Research Article

Impact of *Bacillus amyloliquefaciens* on Immunity of the Broiler Chicken, Shedding Pattern and Organ Colonization of *Salmonella* Enteritidis and *Clostridium perfringens* as Direct Fed Microbial in Broiler Chicken

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Abstract | In Pakistan, poultry is an important sector with a significant contribution to the national GDP. However, there are several diseases that impact poultry health. The *Salmonella* Enteritidis can cause systemic infection in chicks and laying hens with prolonged faecal shedding. *C. perfringens* cause necrotic enteritis (NE) which instigated extreme economic losses and usually occur in broiler chicken. Use of AGP in Poultry feed has several negative implications involving public health concerns and trade restrictions. *Bacillus amyloliquefaciens* has been investigated as potential probiotic. The study involves the evaluation of competitive exclusion ability of *B. amyloliquefaciens* against *S.* Enteritidis and *Cl. perfringens*. A total of 180 commercial broiler chickens were obtained from local hatchery and were reared for 42 days. Three groups (A, B, C) of birds were divided into 3 subgroups with 20 birds in each group. Group A (-ve control) received diet without AGP or probiotic. Group B (+ve control) was fed with AGP. Group C was probiotic feed supplemented with *B. amyloliquefaciens*. All subgroups 1 were kept non-challenged, at day 21, subgroups 2 were challenged with $1x10^5$ CFU of *S.* Enteritidis and subgroup 3 were challenged with $1x10^5$ CFU of *Cl. perfringens*, orally. Humoral immunity was evaluated against Newcastle disease virus by using Hemagglutination inhibition test. ChIFN-gamma ELISA was also performed. Shedding pattern post challenge was evaluated thorough cloacal swabs and evaluation of tissue tropism pattern of bacteria through dilution method. 4 birds/ group were slaughtered at d35 and d42, liver, spleen, intestine, and ceca were collected to observe organ colonization. Use of *Bacillus amyloliquefaciens* as feed additive decreased the shedding pattern of *S.* Enteritidis and *Cl. Perfringens* and may pave way for safe poultry rearing and addressing public health concerns associated with AGP.

Keywords | *Salmonella* Enteritidis, *Clostridium perfringens*, Probiotic, AGP, Evaluation of immunity, Competitive Exclusion, *Bacillus amyloliquefaciens*

Received | May 23, 2022; **Accepted** | January 15, 2023; **Published** | April 10, 2023
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Citation | Iqbal MA, Ghafoor A, Ahmad I, Ijaz M (2023). Impact of *bacillus amyloliquefaciens* on immunity of the broiler chicken, shedding pattern and organ colonization of *salmonella* enteritidis and *clostridium perfringens* as direct fed microbial in broiler chicken. J. Anim. Health Prod. 11(2): 121-128.
DOI | http://dx.doi.org/10.17582/journal.jahp/2023/11.2.121.128
ISSN | 2308-2801

INTRODUCTION

Among the agricultural industry of Pakistan, the poultry sector is an organized and prominent segment (Hussain et al., 2015). The contribution this sector makes in livestock is 11.5% and in agricultural it contributes up to 6.4%. The industry has flourished in the past decades and now it has become 2nd largest industry of Pakistan (Memon, 2012). Poultry production in rupees has increased from 11.14 million to 32.59 million during the last three decades (Hussain et al., 2015). The quality of poultry products, safety, and the emerging disease, foodborne diseases are some major challenges. The control, eradication of these foodborne pathogens is a major challenge offered

to the poultry industry. *Salmonella* Enteritidis in cause systemic infection in chicks and laying hens accompanied by prolonged faecal shedding (Desin et al., 2009). The infected hens' post- mortem revealed the presence of transovarian infection as they contained ovaries/ ovules that had been deformed, shrunken, discolored. The follicles were also found defected that is they were congested, shrunken or extended and malformed containing an attached cyst filled with fluid (Zhao et al., 2017). *Clostridium perfringens* cause necrotic enteritis (NE) which instigated extreme economic losses and usually occur in broiler chicken and involve less utilization of food by the birds, (Keyburn et al., 2008) ruffled feathers, the intestinal lining that has been damaged with focal necrosis, loose bowels, C. perfringens-associated hepatitis, fibrinoid necrosis in the liver and an astonishingly increased mortality rate (Moore et al., 2016).

There has been use of antimicrobial substances for the preventive purposes, but it has now become problematic as many questions has been raised regarding the antimicrobial resistance in many pathogenic microbes (Singer and Hofacre, 2006). Alternative ways of treatment or prevention are the need of the hour for both consumer and manufacturer (Vuong et al., 2016). Probiotics are considered to have potential to fulfil the requirement regarding the disease prevention as they increase beneficial gut commensal bacteria, so have the potential to be an alternative antibiotic (Jin et al., 1997). They can take the digestion of the host to a better state as a result both growth and immune responses are improved and pathogenic microorganisms are also restricted (Abd El-Hack et al., 2020). *Bacillus amyloliquefaciens* inhabiting soil, plants and animals is a member of the genus Bacillus and family Baciliaceae (Priest et al. 1987). *B. amyloliquefaciens* has ability of complex mixtures' (insoluble proteins, sugars, filaments, hemicellulose, and lignans) hydrolysis which make it applicable for practical food processing. It exhibits the extracellular enzyme production capability. The enzyme it produces include those which assist better digestion and food absorption, a-amylases, proteases and metalloproteases (AlGburi et al., 2016). *B. amyloliquefaciens* has been investigated as potential probiotic in laying hens (Yohannes et al., 2020). The present study involves the use of *B. amyloliquefaciens* in broiler to study its effects as probiotic by competitive exclusion.

MATERIALS AND METHODS

Sample Probiotic

Enviva PRO containing three strains of *Bacillus amyloliquefaciens* (BA) was used to study their role in competitive exclusion studies of *Salmonella* Enteritidis (SE) and *Clostridium perfringens* (CP).

Experimental birds

A day-old salmonella free broiler chicks (45g) were obtained from local hatchery and birds (n=180). The confirmation of salmonella free birds was done by random collection of cloacal swabs of 36 birds by direct plating on SS agar. The birds were kept at experimental shed of Institute of Microbiology University of Veterinary and Animal Sciences Lahore. All the groups and replicates were provided uniform environment, space, number of feeders and drinkers. The birds were kept on a unigram layer of 6 inches sawdust.

Experimental Design

Three groups (A, B, C) of birds (60 birds in each) were divided into three subgroups (20 birds each). Group A (-ve control) received feed without addition of Avilamycin (AGP). Group B (+ve control) was fed with broiler feed containing Avilamycin (100gm/ton). Group C was given probiotic feed supplemented with *Bacillus amyloliquefaciens* (60g Enviva Pro/ ton. Subgroups 1 were kept non challenged, subgroups 2 were challenged with $1x10^5$ CFU of *Salmonella* Enteritidis while subgroups 3 were challenged with $1x10^5$ CFU of *Cl. perfringens* orally at day 21.

SE Confirmation

Salmonella Enteritidis was obtained from University Diagnostic lab, UVAS Lahore. The organism was revived by plating on SS agar. It was then cultured in tetrathionate broth. DNA extraction was done and then conformational PCR using gene specific primers was done. Forward primer **5'**-TGTGTTTATCTGATGCAAGAGG-**3'** and reverse primer **5'**- TGAACTACGTTCGTTCTTCTGG-**3'** (de Freitas et al., 2010).

Isolation and Confirmation of CP

Samples for isolation of *Cl. perfringens* were collected in the form of cloacal swabs fresh fecal samples and guts of rural poultry and layers. After enrichment samples were streaked on TSC agar. That were used for streaking of inoculum from cooked meat broth. Identification was done by Gram's staining, spore staining and biochemical tests Lecithinase Test, Blood hemolysis, Catalase Test, Oxidase Test, and Fermentation.

DNA extraction was done and then conformational PCR using gene specific primers was done. Forward primer **5'**-GCTAATGTTACTGCCGTTGA-**3'** and reverse primer **5'**-CCTCTGATACATCGTGTAAG-**3'** (Mwangi et al., 2019).

SE and CP dose preparation

SE was grown in Tetrathionate broth and CP was cultured in cooked meat broth. Both broths containing 1.0 x 10^8 CFU/mL of SE comparable to 0.5 McFarland standard

were obtained and diluted to 1.0 x 10^5 CFU/mL. Subgroups 2 and 3 were challenged with SE and CP respectively.

Vaccination

All birds were vaccinated with NDV at day 1 and 21 with Medivac ND_B & ND Lasota Medivac respectively that was manufactured by Hilton Pharma. For evaluation of immune response of birds in different groups. Blood samples of randomly selected 4 birds from each subgroup and 12 from each group were collected on day 7, 14, 21, 28, 35 and 42.

Evaluation of immunity

Hemagglutination inhibition assay assessed Newcastle disease virus cellular immunity. 2 x serum dilutions Except for the control wells, each 96-well plate received 4 HA units of virus. The platter sat out for 1.5 hours. RBCs were added 30 minutes later at 4°C. Reading wells and recording HI titers examined circulating IGg's ability to neutralize Newcastle disease virus (Sedeik et al., 2019).

ChIFN-gamma was identified using modified ELISA. Observe protocol (Cat no. NB-E60048). The sample solution was put into each well. The control well received 100 µl of sample diluent. Each well received 50 µL of biotinylated IFN gamma working solution. The wells were incubated for 2 hours at room temperature with 500-600 rpm shaking after the adhesive strip was applied. Clean each well with Wash Buffer. This was said four times. Each well got 100 µL of Streptavidin-HRP solution. Covering the plate after 30 minutes of shaking at 500–600 rpm at room temperature. Aspiration and rinsing were repeated. TMB development reagent was added to each well. After 8-30 minutes of incubation, a blue colour gradient appears. 50 µL of Stop Solution was added to each well and stirred. A microtiter plate reader measured 450 nm O.D. Each sample's O.D. 450 was compared to the standard's concentration. Interpolating sample concentrations using the Chicken IFN gamma standard curve.

Evaluation of fecal shedding of SE and CP

Shedding pattern of *S.* Enteritidis *and Cl. Perfringens* post challenge was evaluated through fecal samples. Ten folds dilutions were prepared and plating was done on SS (salmonella shigella agar) agar for SE and TSC (Tryptose sulfite cycloserine) agar for CP.

Three birds from each group were randomly selected from each replicate and slaughtered at Day 35 & Day 42. Various organs like liver, spleen, intestine and caecal contents were collected and transported immediately to lab to evaluate organ colonization. The organ samples (liver, spleen, intestine and ceaca) were taken and weighed. The samples were homogenized and diluted up to 10 mL in peptone water. Ten folds dilutions were prepared and plating was done on SS (salmonella shigella agar) agar for SE and TSC (Tryptose sulfite cycloserine) agar for CP.

RESULTS

96-well plate wells were read, and HI titers were noted for evaluation of humoral immune response through ability of circulating IGg to neutralize the Newcastle disease virus (Figure 1). Antibody titers were noted for 2nd,3rd, 4th, 5th and 6th week post vaccination (Table 1). Protection Level of HI Antibody titers percentages and maximum and minimum titers for each week are also noted (Table 2 and Table 3).

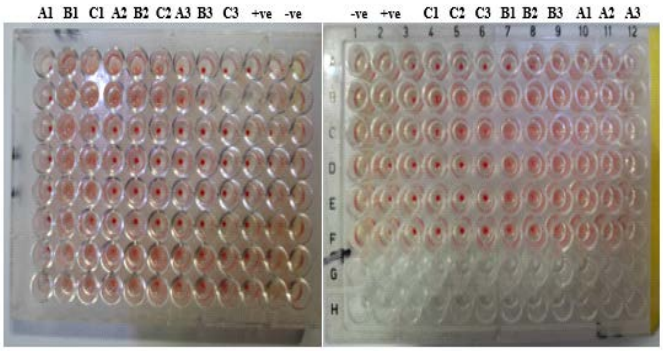

Figure 1: Hemagglutination Inhibition Assay

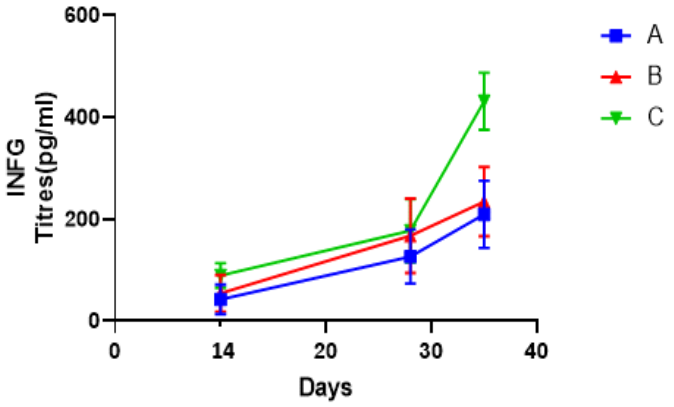

Figure 2: Graphical representation of INFG titres in different groups

Shedding pattern in feces and organ colonization by *S*E (Figure 3) and *Cl. Perferingens* (Figure 4) pre and post challenge was observed and noted through dilution and platting method. Prior to challenge with oral dosing of *S*E and *Cl. perferingens* no shedding was observed on 1st, 2nd and 3rd week from any group (Table 5). Counts for *S*E and *Cl. perferingens* were made per gram of fecal samples for 4th, 5th and 6th week (Table 6 and Table 7). Shedding counts for *S*E in group remained far higher than Group B and Group C in all weeks of post challenge.

Table 1: Antibody titers obtained by HI test

Week Post Vaccination	Group	Antibody titers obtained by HI test									
		2^1	2^2	2^3	2^4	2^5	2^6	2^7	2^8	2^9	2^{10}
2nd	A	3	2	3	4	0	0	0	0	-	-
	B	1	1	2	5	2	1	0	0	-	-
	C	1	2	1	5	3	0	0	0	-	-
3rd	A	2	1	4	3	2	0	0	0	-	-
	B	0	1	1	6	2	1	1	0	-	-
	C	0	2	1	6	1	2	0	0	-	-
4th	A	2	2	2	4	2	0	0	0	-	-
	B	0	0	2	4	1	2	1	2	-	-
	C	0	0	1	2	2	1	2	4	-	-
5th	A	0	1	4	7	0	0	0	0	-	-
	B	0	0	1	3	2	3	1	2	-	-
	C	0	2	2	4	0	2	2	0	-	-
6th	A	1	1	3	5	1	1	0	0	-	-
	B	0	0	2	5	3	1	1	0	-	-
	C	0	1	2	3	4	0	2	0	-	-

Table 2: Protection Level of HI Antibody titers percentage

Week Post Vaccination	Group	Below Protective Level	Marginal Protective Level	Protective Level
		2^1-2^2	2^3	2^4-2^{10}
2nd	A	41%	25%	34%
	B	17%	17%	66%
	C	25%	8%	67%
3rd	A	25%	33%	42%
	B	8%	8%	84%
	C	16%	8%	76%
4th	A	33%	17%	50%
	B	0%	17%	83%
	C	17%	8%	75%
5th	A	8%	33%	58%
	B	0%	8%	92%
	C	0%	17%	83%
6th	A	17%	25%	58%
	B	0%	17%	83%
	C	8%	17%	75%

Table 3: Minimum and Maximum titers

Week Post Vaccination	Group	Minimum and Maximum Titre	
		Minimum	Maximum
2nd	A	2^1	2^4
	B	2^1	2^6
	C	2^1	2^5
3rd	A	2^1	2^5
	B	2^2	2^7
	C	2^2	2^6

4th	A	2^1	2^5
	B	2^3	2^8
	C	2^3	2^8
5th	A	2^2	2^4
	B	2^3	2^8
	C	2^2	2^7
6th	A	2^1	2^6
	B	2^3	2^7
	C	2^3	2^7

Optical density (O.D) of the ELISA plate wells was read at 450 nm using a microtiter plate reader. The charts were made according to the noted values. Group B showed high values of IFN-gamma while Group C also showed comparable values while lowest values were observed in group A.

Table 4: Effect of BA supplementation on INFG level of chickens in different groups

INFG (pg/ml)	Day of sampling post vaccination	A	B	C
	14	42±29	63±36	54±24
	28	126±53	183±73	167±62
	35	283±66	359±68	388±56

The results were expressed as arithmetic means (AVG) ± SD.

Table 5: Shedding pattern prior to challenge

Age (Weeks)	Group					
	A2	B2	C2	A3	B3	C3
	Salmonella Enteritidis detection on SS agar			*Clostridium perfringens* detection on TSC agar		
1st	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
2nd	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
3rd	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected

Dilution of fecal samples were made and plated on SS agar. The growth was observed on 10^{-4} dilution.

In Group A decline in shedding pattern was observed in 6th week as compared to 4th & 5th week (Table 7). But still values remained towards higher side as compared to Group B and Group C. Shedding count was lowest in Group B in every week as compared to Group A and Group C (Table 9). Thus, meaning AGP avilamycin exerted effect on growth suppression on SE (Table 8). Group C had far lesser counts that Group A. But higher than Group B. Findings are suggestive that if birds are left alone without shielding and preventive effect of AGP or suitable DFM, pathogens grow exponentially AGP exerts very visible effect in suppression of harmful microbes. While *B.amyloliquafaciens* has ability to exclude *S*E. But not as efficient as is Avilamycin in this study.

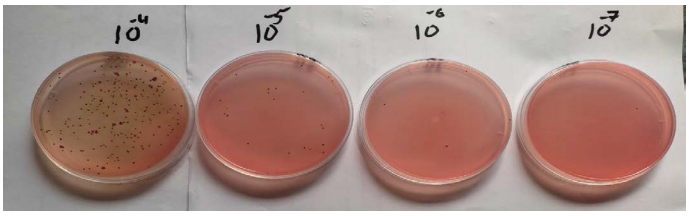

Figure 3: *Salmonella* Enteritidis at different dilutions

Table 6: *Salmonella* Enteritidis Shedding pattern post challenge

Age (Weeks)	A2	B2	C2
4th	$1.4x10^5$	$9.2x10^3$	$3.7x10^3$
5th	$2.7x10^7$	$4.0x10^4$	$2.1x10^5$
6th	$3.1x10^6$	$3.7x10^5$	$7.1x10^5$

Dilution of fecal samples were made and plated on TSC agar. The growth was observed on 10^{-4} dilution.

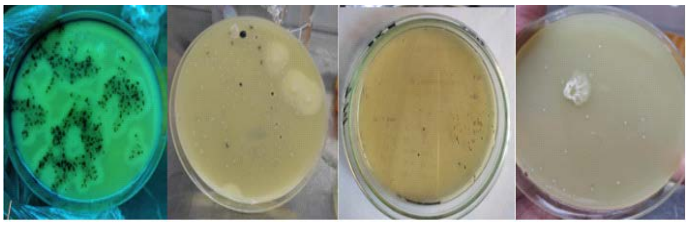

Figure 4: *Clostridium perfringens* at different dilutions

Table 7: *Clostridium perfringens* Shedding pattern post challenge

Age (Weeks)	A3	B3	C3
4th	$1.2x10^5$	$8.3x10^3$	$9.1x10^3$
5th	$2.9x10^8$	$2.3x10^5$	$6.1x10^5$
6th	$5.2x10^6$	$4.2x10^4$	$7.2x10^4$

Table 8: *Salmonella* Enteritidis plate count per 1g sample

Day	Group	Spleen	Liver	Caeca	Intestine
35th	A2	$2.3x10^3$	$7.2x10^3$	$2.1x10^5$	$9.3x10^5$
	B2	$6.0x10^2$	$1.2x10^3$	$3.9x10^4$	$4.2x10^4$
	C2	$8.5x10^2$	$2.7x10^3$	$5.1x10^4$	$8.1x10^4$
42nd	**A2**	$5.5x10^3$	$6.5x10^3$	$9.3x10^4$	$3.1x10^5$
	B2	Not detected	Not detected	$5.5x10^3$	$1.4x10^4$
	C2	$4.9x10^1$	Not detected	$1.1x10^4$	$2.3x10^4$

Table 9: *Clostridium perfringens* plate count per 1g sample

Day	Group	Spleen	Liver	Caeca	Intestine
35th	A3	$1.40x10^3$	$1.2x10^3$	$9.7x10^5$	$8.1x10^5$
	B3	Not detected	$2.5x10^2$	$7.5x10^4$	$7.3x10^4$
	C3	$5.1x10^2$	$3.0x10^2$	$1.4x10^5$	$1.2x10^5$
42nd	**A3**	$5.9x10^2$	$3.1x10^2$	$1.1x10^5$	$9.8x10^4$
	B3	Not detected	Not detected	$9.2x10^3$	$7.7x10^3$
	C3	Not detected	Not detected	$1.9x10^4$	$1.3x10^4$

DISCUSSION

A recent study conducted by Li et al. (2015) supported ours results as *Bacillus amyloliquefaciens* have been reported for improved growth performance and villus morphology, modified cecal microbiota and metabolites and increased serum IgG and IgA concentration of healthy broilers challenged with LPS extracted from Salmonella. Li et al. (2015) have suggested that feed supplemented with BA downregulated the mRNA abundance of TLR-4, INF-γ, and IL-1β, and improved intestinal barrier junction in LPS-challenged broilers (Li et al., 2015).

A multi-species probiotic containing *Lactobacillus crispatus, Lactobacillus salivarius, Lactobacillus gallinarum, Lactobacillus johnsonii, Enterococcus faecalis,* and *Bacillus amyloliquefaciens* was used in this study to colonise one group of newly hatched, Salmonella-free broilers for a period. Instead of the probiotic, broilers in another group were given oxytetracycline (200 mg/kg feed) for 28 days. On days 9 and 10, broilers in both groups were gavaged with 9 107 CFU Salmonella Enteritidis A9, a pathogenic strain isolated from infected broilers, to determine if they were infected or not. In the ceca of broilers treated with the multi-species probiotic on day 14, 95 percent of the birds had Salmonella in their ceca; however, two weeks later, almost half of the birds (45 percent) had no Salmonella in their ceca. After 28 days of treatment with oxytetracycline, researchers observed effects that were similar (Neveling et al., 2020).

In our study, the humoral immune response was assessed by NDV hemagglutination inhibition. Below protective (21-22), marginal protective (23) and protective (24-210). Each group's titer percentages were computed. Each week, the lowest and highest titers were recorded. The results showed that Group B's AGP-fed titers were very protected. Group C fed *B. amyloliquafaciens* showed higher titers than Group A. Group C titers were similar to Group B but lower. The results showed that Group B's AGP-fed titers were very protected. Group C given *B. amyloliquafaciens* as DFM showed high titers. Group C titers were similar to Group B but lower. Several investigations have found that adding *B. amyloliquafaciens* improves humoral immune response. Better gut health and digestion increases overall health of birds and metabolic pathway, which in turn promotes immune system performance to manufacture more antibodies (immunoglobulins). While birds in group A had inadequate immunological responses due to lack of AGP or DFM. The AGP group had the best immunological levels due to active bacterial suppression in the birds' GIT. NovateinBio's Chick Interferon gamma Elisa kit tested chicks' cellular immunity. Serum samples were used to examine cellular immune response in groups fed varied diets (Roberto et al., 2013). Chick Interferon

gamma was measured in 14-, 28-, and 35-day serum samples. IFN is mainly secreted by T cells and natural killer cells. It's a good measure of cellular immunity. Serum values are pg/mL. B had the most IFN. Group C follows. A had the lowest values. Unlike humoral immune response, cellular immunological response increased with age. On Day 35, each group's values peaked. Day 14 had the lowest values. Unlike the fluctuating humoral immune response, the cellular immune response increased with each week of age. On Day 35, each group's values peaked. Day 14 had the lowest values. This quantitative IFN analysis showed that AGP affects avian immunity. But adding adequate Probiotics such *B. amyloliquafaciens* as DFM increases IFN levels compared to Group A.

Groups that had been fed with BA showed significant reduction in shedding and organ colonization of SE and CP when challenged with live SE and CP infection. Although control groups when kept unchallenged showed higher level of SE and CP in fecal contents and organ samples. The study concludes that when *Bacillus amyloliquefaciens* was added in the feed of chicken, *Clostridium perfringens* (CP) and *Salmonella* Enteritidis (SE) were competitively excluded hence leading to a positive impact on overall growth and health of the birds. Hence, *Bacillus amyloliquefaciens* has the ability to serve as a potential probiotic to prevent against *Clostridium perfringens and Salmonella* Enteritidis infections.

CONCLUSION

When *Bacillus amyloliquefaciens* was added in the feed of chicken, *Clostridium perfringens and Salmonella* Enteritidis were competitively excluded and a positive impact on cellular and humoral immunity was also observed.

ACKNOWLEDGEMENTS

The authors are highly thankful to University Diagnostic Laboratory, University of Veterinary and animal Sciences, Lahore.

CONFLICT OF INTEREST

The authors declared no conflict of interest in the publication of data.

NOVELTY STATEMENT

Immunogenic properties of Bacillus amyloliquefaciens using chick interferon gamma haven't been researched prior to this in broiler chicken.

AUTHORS CONTRIBUTION

Muhammad Arshad Iqbal: Writing – original draft, Data curation. Aamir Ghaffor: Conceptualization. Irfan Ahmad: Writing – review & editing. Muhammad Ijaz: Methodology, Formal analysis.

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