ORIGINAL ARTICLE

Evaluation of transgenic event CBH 351 (StarLink) corn in broiler chicks

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ABSTRACT

The influence of transgenic event CBH 351 (StarLink; SL)-derived hybrid corn on the growth performance, health condition and physiological function in broiler chicks, as well as the possible transfer of the *cry9C* gene and Cry9C protein to blood, liver and muscle were examined in comparison with chicks fed on a diet with non-transgenic corn (SL-F). Bodyweight gain and feed conversion ratio in the chicks fed on a diet with SL were significantly greater than in chicks fed on a diet with SL-F during the starter phase (0–3 weeks of age), but this significant difference disappeared during the finisher phase (4–7 weeks of age). No abnormalities in health condition in either SL or SL-F groups were observed, and livability did not differ significantly between SL and SL-F groups. Moreover, no significant differences in serum biochemical and hematological values, histopathological observation and necropsy findings were observed between SL and SL-F groups at the end of the experiment. The *cry9C* gene and Cry9C protein were not detected in blood, liver and muscle of chicks at 3, 5 or 7 weeks of age. The results indicate that feeding SL does not influence growth performance, health condition or physiological function in broiler chicks, and the *cry9C* gene and Cry9C protein are not transferred to the blood, liver and muscles of broilers.

KEYWORDS: broiler chicks, growth performance, StarLink corn, transfer of *cry9C* and Cry9C.

INTRODUCTION

Many varieties of crop plants with improved agronomic characteristics (e.g. insect resistance, herbicide tolerance, disease resistance and climate tolerance) and enhanced consumer benefits (e.g. better taste and texture, longer shelf-life, low-allergen potential and more nutritious) have been developed through agricultural biotechnology. Because genetically modified (GM) crops bring about a profit to the growers through prevention of crop losses by pest control or a reduction in the amount of herbicide applied to the field, the harvested area of GM crops has increased, mainly in the USA, Argentina and Canada, every year since 1996. Genetically modified crops with insect resistance and herbicide tolerance are used in animal feeds, however, only GM crops approved by regulatory agencies are commercialized in each country.

In Japan, the safety assessment of GM crops as feed ingredients is conducted according to the 'Guidelines for Safety Assessment of Feed Produced by the Recombinant DNA Techniques' (Ministry of Agriculture, Forestry and Fisheries, MAFF 1996). These guidelines were prepared on the basis of concepts and principles (namely, substantial equivalence) for safety evaluation of foods derived by modern biotechnology, which were presented by the OECD (1993). At the present time, GM crops that have been confirmed for their safety and approved for the use as a feed ingredient by the Ministry of Agriculture, Forestry and Fisheries total 30 varieties, including corn, soybean, rapeseed, cottonseed and beet.

In October 2000, social unease developed when it was discovered that part of the food and feed

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distributed in Japan contained StarLink (SL). At that time, SL was not approved by regulatory agencies in Japan for food and feed, but was approved by USA regulators for feed. StarLink, an insect-resistant corn variety, contains a novel gene from a naturally occurring soil bacterium, *Bacillus thuringiensis* subsp. *tolworthi*, which codes for a form of the Bt toxin known as Cry9C protein (Schepf *et al*. 1998). Aventis CropScience (MN, USA) subjected SL to safety assessments, but a feeding experiment in domestic animals has not been published.

In this study, the effect of SL on growth performance, health condition and physiological function was examined in broiler chicks. We also wanted to determine if the *cry9C* gene and Cry9C protein contained in SL could be transferred to blood, liver and muscles of the chicks.

MATERIALS AND METHODS

Corn grain

The transgenic corn used was event CBH 351-derived hybrid (SL) and was purchased from Aventis CropScience. This corn grain contained 89.3% of the grain carrying the *cry9C* gene.

The control non-transgenic corn (SL-F) used was a commercial corn imported from the USA. In the SL-F, the *cry9C* gene was not detected by polymerase chain reaction (PCR), and the Cry9C protein was not detected by an enzyme-linked immunosorbent assay (ELISA). Before formulating the diet, both SL and SL-F were ground through 1-mm sieves.

Diet

The composition of the starter and finisher diets is shown in Table 1. Chicks were fed the starter diet for 3 weeks (0–3 weeks of age; starter phase) followed by the finisher diet for the subsequent 4 weeks (4–7 weeks of age; finisher phase). The diet for the SL-F group contained 70% SL-F, and the diet for SL group contained 70% SL. All diets were formulated to meet or exceed recommended nutrient requirements (Ministry of Agriculture, Forestry and Fisheries Research Council Secretariat, MAFF 1997) based on the Standard Table of Feed Composition in Japan (1995) (Ministry of Agriculture, Forestry and Fisheries Research Council Secretariat, MAFF 1995).

Experimental design

A total of 256 1-day-old Chunky chicks (equal number of males and females) were obtained from a local hatchery and were randomly allotted into eight pens according to sex with 16 chicks each (two treatments, four replications). The diets and tap water were available ad libitum. In the starter phase, chicks were maintained in electrically heated batteries in a windowless poultry house. The temperature of the room was set at 34°C and gradually decreased to 25°C. To prevent mutual contamination, the battery for the SL group was separated by a vinyl sheet curtain from that for the SL-F group. In the finisher phase, chicks were maintained in wire-mesh cages in an open-type poultry house. To prevent mutual contamination, the

Table 1 Composition of diets

Ingredients (%)	Starter diet†	Finisher diet‡
Corn§	70.00	70.00
Soybean protein concentrate	7.00	–
Dehulled soybean meal	13.27	20.12
Fish meal (crude protein 65%)	6.00	5.00
Soybean oil	1.00	2.50
Calcium carbonate	0.72	0.73
Dicalcium phosphate	1.30	1.08
Sodium chloride	0.20	0.20
Vitamin B premix¶	0.10	0.10
Vitamin ADE premix††	0.10	0.10
Trace mineral premix‡‡	0.10	0.10
L-Lysine HCL	0.13	0.07
DL-Methionine	0.05	–
L-Tryptophan	0.01	–
L-Threonine	0.01	–
L-Arginine	0.01	–
Calculated analysis§§		
Crude protein (%)	20.9	19.0
Metabolizable energy (Mcal/kg diet)	3.15	3.19
Calcium (%)	0.97	0.80
Non-phytate phosphorus (%)	0.49	0.44
Available lysine (%)	1.08	0.92
Available methionine (%)	0.45	0.37
Available threonine (%)	0.72	0.65

†Starter diets were fed to chicks from 0 to 3 weeks of age. ‡Finisher diets were fed to chicks from 4 to 7 weeks of age. §Transgenic corn (StarLink) was used in the diets for the experimental group and non-transgenic corn was used in the diets for the control group. ¶Contained (g/kg of premix): thiamine nitrate 2.0, riboflavin 10.0, pyridoxine hydrochloride 2.0, nicotinic amide 2.0, D-calcium pantothenic acid 4.35, choline chloride 138.0, folic acid 1.0 and defatted rice bran. ††Contained (IU or mg/kg of premix): vitamin A 10 000 IU, vitamin D3 2000 IU, DL-α-tocopherol acetate 10 mg and defatted rice bran. ‡‡Contained (g/kg of premix): manganese 80, iron 6.0, copper 0.6, zinc 50, iodine 1.0 and defatted rice bran. §§Based on Standard Table of Feed Composition in Japan (1995) (Ministry of Agriculture, Forestry and Fisheries Research Council Secretariat, MAFF 1995).

SL and SL-F groups were separately housed at either end of the room.

Growth performance

Individual bodyweight was determined at 0, 3, 5 and 7 weeks of age. Feed intake and livability were recorded every day for each replication. The feed conversion ratio was calculated for the starter phase, the finisher phase and the entire experimental period. The individual health status of the chicks was monitored every day.

Serum biochemical, hematological and histopathological examination

At the end of the experiment, 20 chicks comprising an equal numbers of males and females were randomly chosen from the SL and SL-F groups. The chicks were anesthetized with ether and then humanely killed.

For the serum biochemical examination, bloods were collected from the jugular vein, and sera were separated. Lactate dehydrogenase (LDH), glutamic-oxaloacetic transaminase (GOT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), neutral lipid (NL), total cholesterol (T-Chol), total protein (TP), albumin (Alb) and glucose (Glu) were determined by an autoanalyzer (JCB MB8 model; Nihon Denshi Co., Tokyo, Japan). Globulin (Glob) and the Alb/Glob (A/G) ratio were calculated on the basis of TP and Alb.

For the hematological examination, blood samples were collected from the jugular vein. Hemoglobin (Hb) (azido-methemoglobin method), hematocrit (Ht) (capillary method), red blood cell count (RBC) (Coulter Counter, model ZF; Coulter Electronics, Florida, USA), and white blood cell (WBC) (indirect method) were determined (Anon 1980).

For the histopathological examination, tissue samples of brain, lung, heart, liver, kidney, spleen, pancreas, duodenum and jejunoileum were collected. The tissue samples were fixed, thin sections were prepared, stained with hematoxylin and eosin, and examined with a light microscope.

Detection of the *cry9C* gene and Cry9C protein in blood, liver and muscle

At 3 and 5 weeks of age, a total of 16 chicks comprising an equal number of males and females were chosen from the SL and SL-F groups. In this case, one chick, with an average bodyweight, was selected from each replicate group. At the end of the experiment, a total of 20 chicks comprising an equal number of males and females were randomly chosen from the SL and SL-F groups. Individual blood, liver and muscle samples were collected.

For detection of the *cry9C* gene by PCR, DNA was isolated from the heparinized bloods using a commercial DNA extraction kit (Dr. GenTLE; Takara Shuzo Co. Ltd, Kyoto, Japan) according to the manufacturer's protocol. Liver and muscle were frozen in liquid nitrogen, and finely ground before DNA extraction. DNA was isolated from the ground tissues using a commercial DNA extraction kit (Isotissue; Nippon Gene Co. Ltd, Toyama, Japan) according to the manufacturer's protocol.

The PCR amplification was conducted in a Takara PCR Thermal Cycler (MP model; Takara Shuzo Co. Ltd) according to the the conditions recommended by Aventis CropScience and by Shen *et al*. (1999) respectively. The amplified fragments were separated on 3% agarose gel. Following separation of the fragments, the gels were stained with ethidium bromide solution (0.5μg/mL). The gel was photographed with a CCD camera (AE-6915 model; Atto Co. Ltd, Tokyo, Japan) under UV irradiation. The PCR amplification was performed using two sets of primers. The PCR primer set specific for amplification of a 379-bp *cry9C* gene region in transgenic DNA was designed by Aventis CropScience. The PCR primer set specific for amplification of a 1075-bp cytochrome b region in mtDNA of chicks was designed by Shen *et al*. (1999).

For the detection of the Cry9C protein by ELISA, blood was mixed with an equal volume of sterile water. After the sample was homogenized, the sample was centrifuged at 500 *g* for 5 min. The supernatant was used as the analytical material. Liver and muscle were frozen in liquid nitrogen and finely ground. After mixing with five parts extraction buffer, the samples were homogenized. The samples were centrifuged at 500 *g* for 5 min for muscle, and at 7000 *g* for 10 min for liver. The supernatants were diluted 4–40-fold with water, and the diluted samples were used as the analytical materials.

The determination of Cry9C protein in the analytical materials was performed using a commercial GMO Bt9 maize test kit and Cry9C standard solution (Strategic diagnostics Inc., Delaware, USA) according to the manufacturer's protocol. After the reaction was stopped, the extinction was measured at 450 nm with a microplate reader (Spectra classic model; TECAN Austria GmbH, Salzburg, Austria).

Statistical analysis

Data was analyzed by a two-way ANOVA (Yoshida 1985), which included the main effect of diet and sex,

and the interaction between diet and sex. The significance of differences between means was tested at the level of $P < 0.05$. The data for chicks that were killed for sampling at 3 and 5 weeks of age, or that died or were culled during the experimental period, were removed from the statistical analyses of bodyweight gain and feed conversion ratio.

RESULTS

Growth performance

Bodyweight gain and livability, and feed intake and feed conversion ratio are presented in Tables 2 and 3, respectively. During the experimental period, one male in the SL-F group died by accident and one female in the SL group died of ascites. In addition, one male in the SL group was culled because of perosis. There were no significant differences in livability between the SL and SL-F groups.

During the starter phase, bodyweight gain in the SL group was significantly greater than that in the SL-F group, and feed intake of the SL group was less than that of the SL-F group. Therefore, the feed conversion ratio of the SL group for the starter phase was significantly greater than that of the SL-F group. However, during the finisher phase and over the entire experimental period, bodyweight gain, feed intake and feed conversion ratio were not significantly different

Table 2 Bodyweight gain and livability of chicks during the starter phase (0–3 weeks of age), finisher phase (4–7 weeks of age) and the entire experimental period

	Bodyweight gain (g)			Livability (%) (0–7 weeks)
	0–3 weeks	4–7 weeks	0–7 weeks	
Diet				
SL-F group	611	1903	2514	99.2
SL group	648	1931	2579	98.5
Sex				
Male	647	1953	2621	98.5
Female	613	1881	2473	99.2
Pooled SE	30	90	108	2.4
Probabilities				
Diet	< 0.01	NS	NS	NS
Sex	< 0.01	NS	< 0.01	NS
Diet × sex	NS	NS	NS	NS

Each value is the mean of four replications. Transgenic corn (StarLink) was used in the diets for the experimental group (SL) and non-transgenic corn was used in the diets for the control group (SL-F). NS, not significant ($P > 0.05$).

Table 3 Feed intake and feed conversion ratio of chicks during the starter phase (0–3 weeks of age), finisher phase (4–7 weeks of age) and the entire experimental period

	Feed intake (g)			Feed conversion ratio		
	0–3 weeks	4–7 weeks	0–7 weeks	0–3 weeks	4–7 weeks	0–7 weeks
Diet						
SL-F group	820	3781	4601	1.34	1.99	1.83
SL group	796	3872	4668	1.23	2.01	1.81
Sex						
Male	824	3914	4738	1.27	2.01	1.82
Female	793	3739	4531	1.30	1.99	1.82
Pooled SE	26	150	165	0.06	0.06	0.04
Probabilities						
Diet	0.03	NS	NS	< 0.01	NS	NS
Sex	0.01	0.02	0.01	0.01	NS	NS
Diet × sex	NS	NS	NS	NS	NS	NS

Each value is the mean for four replications. Transgenic corn (StarLink) was used in the diets for the experimental group (SL) and non-transgenic corn was used in the diets for the control group (SL-F). NS, not significant ($P > 0.05$).

between the SL and SL-F groups. The interactions were not significant for livability, bodyweight gain, feed intake and feed conversion ratio in the starter phase, the finisher phase or the entire experimental period.

Serum biochemical values

The serum biochemical values are presented in Table 4. No significant differences between SL and SL-F groups were observed for LDH, GOT, ALP, BUN, NL, T-Chol, Glu, TP, Alb and Glob. However, LDH, GOT, TP, Alb and Glob were significantly higher in females than in males. Conversely, T-Chol and Glu were significantly higher in males than in females. For other measurements, significant differences by sex were not observed.

Hematological values

The hematological values for both groups are presented in Table 5. No significant differences between SL and SL-F groups were observed for the values of Hb, Ht, RBC and WBC. However, RBC was significantly higher in females than in males. The interactions were not significant for the hematological values examined.

Histopathological observations

Histopathological observations were performed on samples from males and females of both SL and SL-F groups. No significant changes in tissue samples of brain, lung, heart, liver, kidney, spleen, pancreas, duodenum and jejunoileum of chicks were observed in

Table 4 Serum biochemical values for chicks at the end of the experiment

	LDH (IU/dL)	GOT (IU/dL)	ALP (IU/dL)	BUN (mg/dL)	NL (mg/dL)	T-Chol (mg/dL)	Glu (mg/dL)	TP (g/dL)	Alb (g/dL)	Glob (g/dL)	A/G
Diet											
SL-F group	1411.0	249.9	6231.1	0.42	99.1	116.5	233.0	3.65	1.39	2.26	0.62
SL group	1476.6	255.2	5949.8	0.60	91.1	109.7	226.9	3.60	1.39	2.21	0.63
Sex											
Male	1225.3	240.2	6530.4	0.44	94.3	117.9	235.1	3.51	1.34	2.17	0.62
Female	1662.3	264.9	5650.4	0.59	95.9	106.8	224.7	3.73	1.44	2.29	0.63
Pooled SE	402.7	31.9	805.4	0.31	22.2	10.9	14.6	0.23	0.13	0.15	0.06
Probabilities											
Diet	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Sex	< 0.01	0.01	NS	NS	NS	< 0.01	0.02	< 0.01	0.01	0.01	NS
Diet × sex	NS	0.05	NS	NS	NS	NS	NS	NS	NS	0.04	NS

Values are means, $n = 10$ chicks/sex/group (i.e. total $n = 40$). Transgenic corn (StarLink) was used in the diets for the experimental group (SL) and non-transgenic corn was used in the diets for the control group (SL-F). A/G, albumin/globulin ratio; Alb, albumin; ALP, alkaline phosphatase; BUN, blood urea nitrogen; Glob, globulin; Glu, glucose; GOT, glutamic-oxaloacetic transaminase; LDH, lactate dehydrogenase; NL, neutral lipid; NS, not significant; T-Chol, total cholesterol; TP, total protein.

Table 5 Hematological values for chicks at the end of the experiment

	Hb (g/dL)	Ht (%)	RBC ($\times 10^6$/mm^3)	WBC ($\times 10^3$/mm^3)
Diet				
SL-F group	8.9	31.7	3.34	30.0
SL group	8.5	31.5	3.21	25.8
Sex				
Male	9.0	30.9	3.18	26.3
Female	8.4	32.4	3.38	29.5
Pooled SE	1.1	2.7	0.25	7.6
Probabilities				
Diet	NS	NS	NS	NS
Sex	NS	NS	0.03	NS
Diet × sex	0.03	NS	NS	NS

Values are means, $n = 10$ chicks/sex/group (i.e. total $n = 40$). Transgenic corn (StarLink) was used in the diets for the experimental group (SL) and non-transgenic corn was used in the diets for the control group (SL-F). Hb, hemoglobin; Ht, hematocrit; NS, not significant. RBC, red blood cell count; WBC, white blood cell.

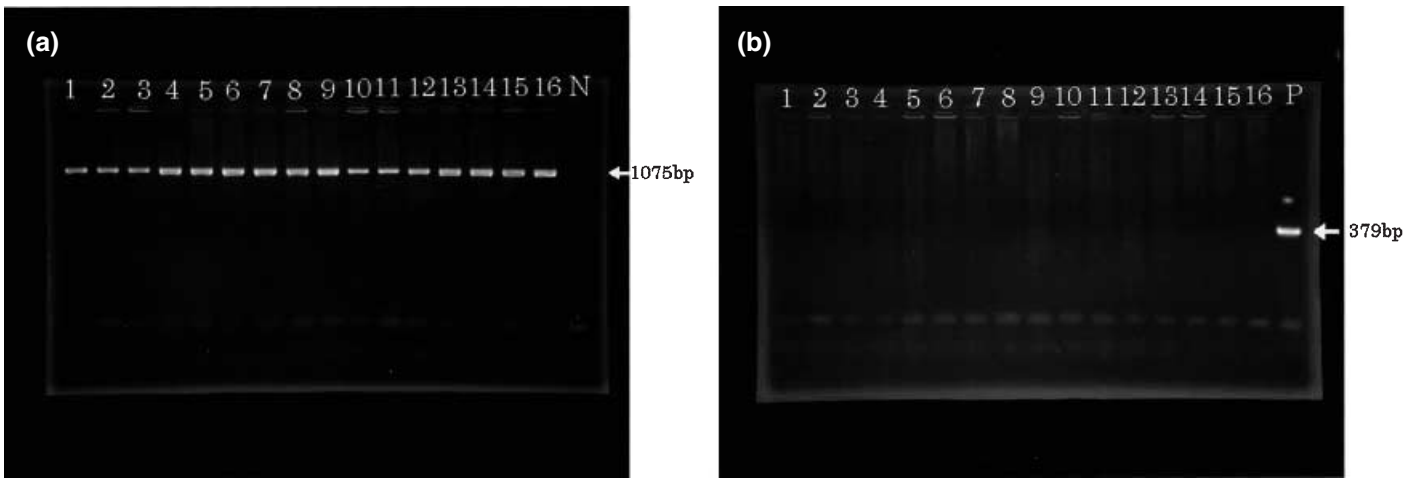

Fig. 1 Detection of polymerase chain reaction products specific for (a) the cytochrome b region and (b) *cry9C* gene in the liver of broiler chickens. Lanes 1–8, livers of broiler chickens from the control group. Lanes 9–16, livers of broiler chickens from the experimental group. N, no liver DNA; P, positive control.

either group (data not shown). There were no abnormalities observed in the main organs of the chicks.

Possibility of *cry9C* gene and Cry9C protein transfer to blood, liver and muscles

At 3, 5 and 7 weeks of age, the *cry9C* gene and Cry9C protein were not detectable in blood, liver and muscles in SL and SL-F groups as determined by PCR and ELISA, respectively (data not shown). In all cases, DNA strands of the 1075-bp cytochrome b region were amplified by PCR from DNA obtained from blood, liver and muscles in both groups, whereas DNA strands of the 379-bp *cry9C* region were not amplified. The PCR products amplified from liver DNA at 3 weeks of age are shown in Figure 1. The same results were obtained for samples from blood, liver and muscles at 5 and 7 weeks of age (data not shown). These results indicate that the *cry9C* gene and Cry9C protein contained in SL are not transferred to blood, liver and muscle of chicks after ingestion.

DISCUSSION

The influence of SL on growth and health, as well as the possibility of transfer of the *cry9C* gene and Cry9C protein to blood, liver and muscle were examined. The diets used in this study contained 70% SL or SL-F, which is accepted as the upper limit of the formula for broiler chicks from the viewpoint of animal nutrition. Since SL contained 89.3% corn grain carrying *cry9C*, eventually chicks in the experimental group continued to take a diet containing about 63% SL from the start to the end of the experiment.

The present data showed that bodyweight gain and feed conversion ratio in the SL group during the starter phase were significantly greater than in the SL-F group, but this significant difference was not present during the finishing phase or over the entire experimental period. There may be two possible reasons for this. First, there may be a difference in the nutritive value between SL and SL-F, influencing the nutritive value of the diets. Second, the battery of the SL-F group was separated from the battery of the SL group by a vinyl sheet curtain in a windowless poultry house to prevent mutual contamination, possibly resulting in a difference in the environmental conditions between the two batteries.

In order to examine the first possibility, the crude protein content and amino acid composition of each diet used in this study were determined by a standard method. In addition, apparent metabolizable energy values of each diet were determined by the chromium oxide-indicator method using three 35-day-old broiler chicks for each diet. As shown in Table 6, there were no significant differences in crude protein content, amino acid composition and apparent metabolizable energy value between SL and SL-F diets for both starter and finisher diets. Therefore, it was assumed that the significant differences in bodyweight gain and feed conversion ratio between SL and SL-F groups during the starter phase were not caused by SL intake,

Table 6 Crude protein content, apparent metabolizable energy value and animal acid composition of diets

	Starter diet		Finisher diet	
	SL-F diet	SL diet	SL-F diet	SL diet
Crude protein (%)	20.3	20.6	18.7	18.4
Metabolizable energy (Mcal/kg diet)	3.09	3.11	3.12	3.11
Arginine (%)	1.15	1.14	1.09	1.01
Glycine (%)	0.90	0.91	0.89	0.82
Serine (%)	0.92	0.93	0.90	0.83
Histidine (%)	0.52	0.53	0.50	0.49
Isoleucine (%)	0.77	0.77	0.75	0.70
Leucine (%)	1.72	1.65	1.64	1.52
Lysine (%)	1.11	1.10	1.03	0.99
Methionine (%)	0.43	0.43	0.36	0.37
Cystine (%)	0.30	0.31	0.28	0.29
Phenylalanine (%)	0.90	0.88	0.88	0.81
Threonine (%)	0.76	0.77	0.72	0.67
Tryptophan (%)	0.23	0.24	0.21	0.20
Valine (%)	0.85	0.86	0.84	0.77

Data are measured values. Transgenic corn (StarLink) was used in the experimental diet (SL) and non-transgenic corn was used in the control diet (SL-F).

but, more likely, by environmental factors. This supposition may be supported by the fact that bodyweight gain and feed conversion ratio of both groups during the finisher phase were approximately the same, and the cages housing chicks in control and experimental groups during the finishing phase were settled separately at both ends in an open-type poultry house in which environmental conditions were almost uniform.

Abnormalities in health condition for chicks in the SL and SL-F groups were not observed, and the livability did not differ significantly between the two groups. Moreover, significant differences in serum biochemical and hematological values of chicks examined on the last day of the experiment were not found between the SL and SL-F groups, and no abnormalities were observed after histopathological observation or in necropsy findings for chicks in each group.

Although some feeding experiments of GM corn, excluding SL, have been done with broiler chicks, there was no difference in broiler performance owing to the consumption of the GM corn compared with the conventional non-GM corn (Brake & Vlachos 1998; Sidhu *et al*. 2000; Einspanier *et al*. 2001).

In this study, the *cry9C* gene and Cry9C protein were not detected by PCR and ELISA in blood, liver and muscle of chicks at any sampling period. Duggan *et al*. (2000) showed that corn chromosomal DNA, including a 1914-bp fragment of the *cry1A (b)* gene was degraded after just a 1-min incubation with ovine rumen fluid. Thus, there is a possibility that the Cry9C protein was degraded in the gastrointestinal tract of broiler chicks and was not transferred to blood, liver and muscles.

In conclusion, the results obtained in this study indicate that feeding a diet containing SL up to the nutritional upper limit of the formulation, does not influence growth performance, health condition and physiological function in broiler chicks between hatching and the end of fattening, compared with chicks fed on a diet containing SL-F. The *cry9C* gene and Cry9C protein are not transferred to blood, liver and muscles of broiler chicks.

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