorylation and the electron transport system (Cowan, 1999: Scalbert, 1991). Or Probiotics have antimicrobial enzymes/proteins i.e. serine protease, clausin or reuterin (Bouhss *et al*., 2009: Kazan *et al*., 2005: Talarico *et al*., 1989) which utilized glycerol as a substrate to enhance the antibacterial effects (detoxification of pathogenic toxin). In this regard, the inhibition of pathogens might be due to phytobiotics that have high glycerol compounds (Vira *et al*., 2018) which are significantly utilized by proteolytic enzymes (Gabrielle *et al*., 2016) from probiotics to arrest the growth of microbiota. So, phytoprobiotics comically work together against the pathogens by the described above phenomenon.

The findings of the present study are in line with the results of Fadareabcd *et al*. (2022) who documented that combination of probiotics with phytobiotics significantly arrests salmonella growth in an *in-vitro* study. The best antibacterial activities of petroleum ether extract of fenugreek seed against *E. coli* at the highest concentration (250 mg/ml) was 17 mm (zone of inhibition) while 10 mm inhibition was recorded in methanolic fraction as reported by Mawahib *et al*. (2015). The present investigation is supported by the work of Qureshi *et al*. (2015) who investigated the ethanolic extract of fenugreek seed at the rate of 0.5 mg/ml against *E. coli* and found 2.1mm inhibition. An antimutagenic and chemo preventive study was carried out by Chatterjee *et al*. (2013) in mice by using skin papilloma as a model. They observed that a water-based extract of fenugreek seeds at the rate of 20 g/ml showed strong inhibition against the mutagens in different strains of *Salmonella*. Nandagopal *et al*. (2012) evaluated the antimicrobial activities of the different organic solvents fenugreek seed extract against the different microbes including salmonella. Aneja *et al*. (2012) worked on the different solvent extraction of *T. arjuna* against the different bacteria with a main focus on *E. coli*. They notice the antibacterial activities of *T. arjuna* against the *E. coli* (14.6 mm of inhibition zone) as reported by the present study. Significant antibacterial activity of the methanolic extract of fenugreek seed against *E. coli* was reported by Dash *et al*. (2011). Screening of methanolic and acetone extract of fenugreek and coriander against the various gram-negative bacteria including *Salmonella* was

performed by Dash *et al*. (2011). They revealed the significance of fenugreek as an antibacterial agent and concluded that the extraction should be used to develop a novel broad spectrum of the herbal antibacterial formulation. The ethanolic fraction of *B. lycium* was carried out by Hussain *et al*. (2011) against the different microbes including *E. coli*. The ethanolic fraction was found more effective as an antibacterial against *E. coli* including all other tested bacteria. The investigation of the present regarding the antibacterial activities of *T. arjuna* is supported by the finding of previous researchers Ramya *et al*. (2008) that the plants possess antibacterial efficacy against *E. coli*. Similar findings were observed as reported by the present study. The study of Gulfraz *et al*. (2007) reported the *B. lycium* activities against gram-negative bacteria including *E. coli*, and they do notice that the methanolic extract of *B. lycium* has good antibacterial activities as it possesses some phytochemicals. Findings in this study indicated a similar pattern to results obtained by (Rees *et al*., 1993) where the phytobiotics extracts included in the mixed culture of *E. coli* and probiotics selectively affected the bactericidal on *E. coli*. Mortality and morbidity in the treated groups were significantly different from the positive control. All the probiotics containing medicinal plants showed good results by reducing mortality and morbidity in the experimental trial against the positive and standard groups. Reduction in mortality and morbidity during induced pathogenic infection in egg-type birds might be due to the incorporation of probiotics with phytobiotics that resulting in reducing the pathogenic/disease/health stress by improving animal welfare (Yazhini *et al*., 2018; Vase-Khavari *et al*., 2019) or this improvement is due to the phytogenic and probiotic effects on the health status of GIT of the birds. Healthy GIT improves digestion and inhibits the adhesion of pathogens by decreasing the inflammation at the site of infection (Mahmood *et al*., 2015). So, it revealed that mortality was controlled due to healthy GIT with mild morbidity due to mixed infection. The findings of the current study are in agreement with the previous researcher (Sokale *et al*., 2019: Bortoluzzi *et al*., 2019) that necrotic enteritis (NE) in poultry production leads to high mortality and morbidity which can be significantly reduced or minimized with the use of probiotics in poultry production. The results of the present study are in line with the findings of Motawe *et al*. (2014) that aflatoxin-induced chicken significantly recovered by using probiotics in their diet. As *E. coli* and *Salmonella* infection is considered as cross-infection (zoonotic infection) between animals and humans. Therefore, the birds were challenged with a mix of infections of *E. coli* and *Salmonella* in the present study at the dose rate of $1.0x10^9$. To know the beneficial effects of phytobiotics with the combination of probiotics in drinking water against the induced mix infection were carried out in egg-type birds. As per the results of the present study, the combination of phytobiotics methanolic extracts with probiotics at the highest level of incorporation in drinking water of egg-type birds showed a significant reduction in the intestinal microflora count as compared to positive and other treatment groups. The possible phenomenon behind the reduction of pathogenic intestinal microflora might be attributed to the different protection, activation, and immunomodulatory activities of phytobiotics and probiotics. Bioactive compounds of phytobiotics are considered potential agents by promoting beneficial intestinal microbiota (probiotics) without influencing the growth of pathogenic microflora spp. This can provide an optimal precondition for effective protection against zoonotic infectious pathogens and involve in host gut-immune/defense system (Wenk, 2003). Probiotics help in the alleviation/inhibition of inflammatory reactions of pathogenic microflora and improved the host gut health immune system by modulating the cytokines expression (Wang *et al*., 2017; Lee *et al*., 2012) or secretion of antimicrobial substances, modulation of GIT-immune response, adherence to the spaces at intestinal mucosa, and improved intestinal epithelial barrier function (Broom and Kogut, 2018; Tejeero *et al*., 2012; Lin *et al*., 2008). Probiotics can reduce pathogenic activities either by lower the pathogenic-phospholipase activities or by blocking pathogens' adherence space (Ohashi and Ushida, 2009; Fuller, 1991). Probiotics reduce the pH of the gut through the production of volatile fatty acids that lead to prohibiting the growth of pathogens by reducing the pathogenic-phospholipase activities (Chichlowski *et al*., 2007; Marteau *et al*., 1997; Zentler *et al*., 1984). These are all mechanisms of phyto-pro-biotics resulting in a reduced load of pathogenic bacteria and improving gut health status. Assessment of phytobiotics and probiotics as antibacterial agents, significantly reduced the load of *E. coli* and *Salmonella* in the intestinal digesta as presented in the current is supported by the results of Faisal *et al*. (2019). That basal diet supplemented with pro-phyto-biotics significantly reduced the pathogenic strains of bacteria in intestinal digesta. The findings of the present study are in agreement with the results of (Li *et al*., 2018) who documented that birds fed with probiotics can result in a decrease in ileocecal pathogens. Decreased pathogenic load in intestinal digesta in the present study by phyto-pro-biotics combination is in-line with the results of Guo *et al*. (2004) who documented that plant extracts with probiotics feeding to broiler chicks, significantly increased the total viable count of beneficial microflora like lactobacilli and reduced the number of pathogenic microbiota in meat-type

birds. Immune-globulin (Ig) is to be considered the first isotype of antibodies that are produced by mature B-cell in the primary humoral immune system in response to the antigenic agent. In response to antigenic stimulation or signals received by helper T-cells in the immune system, it promotes the production of Ig by B-cells to defend the host body from the pathogenicity of antigens (Zhangke *et al*., 2021). The presence of immune globulin in the host serum is the indicator of long-term exposure of the host (animals or humans) to foreign antigens. As per the present results of the project, phyto-pro-biotics significantly affect the serum Ig level in egg-type birds after induced mixed infection (*E. coli* + *Salmonella*) as compared to the positive control group. The highest supplementation of phyto-pro-biotics in the drinking water of egg-type birds significantly activated the humoral immune response against the induced foreign antigens. This might be attributed to the reduce the number of pathogenic microflora in the intestinal mucosa by phyto-pro-biotics which results in less response of induced pathogenic agents to stimulate the host humoral immune response to promote more production of Ig B-cells (Zhengke *et al*., 2021). Supplementation of probiotics in the meat type bird's ration significantly promotes the immune system response (Ig) (Sefcova *et al*., 2020). Immunoglobulin-G in the present study indicated that birds were long-term/ chronic exposure to different pathogenic agents or switching of acute to chronic inflammation, resulting in the activation of B-cells by the pathogenic agent differentiate into plasmocytes which later on make the part of plasma cells that are responsible for the secretion of IgG in the plasma to neutralize the pathogenic agent toxicity/ count and become long-lived plasma memory B-cells (Chen *et al*., 2017). The findings of the present study are in line with the results of Wenk (2003) who documented that phytobiotics bioactive agents promote gut-beneficial microbiota which leads to a remarkable reduction in the pathogenic microflora. Pathogens cause an inflammatory reaction at the intestinal level which results in low or upset growth performance and alteration in immunological response by the intestinal immune system due to high count. Probiotics help the host immune system (Ig) to inhibit the inflammatory reactions (modulation in expression of cytokines) and also secret antimicrobial substances, modulation in GIT immune responses (Ig), promote the intestinal mucosal response and epithelial barrier function in the response of pathogenic-inflammatory reactions (Broom and Kogut, 2018; Wang *et al*., 2017; Lee *et al*., 2012; Tejeero *et al*., 2012; Lin *et al*., 2008). The synergistic effects of phytoprobiotics can also stimulate the intestinal immune response (Faisal *et al*., 2019). The findings of the present suggested that incorporation of phyto-pro-biotics in the egg types of birds leads to reduce the count of induced pathogenic agents which would be considered insufficient to promote/stimulate the intestinal immune system to produce a more specific immune response (Ig). The findings of the present study are in-line with the Zeng *et al*. (2015) results that essential oils from the plant kingdom reinforce the poultry birds' immune system to promote the rate of lymphocyte proliferation and phagocytosis and also improve the serum immunoglobulin. All these observations encourage the assumption that these additives may favorably affect gut functions, but the number of in vivo studies in poultry is still limited.

CONCLUSION

Based on the *in-vitro* and *in-vivo* analyses, *Trigonella foenum graecum*, *Berberis lycium* and *Terminalia arjuna* with *Bacillus clausii* (probiotics) possess strong antibacterial and immunomodulatory effects against multi drug resistant *Salmonella* and *E. coli* infection.

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IRB approval

The experimental work was approved by the Advanced Studies and Research Board (ASRB) (No: 5214-A/UAP, Dated: 31/12/2019), The University of Agriculture Peshawar, KP, Pakistan

Ethical statement

The experimental procedures used in the study were according to the guidelines of the Ethical Review Committee of the Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture Peshawar. Proper approval was taken by the aforementioned authority before the start of the experimental trial.

Statement of conflict of interest

All authors thoroughly go through the research materials and they have no conflict of interest.

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