INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
22–0268/2022/28–5–333–340
DOI: 10.17957/IJAB/15.1986
http://www.fspublishers.org

Full Length Article

Potential Effects of CEMB Bt Corn on Immunology and Hormonal Metabolism in Broiler Chicken

Zakiya Javed[1,6], Fazeel Laraib[2,6], Shafique Ahmed[3,6], Arfan Ali[4], Bushra Tabassum[5,6], Abdul Munim Farooq[6], Muhammad Tariq[6*], Asmatullah[1] and Idrees Ahmad Nasir[4,6]
[1]*Institute of Zoology, University of the Punjab Lahore, Pakistan*
[2]*Department of Biotechnology, Virtual University of Pakistan*
[3]*The Superior University, Lahore, Pakistan*
[4]*FB Genetics (Pvt) Ltd, Four Brothers Group Lahore, Pakistan*
[5]*School of Biological Sciences, University of the Punjab, Lahore, Pakistan*
[6]*Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan*
[*]For correspondence: m.tariq@cemb.edu.pk
Received 24 June 2022; Accepted 05 November 2022; Published 28 November 2022

Abstract

Genetically modified crops are being grown and consumed in the world at large scale. A comprehensive biosafety assessment to reveal any potential risks associated with the developed GM crop is mandatory before commercial release. The current study was designed to analyze the potential toxic effects of CEMB-Bt corn (expressing *cry1Ac* and *cry2A* insecticidal genes derived from soil bacterium, *Bacillus thuringenesis*) on broiler chicken through *in-vitro* feeding assay. A total of 60 birds were procured, vaccinated and randomly divided into four groups for a 45-day feeding assay; control group, T50, T40 and T30 were fed 0 50, 40 and 30% GM corn of total diet, respectively along with the commercial diet. Each animal of T50, T40 and T30 group total Bt protein (Cry1Ac and Cry2A) intake along with diet was 6.09 mg, 5.14 mg and 3.64 mg, respectively. The nutritional analyses of diet fed to four chicken groups revealed no significant difference. Birds were sacrificed and sampled for biochemical and molecular investigations. The evaluation of biochemical parameters (LFT; Liver function tests, TP; total protein and RFT; Renal Function Test) exhibited no significant difference among treated and control group. The mRNA expression of growth-related genes (*cGH*; chicken growth hormone, *IGF*; Insulin like Growth Factor I & II) and immune response genes (*IL-2, IL- β, TLR-04, TLR-15 and iNOS*) were also analyzed through real time PCR. No significant effects on the relative expression of growth and immune related genes were observed except *cGH* gene. The mRNA expression of *cGH* gene in control group was reduced and significantly different from the experimental groups. Conclusively, the consumption of GM Bt corn does not induce any toxic effects on growth and health of broiler chickens.

Keywords: Genetically modified; Food security; Chicken feed; CEMB-Bt corn; Growth and immune related genes

Introduction

GM crops evolved the agriculture by improving the crop productivity to ensure adequate food supply, enhanced nutritional quality, taste, resistance to herbicides, pests, increased shelf life etc. (Napier *et al.* 2019; Mbabazi *et al.* 2021). The first GM plant (*Nicotiana tabacum*) was developed in 1983 with antibiotic resistance trait (Woolsey 2012). Currently, GM crops are being cultivated on an area of approximately 190 million hectares in the world, with GM cotton, corn and soybean are being the prominent crops (Turnbull *et al.* 2021). Numerous studies have documented an expansion in the development of the R&D sector for GM crops (Graff *et al.* 2009; Miller and Bradford 2010). The GM corn has more approved events than any other transgenic crop. It was planted around the globe at 53.6 million hectares in 2015 which was about one-third of the total cultivated area with global net worth of transgenic corn is 8.1 billion US dollars (Pellegrino *et al.* 2018).

The cultivation of GM corn has not yet been approved in Pakistan. A comprehensive biosafety study to assess any of its potential effects is pre-requisite for commercialization. Centre of Excellence in Molecular Biology (CEMB), University of the Punjab-Pakistan has developed a Bt transgenic corn event expressing two Cry toxins (Cry1Ac & Cry2A) and is in pipeline for the commercialization. Possible risks associated with the GM food could be allergenicity (Gabol *et al.* 2012), harmful effect on non-targeted organisms (Losey *et al.* 1999), adverse health effects on the experimental animals (Sánchez and Parrott 2017) and any hematological, immunologic and biochemical effect (Dona and Arvanitoyannis 2009). Inconsistent reports and

To cite this paper: Javed Z, F Laraib, S Ahmed, A Ali, B Tabassum, AM Farooq, M Tariq, Asmatullah, IA Nasir (2022). Potential effects of CEMB Bt corn on immunology and hormonal metabolism in broiler chicken. *Intl J Agric Biol* 28:333–340

contradictory opinions regarding the possible dangers of GM crops to human health further sensitize the risks associated with GM crops. Moreover, non-availability of authentic information regarding the legitimacy of safety assessment tests pose bottleneck in consumer acceptance towards GM foods (Ibrahim and Okasha 2016).

The current biosafety study was designed on broiler chicken that were fed with CEMB-Bt corn in varied concentrations to assess any potential risk associated with the CEMB-Bt corn. After feeding diet containing CEMB-Bt corn, different health related parameters; feed consumption, serum biochemistry, immune and growth-related genes expression were evaluated. Corn is the basic ingredient of poultry feed, therefore chickens were selected in the current study (Anami and Widanarti 2020). In Pakistan, consumption of total annual grain in commercial poultry industry is estimated at 4.23 million tons out of which 2.42 million tons (57%) is met from corn (Habib *et al.* 2016). Moreover, the broiler chicken has high tendency of gain weight, making them highly responsive to any modification or toxins associated with diet.

Materials and Method

Test material

The test substance was genetically modified corn event, CEMB-Bt corn. Two insecticidal Cry toxin genes (c*ry1A*c & c*ry2A*) derived from *Bacillus thuringenesis* were codon optimized for enhanced expression in maize. The illustration of the binary construct harboring *cry* genes is depicted as Fig. 1.

Confirmation of *Bt* gene insertion in CEMB Bt-corn event

The insecticidal Cry toxin genes; c*ry1Ac* and *cry2A* were expressed as a single T-DNA insertion in the transgenic event, hence transgene insertion could be achieved either through *cry1Ac* or *cry2A* gene amplification (Lee and Gelvin 2008). For this, genomic DNA was extracted from the seeds of GM and non-GM corn samples through the modified CTAB method. A 2X CTAB buffer (2% CTAB, 1% PVP, 1.4 M NaCl, 100 m*M* Tris HCl pH 8.0, 20 m*M* EDTA pH 8.0 and H_2O) and extraction Buffer (20 m*M* Tris HCl pH 8.0, 25 m*M* EDTA pH 8.0, 200 m*M* NaCl, 0.5% SDS and H_2O) was heated at 65°C in the water bath prior to extraction. A 100 mg seed sample was ground in 600 μL pre-heated extraction buffer. The mixture was transferred to a tube and 400 μL of 2X CTAB buffer along with 5 μL of β-Mercaptoethanol, samples were vortexed for 1 to 2 min. Then samples were incubated at 65ºC in the water bath for 1 h accompanied by occasional shaking. Samples were cooled down on the ice for 5 min and added the 400 μL of chilled Chloroform: Isoamyl alcohol (24:1). Later, the samples were centrifuged at 13000 rpm for 20 min at 4ºC. The supernatant was shifted to a new tube and 2/3 volume of ice-cold isopropanol was added and mixed by inversions. The samples were incubated for 1 h at -20ºC and later centrifuged at 13000 rpm for 15 min at 4ºC. The supernatant was removed and the pellet was washed with 500 μL of 70% Ethanol (Merk). The DNA pellet was air-dried and resuspended in the 30 μL of nuclease free water. The DNA was treated with 0.5 μL RNAse (10 mg/mL) to remove any RNA.

PCR amplification for transgene insertion

PCR amplifications were performed to reveal insertion of *cry* genes in CEMB inbred corn line. The gene specific primers (Table 1) were used for amplification. Genomic DNA isolated from non-GM corn seeds was used as negative control while for positive control; binary construct containing *cry* genes was used. The PCR reaction mixture contained 100 ng of DNA template, 1X PCR Buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs mix, 1 µM of each forward and reverse primer, 0.5 units of Taq DNA polymerase (Thermo Scientific) and nuclease free water to make volume up to 20 μL. The amplification was performed in Veriti thermal cycler (ABI) and the conditions were; denaturation at 94ºC for 5 min followed by 35 cycles of denaturation at 95ºC for 30 sec, annealing at 60ºC for 30 sec and extension at 72ºC for 1 min. Final extension was at 72ºC for 10 min and amplified products were resolved on 1% agarose gel at 80 volts for 15–20 min.

Diet Formulation for *in-vitro* feeding assay

For feeding of the subjected birds, four different diets based on corn seed content by mixing defined quantity of non-GM and CEMB-Bt transgenic corn. The diet formulation for control and experimental groups were prepared as described by Hameed *et al*. (2016) with slight modifications. In the control group, the chickens were fed 50% non-GM corn and 50% commercial feed. While the experimental group, labeled as T50, T40 and T30 contained 50, 40 and 30% of CEMB-Bt corn, respectively in addition to 50% commercial feed (Table 2). Each diet formulation was ground to form porridge.

Nutritional analysis of diets

The diet formulations were analyzed for their nutritional contents from Provincial Animal Research Laboratory affiliated with Veterinary Research Institute, Lahore-Pakistan. Three random samples from each diet were used for analysis as biological replicates. The various dietary parameters (dry matter, crude protein, crude fat, crude fiber, ash, phosphorus, Nitrogen-Free Extract (NFE) starch and soluble sugars) were measured.

Enzyme Linked Immunosorbent Assay (ELISA) to measure Cry toxins (Cry1Ac and Cry2A) in CEMB-Bt corn

ELISA technique was used to measure the concentration of Cry toxin in all four diets (T30, T40, T50 and control).

Table 1: List of the primers used for Quantitative Real-Time PCR

Primers name	Primer Sequence 5' to 3'	Annealing temperature (°C)	Product size (bp)	Accession No.
	Bt gene			
Bt	F: ATCTTCACCTCAGCGTGCTT R: GGTGGCACATTGTTGTTCTG	62	769bp	
	Major Growth-Related Gene			
IGF-I	F: GGTGCTGAGCTGGTTGATGC R: CGTACAGAGCGTGCAGATTTAGGT	58	203	JN942578
IGF-II	F: GGCGGCAGGCACCATCA R: CCCGGCAGCAAAAAGTTCAAG	58	215	JN942579
cGH	F: CACCACAGCTAGAGACCCACATC R: CCCACCGGCTCAAACTGC	58	201	HE608816
	***Mucin* Gene**			
Muc-	F: CTGGCTCCTTGTGGCTCCTC R: AGCTGCATGACTGGAGACAACTG	58	242	JN639849
	Immune Response Gene			
IL-2	F: CCCGTGGCTAACTAATCTGCTG C: TGAGACACCAGTGGGAAACAGT	57	287	HE608819
TLR-04	F: GTTCCTGCTGAAATCCCAAACACC R: GCCAAGAGCCACGAGACTCCAAA	58	239	NC_006104.5
TLR-15	F: GTGAGAATGGGCTGGTACTGGTG R: CCAAGTACAGGATGCCCTGGT	58	203	NC_006090.5
IL- 1β	F: CATGTCGTGTGTGATGAGCGG R: GCTGTCCAGGCGGTAGAAGATGAA	57	208	AJ245728
iNOS	F: GTGTTGTGTGCTTCCACTGC R: AACACCTCCAAAGCCCTAGC	59	215	NC_006106.5
	Reference Gene			
28s	F: CAGGTGCAGATCTTGGTGGTAGTA R: GCTCCCGCTGGCTTCTCC	58	273	JN639848

Note: Bt (*Bacillus Thuringiensis*; *cry1Ac* and *cry2A* genes) IGF (Insulin-like Growth factor), GH (chicken Growth Hormone), Muc (mucin), IL (interleukin), iNOS (inducible Nitric Oxide Synthase), TLR (Toll-like receptor) and 28S = 28S rRNA

Table 2: Experimental diets GM corn compositions for control and transgenic groups

Diet groups	Commercial diet	Non-GM corn seeds	CEMB-Bt corn seeds
Control	50%	50%	0%
T50	50%	0%	50%
T40	50%	10%	40%
T30	50%	20%	30%

Note: Commercial diet for broiler chicken purchased from local market

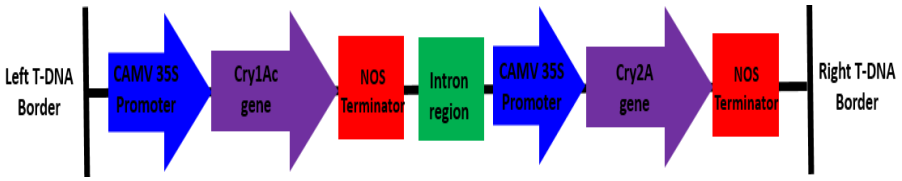

Fig. 1: Construct map transformed in to the CEMB Bt corn variety showing *cry1Ac* and *cry2A* genes

The concentration of Cry toxin in each diet formulation was revealed by using QuantiPlate for Cry1A & Cry2A Kit by Envirologix as per manual. Each sample was used in triplicate and OD was measured at 450 nm through SpectraMax® Plus 384 microplate reader with SoftNax Pro® software. The concentration of the bound Cry toxin was calculated by using the following formula:

$$\text{Concentration of}\frac{\text{Cry1Ac}}{\text{2A}}(\mu g) = \frac{\text{O. D. of the samples}}{\text{Known Concentration (Calibrator)}} * \text{Calibrator O. D}$$

Management of experimental birds and feeding trial

For the *in-vitro* feeding assay, 60 one-day old broiler chicks were procured by the courtesy of Sabir's group, Faisalabad Road, Sheikhupura-Pakistan. All the chickens were vaccinated against New castle Disease (ND) at 8th day. Later, at 30th day, vaccination against infectious bursal disease was administered . The chickens were kept in a temperature controlled unit according to the protocol described by (Brake and Vlachos 1998). Further, incandescent lighting was provided for the first seven days of the experiment (Taylor *et al.* 2003). Chickens were kept at 32°C and the temperature was gradually decreased to 24°C until the completion of the trial. This trial was conducted in the month of February, 2019. Heaters were utilized to maintain the temperature of the chamber. The animals were kept in standard laboratory environments *i.e.,* on floor covered with wooden shavings (Brake and Vlachos 1998). For each experimental group that

was fed on control, T30, T40 and T50 diet formulations, 15 birds were randomly assigned to one group. The birds were fed for a period of 45 days.

Total feed and protein consumption

After completion of the 45-day feeding period, total feed consumed by each group was calculated. On the basis of feed consumption data was calculated and total Cry protein intake by each bird by using the following formula:

$$\text{Total Cry protein intake by chicken (mg)} = \frac{\text{Total feed consumption (kg)} * \text{total protein (µg)}}{\text{total number of animals}}$$

Tissue sample collection for RNA isolation post feeding assay

Post 45-day feeding assay, blood and organ samples of birds were collected. Three birds were selected randomly from control and experimental groups for sampling. Blood was collected from the wing vein in Vacutainer (Rossi *et al.* 2005) for serum biochemistry. Total 3 mL blood was drawn from three animals per group and taken into the sterile serum separator tube. Blood samples were centrifuged at 3000 rpm for 10 min and serum was collected in new tubes and stored at 4ºC for further analysis. Tissue samples of the vital organs for RNA extraction were preserved in liquid nitrogen to preserve and were stored at -80ºC until use.

Serum biochemistry

Serum biochemistry was performed by the Diagnostic Lab., University of Veterinary and Animals Science, Lahore-Pakistan. The tests were liver function test (LFTs), AST **(**Aspartate aminotransferase), ALT **(**Alanine Aminotransferase), ALP (Alkaline Phosphatase) and total protein (TP) along with albumin and globulin. Levels of creatinine (CREA), Urea and Uric acid were also investigated for renal function test (RFTs).

Expression of immune and growth-related genes through Real-Time qPCR

The relative mRNA expression of selected marker genes for liver (*cGH;* chicken growth hormone gene, *IGF-I* & *II*; Insulin-like Growth Factor), spleen (interleukins; *IL-2* and *IL-β*, *iNOS;* inducible Nitric Oxide Synthase, toll-like receptors; (*TLR-05* and *TLR-15*) and intestine (*mucin* gene) was measured. For normalization, *28S rRNA* was used as reference gene (Bhanja *et al.* 2014). The assays were performed in PikoReal PCR systems (Thermo Scientific). Primers sequences and details are depicted in the Table 1. For total RNA extraction from liver, spleen and intestine, the samples processed as described by Toni *et al.* (2018). A 100 mg tissue sample was taken from cryogenic vial and ground in liquid nitrogen for RNA isolation. Concentration of total RNA was measured by using NanoDrop (ND1000) by Biocompare. The cDNA was synthesized using Revertaid First strand cDNA synthesis kit (Thermo Scientific) as per manual. The mRNA expression of *mucin,* growth related and immune response genes from intestine, liver and spleen were revealed through qPCR. The reaction mixture comprised of 1 μM of each primer, 5.5 μL Maxima SYBR Green master mix (2X), 50 ng of cDNA and water (nuclease-free) to make up volume 10 μL. *28S rRNA* gene was used as internal control for normalization. The real time PCR reaction conditions were denaturation at 95ºC for 3 min, 35 cycles of denaturation at 95ºC for 30 sec, annealing at 59ºC for 30 sec and extension at 72ºC for 30 sec. The gene expression data was analyzed using Livak method (Livak *et al.* 2013).

Statistical Analysis

All the data of the compositional diet analysis, various biochemical tests (LFTs and RFTs) and gene expression (growth and immune response genes) were subjected to statistical analysis and made comparison between the control and experimental groups. The mean and standard deviation of each replicate was calculated by using Microsoft Excel while 1-way ANOVA was applied by using Graph Pad Prism (version 5.00.288). The Post-test applied was Dunnet's test if $p>0.05$ then values are non-significant and vice versa whereas "*" and "ns" representing the significant and non-significant difference, respectively.

Results

Transgenes (*cry1Ac/cry2A*) insertion and protein concentration in CEMB-Bt Corn seeds

The transgenes *cry1Ac/cry2A* were amplified from the CEMB-Bt corn samples to confirm their insertion in the corn genome. It was found that a specific fragment of ~769bp was amplified in GM samples while no such amplification was observed in samples taken from the non-transgenic corn seeds (Fig. 2). The concentration of Cry2A + Cry1Ac protein in CEMB-Bt corn seeds present in diet formulations were 1.62, 1.32, 0.96 and 0 μg/gm in T50, T40, T30 and control group, respectively (Table 3).

Nutritional Analyses of diet fed to birds during feeding assay

The percentage of the dry matter, crude protein, crude fat, NFE starch+ soluble sugar and phosphorus were measured. The data confirmed that the nutritional composition of each of the four diet formulations (control, T30, T40, T50) didn't exhibit any significant differences in dietary components among control and experimental groups (Table 4).

Total feed and Cry protein consumption

During the 45-day feeding trial, no significant difference was observed in feed consumed by subjected chicken in all four

Table 3: Quantification of Cry1Ac and Cry2A protein in the GM corn seeds and experimental diets

	Transgenic corn (%)	Cry1Ac	Cry2A	Cry1Ac+Cry2A
GM corn Seeds	100%	1.4 µg/gm of seeds	1.82 µg/gm of seeds	2.22 µg/gm of seeds
Control (diet)	0%	0 µg/gm of diet	0 µg/gm of diet	0 µg/gm of diet
T50 (diet)	50%	0.70 µg/gm of diet	0.92 µg/gm of diet	1.62 µg/gm of diet
T40(diet)	40%	0.58 µg/gm of diet	0.74 µg/gm of diet	1.32 µg/gm of diet
T30 (diet)	30%	0.42 µg/gm of diet	0.54 µg/gm of diet	0.96 µg/gm of diet

Table 4: Nutritional Analyses of diet for each group

Components (%)	Treatments				p-value
	Control	T50	T40	T30	
Dry Matter	91.52±1.24	93.02±3.04	91.85±4.38	91.94±4.49	0.9571
Ash	3.84±0.96	3.97±1.19	3.81±1.13	3.84±0.37	0.9967
Crude Protein	12.20±1.37	13.40±0.71	12.80±1.67	12.83±1.37	0.7516
Crude Fat	1.74±0.23	1.63±0.34	1.67±0.25	1.68±0.26	0.9716
Crude Fiber	7.48±0.83	7.37±0.95	7.40±1.06	7.37±0.95	0.9987
NFE (%) Starch + Soluble Sugars	64.60±1.73	64.34±4.64	63.83±4.10	63.62±4.23	0.9882
Phosphorus	0.16±0.08	0.15±0.06	0.16±0.05	0.15±0.07	0.9955

Values in the table are the mean of the three replicates. Control, T50, T40 and T30 containing 0%, 50%, 40% and 30% GM corn respectively along 50% with commercial chicken feed. All nutritional values of control diet were non-significantly different with transgenic diet (p>0.05)

Table 5: Approximate intake of Bt protein by per animal of the groups

	A: (total protein) (µg/gm of diet)	B: Total feed consumption of Feed (kg)	Total protein intake per bird = $\frac{A\,X\,B}{(Total\ no.of\ animals\ in\ each\ group)}$
Control	0	57.85	0 mg
T50	1.62	56.36	6.09 mg
T40	1.32	58.36	5.14 mg
T30	0.96	56.95	3.64 mg

Table 6: Liver function parameters of control and transgenic groups

Liver function parameters	Treatments				p-Values
	Control	T50	T40	T30	
AST (U/L)	290.00±58.85	294.00±61.61	280.33±29.19	281.33±57.01	0.9855
ALT (U/L)	21.00±2.00	21.00±3.46	21.00±1.00	20.67±2.08	0.9972
ALP (U/L)	2714.6±206.3	2715.3±95.0	2603.7±183.9	2757.3±236.7	0.7787
Total Protein (g/dL)	2.97±0.31	2.97±0.15	3.00±0.36	3.04±0.25	0.9875
Albumin (g/dL)	1.63±0.15	1.77±0.22	1.70±0.40	1.67±0.21	0.9237
Globulin g/dL	1.37±0.15	1.43±0.23	1.50±0.26	1.57±0.21	0.7048

Note: Liver function parameters (ALT, AST and ALP, total protein, Albumin, and Globulin) values were non-significant of control and transgenic groups (p>0.05; n=3)

Table 7: Renal function parameters of control and transgenic groups

Renal function parameters	Treatments				p-Values
	Control	T50	T40	T30	
UR (mg/dL)	1.73±0.25	1.60±0.300	1.47±0.15	1.63±0.32	0.6944
CR (mg/dL)	0.23±0.06	0.20±0.10	0.17±0.06	0.20±0.10	0.8490
UA (mg/dL)	5.40±1.08	5.27±0.74	5.17±1.31	5.33±0.75	0.9930

Note: Renal function test of control and transgenic groups animals. Values of UR; Urea, CR: Cretinine and UA; Uric Acid (UA) showing no significant difference among control and transgenic groups. (p>0.05; n=3)

groups. Specifically, the control group animals consumed about 57.85 kg of feed while the average feed consumed by T50, T40 and T30 group was 56.36, 58.36 and 56.95 kg, respectively. On the basis of the feed consumption and average Cry protein concentration in diet fed to bird groups control, T50, T40 and T30 group, the estimated Bt protein intake by each animal was 0 mg, 6.09, 5.14 and 3.64 mg, respectively (Table 5).

Serum biochemistry

Serum biochemistry analyses include LFTs (Liver function tests) and RFTs (Renal Function tests). For liver function test, no statistical difference between the values of enzymes expressed by liver; ALT, AST and ALP was found (p>0.05) (Table 6). Results of serum protein analysis parameters *i.e.*, total protein, Albumin and Globulin were also in the normal range and showed non-significant difference among the values of the control and transgenic groups (p>0.05) as shown in Table 6. In renal function test, non-significant difference was observed between the values of Creatinine (CR) and Uric acid (UA) in different groups (p>0.05) as shown in Table 7.

Expression of mucin, growth-related and immune response genes

The mRNA expression of mucin, growth-related and immune response genes were analyzed using 2^(-ΔΔCt)

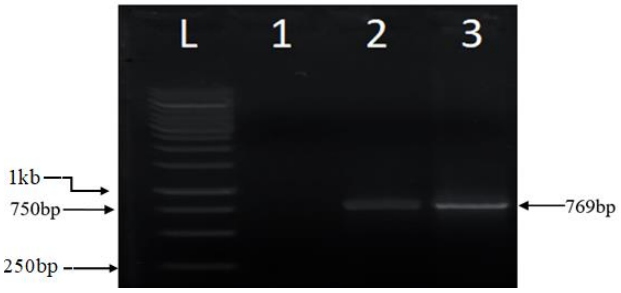

Fig. 2: Transgene (*Bt*) detection in GM and non-GM corn. L; 1kb DNA Ladder, 1; Non-GM Corn and 2; GM Corn, 3; positive Control

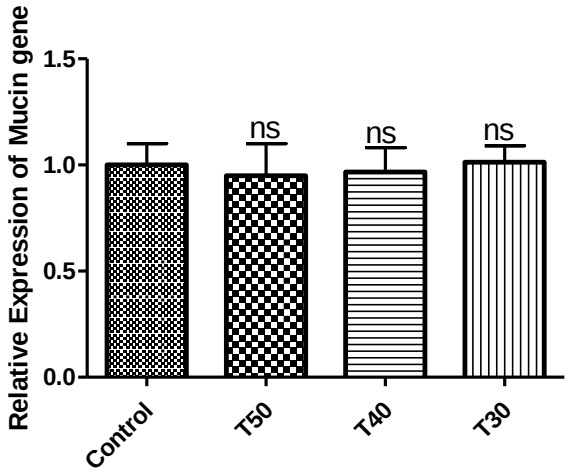

Fig. 3: Relative expression of Mucin gene related to intestinal tract development of control and transgenic corn fed groups chicken (p>0.05; n=3)

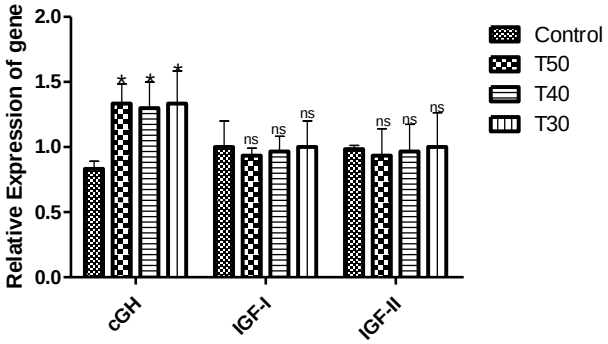

Fig. 4: Relative mRNA expression of chicken growth related gene (*cGH*; growth hormone *IGF*; Insuline like Growth Factor I and II). *GH* expression of control group is slightly low values and significant different to the transgenic groups (p<0.05; n=3). Expression of *IGF-I* and *II* gene shown no significant difference among control and transgenic groups (p<0.05; n=3)

Livak method. No significant difference was found in the relative gene expression among control and experimental group (p>0.05) as shown in the figures (Figs. 3, 4, 5) except

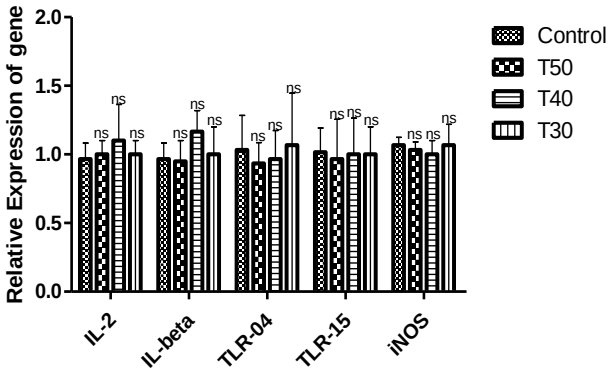

Fig. 5: Relative expression of chicken immune related gene. Expression *of IL-2, IL- β, TLR-04, TLR-15* and *iNOS* genes showed non-significant difference comparing to control and transgenic group (p>0.05; n=3)

cGH gene expression in control group that was recorded as slightly reduced and significantly differ from the transgenic groups (p<0.05).

Discussion

Agriculture has revolutionized by the transgenic crops in the recent years, however adaptation at commercial level require minimized biosafety concerns. In the present study, 45-day feeding trial of locally developed insect resistant CEMB-Bt corn on broiler chicken was performed and any potential effect of transgene on growth, development, immunity and serum chemistry of subjected bird is reported. In this study, we fed a defined concentration of the insecticidal Cry toxins (Cry1Ac and Cry2A) to broiler chicken that were divided in four different groups. Concentration of the transgenic protein in the diet was estimated through ELISA which is a common technique used for the quantification of the transgenic proteins in various crops (Bashir *et al.* 2005; Zhang *et al.* 2016).

Rigorous safety evaluation of a GM crop is warranted if the nutritional attributes has been significantly altered in transgenic line. Hence, to overcome this, we measured the nutritional composition of each diet prepared and found no significant difference when compared with control diet. Castillo *et al.* (2004) compared nutritional compositions of the diets with their considerable equivalences in a similar study. It has been reported from the previous study that *B. thuringenesis* derived insecticidal gene (*cry1Ac*) expressing insect resistance proteins could not change the dietary compositions of cotton (Tripathi *et al.* 2011). Few other studies have reported that the insertion of foreign DNA into plants did not alter the nutritional values of their seeds (Chrenková *et al.* 2002; Salisu *et al.* 2018). We also reported that the inclusion of transgenic corn in broiler chicken feed did not change the nutritional compositions when compared to the diet containing non-transgenic corn. Previous study also showed no significant difference was observed in the

composition and nutrition analysis of Bt cotton (c*ry1Ac* + c*ry2Ab2* gene) and non-Bt cotton seeds (Hamilton *et al.* 2004). Another study in which comprehensive compositional analysis of the transgenic corn (*zmm28* gene) forage and corn was substantially equal to isogenic corn (Anderson *et al.* 2019).

It has been documented that chronic feeding of the GM feed might have more authentic outcome as compared to short term trials. We conducted 45 day feeding trial with mixed population of the broiler chickens which is comparable with others feeding bioassays conducted by (Řehout *et al*. (2009) on mixed population of broiler chickens fed Bt corn for 42 days. Another study performed in Poland on broiler chickens of mixed population fed Bt corn or RR soybean for 42 days (Reichert *et al.* 2012). A comparable feeding trial was performed with mixed population of broiler chicken through feeding of Bt sugarcane with commercial diet but the duration of feeding assay was 120 days (Hameed *et al.* 2016). In numerous studies mixed population of the broiler chicken were used in the studies for the comparison (Bashir *et al.* 2020; Onunkwo *et al.* 2021).

The serum biochemistry analysis reveals the liver and kidney functions (Harper 1971). Most of the serum proteins are generally produced in the liver and perform various tasks like maintenance of blood volume, hormonal transportation, metabolic regulation and providing protection against foreign invaders (Rezende *et al.* 2017). In current study, we measured serum biochemistry (LFTs, RFTs and lipid profile) parameters and found non-significant difference when compared to the control values. Řehout *et al*. (2009) conducted a feeding assay of Bt corn and found non-significant difference among control and treated birds for liver enzyme and total protein content of broiler chicken and fall within physiological range. In another study, broiler chickens fed on GM sugarcane (c*ry1Ac*) exhibit non-significant difference in the ALT, AST, ALP, creatinine and urea (Hameed *et al.* 2016) among control and treatment groups.

The pathological process caused by any toxicant regulates the expression of number of genes including immunity associated genes. In the current study, feeding Bt corn did not cause any observable effects on splenic relative expression of selected genes *IL-1β, IL-2, iNOS, TLR4* and *TLR15* of the broiler chicken up to 50% along with commercial diet. We found relative reduced *cGH* gene expression that was significantly different ($p<0.5$) while non-significant difference was observed in relative mRNA expression of *IGF-I* and *IGF-II* genes among control and treated groups. Nutrient digestion and absorption is influenced by the Mucin which is major constituent of the mucus layer. Dietary components have the potential to induce changes in mucin dynamics. Higher expression of mucin gene triggered the number of goblet cells and production of acidic mucin in the GIT of a chick (Bhanja *et al.* 2014). We reported a non-significant difference in the relative expression of mucin gene among control and treated groups.

Conclusion

CEMB-Bt corn fed to broiler chicken in a 45 day long feeding trial didn't exhibit any potential effect on growth and performance of subjected birds. Moreover, no statistically significant difference was observed in gene expression profile of specific growth related and immune responses in the chicken and no harmful effects were detected on monitoring the changes in specific biochemical parameters.

Acknowledgments

The authors acknowledge Pakistan Agricultural Research Board (PARB) for financial assistance under PARB Project No. 235.

Author Contributions

Zakiya Javed: Investigation. Fazeel Laraib: Investigation. Shafique Ahmed: Review and editing the draft. Arfan Ali: Helped in data interpretation. Bushra Tabassum: Validation. Abdul Munim Farooq: Resources. Muhammad Tariq: Data curation, original draft preparation. Asmatullah: Supervision. Idrees Ahmad Nasir: Supervision and Conceptualization.

Conflicts of Interests

No potential conflict of interest relevant to this article does exist.

Data Availability

All data has been presented in this article.

Ethics Approval

In this study, approved protocols were in accordance with the guidelines approved by the Institutional Animals Ethics committee of Center of Excellence in Molecular Biology, University of the Punjab, Lahore-Pakistan.

References

Anami M, I Widanarti (2020). Design and construction of corn sheller machine to the suitability of grain particle size for poultry feed raw materials. *Musamus AE Featur J* 2:52–60

Anderson JA, B Hong, E Moellring, S TeRonde, C Walker, Y Wang, C Maxwell (2019). Composition of forage and grain from genetically modified DP202216 maize is equivalent to non-modified conventional maize (*Zea mays* L.). *GM Crops Food* 10:77–89

Bashir K, T Husnain, T Fatima, N Riaz, R Makhdoom, S Riazuddin (2005). Novel indica basmati line (B-370) expressing two unrelated genes of *Bacillus thuringiensis* is highly resistant to two lepidopteran insects in the field. *Crop Prot* 24:870–879

Bashir MK, M Ashraf, S Ur-Rehman, S Razzaq, MQ Bilal, S Mariam, M Tabbasum (2020). Effects of carica papaya leaf extract on blood hematology, serum biochemistry and immune response of broilers. *Adv Life Sci* 7:252–256

Bhanja SK, M Sudhagar, A Goel, N Pandey, M Mehra, SK Agarwal, A Mandal (2014). Differential expression of growth and immunity related genes influenced by in ovo supplementation of amino acids in broiler chickens. *Czech J Anim Sci* 59:399–408

Brake J, D Vlachos (1998). Evaluation of transgenic event 176 "Bt" corn in broiler chickens. *Poult Sci* 77:648–653

Castillo AR, MR Gallardo, M Maciel, JM Giordano, GA Conti, MC Gaggiotti, O Quaino, C Gianni, GF Hartnell (2004). Effects of feeding rations with genetically modified whole cottonseed to lactating Holstein cows. *J Dairy Sci* 87:1778–1785

Chrenková M, A Sommer, Z Čerešňáková, S Nitrayová, M Prostredná (2002). Nutritional evaluation of genetically modified maize corn performed on rats. *Arch Anim Nutr* 56:229–235

Dona A, IS Arvanitoyannis (2009). Health risks of genetically modified foods. *Crit Rev Food Sci Nutr* 49:164–175

Gabol WA, A Ahmed, H Bux, K Ahmed, A Mahar, S Laghari (2012). Genetically modified organisms (GMOs) in Pakistan. *Afr J Biotechnol* 11:2807–2813

Graff GD, D Zilberman, AB Bennett (2009). The contraction of agbiotech product quality innovation. *Nat Biotechnol* 27:702–704

Habib G, MFU Khan, S Javaid, M Saleem (2016). Assessment of feed supply and demand for livestock in Pakistan. *J Agric Sci Technol* A 6:191–202

Hameed A, IA Nasir, B Tabassum, Z Qamar, M Zameer, M Younus, AQ Rao, B Rashid, M Tariq, GA Khan (2016). Biosafety assessment of locally developed transgenic sugarcane. *J Anim Plant Sci* 26:1124–1132

Hamilton KA, PD Pyla, M Breeze, T Olson, M Li, E Robinson, SP Gallagher, R Sorbet, Y Chen (2004). Bollgard II cotton: Compositional analysis and feeding studies of cottonseed from insect-protected cotton (*Gossypium hirsutum* L.) producing the Cry1Ac and Cry2Ab2 proteins. *J Agric Food Chem* 52:6969–6976

Harper H (1971). *Review of Physiological Chemistry*, 16th edn. Lange Medical Books, New York, USA

Ibrahim MAA, EF Okasha (2016). Effect of genetically modified corn on the jejunal mucosa of adult male albino rat. *Exp Toxicol Pathol* 68:579–588

Lee L-Y, SB Gelvin (2008). T-DNA binary vectors and systems. *Plant Physiol* 146:325–332

Livak KJ, QF Wills, AJ Tipping, K Datta, R Mittal, AJ Goldson, DW Sexton, CC Holmes (2013). Methods for qPCR gene expression profiling applied to 1440 lymphoblastoid single cells. *Methods* 59:71–79

Losey JE, LS Rayor, ME Carter (1999). Transgenic pollen harms monarch larvae. *Nature* 399:214

Mbabazi R, M Koch, K Maredia, J Guenthner (2021). Crop Biotechnology and Product Stewardship. *GM Crop Food* 12:106–114

Miller JK, KJ Bradford (2010). The regulatory bottleneck for biotech specialty crops. *Nat Biotechnol* 28:1012–1014

Napier JA, RP Haslam, M Tsalavouta, O Sayanova (2019). The challenges of delivering genetically modified crops with nutritional enhancement traits. *Nat Plants* 5:563–567

Onunkwo DN, A Jabbar, M Talha, A Rauf, H Javaid, MU Munir, N Irm, MH Saleem (2021). Response of starter broiler chickens to feed diets treated with organic acids. *Adv Life Sci* 8:257–261

Pellegrino E, S Bedini, M Nuti, L Ercoli (2018). Impact of genetically engineered maize on agronomic, environmental and toxicological traits: A meta-analysis of 21 years of field data. *Sci Rep* 8:1–12

Řehout V, J Kadlec, J Čítek, E Hradecká, L Hanusová, B Hosnedlová, F Lád (2009). The influence of genetically modified Bt maize MON 810 in feed mixtures on slaughter, haematological and biochemical indices of broiler chickens. *J Anim Feed Sci* 18:490–498

Reichert M, W Kozaczyński, TA Karpińska, Ł Bocian, A Jasik, A Kycko, M Świątkiewicz, S Świątkiewicz, I Furgał-Dierżuk, A Arczewska-Włosek (2012). Histopathology of internal organs of farm animals fed genetically modified corn and soybean meal. *Bull Vet Inst Pulawy* 56:617–622

Rezende MS, AV Mundim, BB Fonseca, RL Miranda, W Oliveira, CG Lellis (2017). Profile of serum metabolites and proteins of broiler breeders in rearing age. *Braz J Poult Sci* 19:583–586

Rossi F, M Morlacchini, G Fusconi, A Pietri, R Mazza, G Piva (2005). Effect of Bt corn on broiler growth performance and fate of feed-derived DNA in the digestive tract. *Poult Sci* 84:1022–1030

Salisu IB, AA Shahid, Q Ali, AQ Rao, T Husnain (2018). Nutritional assessment of dietary Bt and CP4EPSPS proteins on the serum biochemical changes of rabbits at different developmental stages. *Front Nutr* 5:49

Sánchez MA, WA Parrott (2017). Characterization of scientific studies usually cited as evidence of adverse effects of GM food/feed. *Plant Biotechnol J* 15:1227–1234

Taylor ML, Y Hyun, GF Hartnell, SG Riordan, MA Nemeth, K Karunanandaa, B George, JD Astwood (2003). Comparison of broiler performance when fed diets containing grain from YieldGard Rootworm (MON863), YieldGard Plus (MON810× MON863), nontransgenic control, or commercial reference corn hybrids. *Poult Sci* 82:1948–1956

Toni LS, AM Garcia, DA Jeffrey, X Jiang, BL Stauffer, SD Miyamoto, CC Sucharov (2018). Optimization of phenol-chloroform RNA extraction. *MethodsX* 5:599–608

Tripathi MK, D Mondal, R Somvanshi, SA Karim (2011). Haematology, blood biochemistry and tissue histopathology of lambs maintained on diets containing an insect controlling protein (Cry1Ac) in Bt-cottonseed. *J Anim Physiol Anim Nutr (Berl)* 95:545–555

Turnbull C, M Lillemo, TAK Hvoslef-Eide (2021). Global regulation of genetically modified crops amid the gene edited crop boom–a review. *Front Plant Sci* 12:258

Woolsey GL (2012). *GMO timeline: A history of Genetically Modified Foods.* GMO Inside, Washington DC, USA

Zhang Y, J Yang, J Nie, J Yang, D Gao, L Zhang, J Li (2016). Enhanced ELISA using a handheld pH meter and enzyme-coated microparticles for the portable, sensitive detection of proteins. *Chem Commun* 52:3474–3477