

Original study

Evaluation of the effect of genetically modified Roundup Ready soya bean and MON 810 maize in the diet of Japanese quail on chosen aspects of their productivity and retention of transgenic DNA in tissues

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Abstract

The objective of the study was to evaluate the impact of genetically modified (GM) ingredients (soya bean meal and maize grain) used in poultry diets on birds' performance, as well as the accumulation of the transgenic DNA in eggs, breast muscle and internal organs. In the present experiment four generations of Japanese quails, which were subject to three different diets, two containing genetically modified organisms (GMO, soya and maize) and one GMO-free control, were analysed. Birds' performance traits were monitored along the trial. A screening molecular method – polymerase chain reaction amplification (PCR) – was used to detect *CaMV* 35S promoter and *nos* terminator in the collected samples. Results showed no presence of modified DNA in the analysed products. According to the obtained results, it was concluded that there was no negative effect of the use of genetically modified soya bean meal or maize grain found with regard to final product safety for consumers or to birds' productivity.

Keywords: Japanese quail, Roundup Ready soya bean, MON 810 maize, DNA retention

Abbreviations: EU: European Union, GMO: genetically modified organism, LSM: least square means, PCR: polymerase chain reaction, Bt: *Bacillus thuringiensis*

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Introduction

As defined by the European Union (EU), genetically modified organisms (GMOs) are organisms such as plants and animals whose genetic characteristics are being modified artificially in order to give them new properties. Food and feed which contain or consist of such GMOs or are produced from GMOs are called genetically modified food or feed (European Commission 2012).

Farm animals in Europe are fed with GMOs. Their feed usually contains ingredients derived from genetically modified plants, most frequently soya bean meal and maize grain. Soya bean meal is extremely important as a relatively inexpensive source of protein. Most of the soya grown around the world is genetically modified. It is imported to Europe from Brazil, the United States and Argentina, which are the world's leading producers of this plant. The Member States of the EU import approximately 40 million tons of raw soya products annually (European Commission 2010). The situation is completely different when it comes to maize, as Europe is quite self-sufficient. Almost two thirds of the maize production in the EU is used for animal feed. The genetically modified crops currently available at the market are mainly aimed at an increased level of crop protection through the introduction of resistance against plant diseases caused by viruses or insects (referred to as Bt crops, since the gene-conferring resistance comes from the bacterium *Bacillus thuringiensis* – e.g. MON 810 maize) or through increased tolerance towards herbicides (glyphosate and gluphosinate, e.g. Roundup Ready soya; World Health Organization 2012).

The use of ingredients and products from genetically modified plants in animal nutrition raises many questions and issues such as the role of a nutritional assessment of the modified feed or feed additive as part of safety assessment and the possible influence of genetically modified products on animal health and product quality. Other important issues are the persistence of the recombinant DNA and of the 'novel' protein in the digestive tract and tissues of food-producing animals (Flachowsky *et al.* 2005). The European public opinion is clearly concerned about potential risks for human health and the environment. In relation to this discussion, an experiment was conducted based on four generations of Japanese quails which were subjected to three different diets: two containing GMOs and one control group fed without GMOs.

The objective of the study was to perform a comprehensive research excluding or confirming the negative impact of genetically modified plant feed on the birds' performance and as a second step on animal products' safety for consumers. To achieve this goal, the following actions were taken: comparison of the productivity traits of the birds fed with one of three diets and examination of the accumulation of the transgenic DNA in eggs, breast muscle and internal organs in subsequent generations of Japanese quails (treated as model animals).

Material and methods

Animals, diets and management

The current trial consisted of four generations of Japanese quails (*Coturnix cot. japonica*) bred in three parallel feeding groups. In total, 4 628 eggs were incubated and 3 684 healthy chicks

entered the trial. At the age of 6 weeks, birds were divided into final flocks of 17 females and 5 males in each cage. Each group consisted of 6 repetitions of such flocks resulting in 102 females and 30 males in each of three groups in four repetitions - generations. In total, 1 584 adult birds were used in the trial. During the early laying period (from 7 to 16 weeks of age), eggs were counted daily and the average egg mass was measured once a week in each cage. At the age of 16 weeks, eggs were collected for incubation in order to form the next generation.

Following feeds were used:

- A – genetically modified soya bean meal included
- B – genetically modified maize grain included,
- C – non- genetically modified materials.

Genetically modified soya bean meal used was produced from Roundup Ready soya beans (*Glycine max. L cv A 5403*). Genetically modified maize grain was MON 810 maize with the specific *Bt*-gene construct. Birds received two types of diet: grower (1-6 weeks) and layer (7-17 weeks) with the same pattern in all four generations. Experimental feeds were prepared by the feed company Agro-Kocięba (Bogdan Kocięba, Czarnocin, Poland). Details of feed composition are presented in Table 1.

The level of basic nutrients in the above mentioned feeds was analysed in each generation according to methods of AOAC (2005) and it was confirmed to be as planned. Birds in each cage received the same amount of feed and leftovers were monitored weekly.

The content of modified DNA in feeds was examined by a reference GMO laboratory in the Plant Breeding and Acclimatisation Institute (National Research Institute, Radzików, Poland) by a PCR method.

Results confirmed the presence of Roundup Ready soya DNA in group A feed, as well as the presence of MON 810 maize DNA in group B feed but not in other feeds. Group C feed was proven to be free from the above modifications.

Performance traits evaluation

Basic production performance was recorded in the course of the trial. Weekly body weight was evaluated as average for birds in each cage: weeks 1-4 (brooding period) – all chicks together; weeks 7, 11 and 15 (laying period) – separately for females and males. Weekly laying performance was measured by number of eggs laid divided by number of females in each cage and calculated into laying percentage. Average egg mass was measured on a chosen day in the middle of the week. The remaining part, including incubation, hatching, feed intake, mortality, carcass characteristics, breast muscle composition and egg yolk composition is the subject of earlier work of the current research team (Sartowska *et al.* 2012). However, the paper covers results of the first two generations.

Sample collection and molecular analysis

Experimental dissection took place in each generation at the end of the laying period (in week 17). Birds were sacrificed by decapitation. Tissue samples for DNA analysis were collected from a part of the breast muscle, gizzard, liver, spleen, duodenum, kidney and heart from 12 females and 3 males from each feeding group and stored at –80 °C. Moreover,

Table 1
Feed composition and nutritional value of feeds used in the experimental diets A, B and C

Indices	Grower feed			Layer feed		
	group			group		
	A	B	C	A	B	C
Ingredients, g/kg						
Soya bean meal non-GMO	0.0	390.0	390.0	0.0	295.0	295.0
Roundup Ready soya bean meal GMO	390.0	0.0	0.0	295.0	0.0	0.0
Maize grain MON 810 GMO	0.0	250.0	0.0	0.0	250.0	0.0
Maize grain non-GMO	250.0	0.0	250.0	250.0	0.0	250.0
Wheat	196.7	222.0	196.7	233.3	233.8	233.8
Triticale	25.0	0.0	25.0	50.0	50.0	50.0
Rapeseed meal	41.7	41.7	41.7	0.0	0.0	0.0
Rape seed	0.0	0.0	0.0	55.6	55.6	55.6
Soya oil	22.3	21.9	22.3	29.8	30.0	30.0
Potato protein	25.3	23.1	25.3	0.0	0.0	0.0
Maize gluten	15.5	17.8	15.5	3.1	0.0	0.0
Limestone	13.4	13.4	13.4	62.6	62.6	62.6
Monocalcium phosphate	9.0	9.0	9.0	9.5	9.5	9.5
Additives*						
Nutritional value (calculated)						
Energy ($\times 10^3$ kcal)**	2.95	2.95	2.95	2.80	2.80	2.80
Crude protein, %	26.50	26.50	26.50	20.86	20.84	20.84
Crude fat, %	6.10	6.04	6.10	5.54	5.56	5.56
Crude fibre, %	2.79	2.80	2.79	3.01	3.01	3.01
Ash, %	5.85	5.85	5.85	10.39	10.39	10.39
Ca total, %	0.97	0.97	0.97	2.78	2.77	2.77
P available, %	0.45	0.45	0.45	0.45	0.45	0.45

*The following additives were included in the diets in grower/layer feed (g/kg): vitamin-mineral premix 3.2/5, NaCl 2.5/2.5, sodium bicarbonate 1.4/2.1, phyzyme 0.1/0.1, ronozyme 0/0.2, methionine 1.8/1, L-lysine 0/0.4 and threonine 0/2.3, **AME: apparent metabolic energy

during week 17 six germinal shields from eggs from each group were collected and stored at -80°C . Tissue samples and germinal shields from eggs from each of the feeding groups (A, B and C) were prepared for analyses of specific gene constructs (*CaMV* 35S promoter and *nos* terminator) with the use of PCR analysis. The genomic DNA was extracted from the thawed tissues with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The concentration of DNA was determined by Pico drop Microliter UV/Vis Spectrophotometer (Picodrop Ltd., Hinxton, UK) and the quality of the DNA was checked with an ethidium bromide-stained agarose gel.

In the first step, the DNA obtained from tissues was tested for integrity by amplification of the species-specific gene. The *IGF1* (Insulin growth factor 1) gene fragment specific for avian DNA was amplified using the PCR-RFLP method according to Moody et al. (2003) and Zhou et al. (2005).

The International Standard provides the overall framework of qualitative methods for detection of GMOs in foodstuffs using the PCR method. Qualitative analysis consists of screening and/or specific detection of target nucleic acid sequences in the test samples. The above mentioned method is also used for specific detection and amplification of the ›specific construct‹ of genetically modified plants: *CaMV* 35S promoter or *nos* terminator. Due to the presence of these constructs in many genetically modified plants, this method can be used to screen for the presence of genetically modified plant-derived components in animal tissue.

In the second step, the PCR amplification of 1 332 examined probes (15 animals \times 3 feeding groups \times 7 tissues \times 4 generation and 6 germinal shields \times 3 feeding groups \times 4 generations) was used for the detection of *CaMV* 35S promoter and *nos* terminator.

Specific primers have been used for the detection of *CaMV* 35S promoter (*p35S-cf3*, *p35S-cr4*) and *nos* terminator (*HA-nos188f*, *HA-nos118r*) according to Lipp et al. (2001, Table 2). The protocol of the PCR-based method, allowing the screening of GMOs in analysed material according to Querci et al. (2006), was used. The following reagents were mixed in the preparation of a MasterMix: 0.5 μ M of each primer, 0.025 U/ μ l Red Taq polymerase and nuclease-free water aliquots. After amplification of the target sequence, the PCR products were analysed by agarose gel electrophoresis in the presence of ethidium bromide. The gel was photographed to provide a permanent record of the result of the experiment. As positive control, a reference DNA extracted from a certified European Reference Material, Roundup Ready soya (ERM-BF410gk and ERM-BF410dk) and MON810 maize (ERM-BF413f) containing 2 % and 5 % of genetically modified plant ingredients, has been used. The reference material is prepared by JRC-IRMM (Institute for Reference Material and Measurements) which is one of the seven Institutes of the Joint Research Centre (JRC). The DNA from the reference material was extracted using the NucleoSpin Food Kit (Macherey-Nagel, Duren, Germany). The characteristic fragments were amplified by PCR and detected after separation by agarose gel electrophoresis. The positive control is intended to demonstrate what the result of analyses of test samples containing the target sequence will be. As a negative control, IRMM Roundup Ready soya (ERM-BF410a) and IRMM maize (ERM-BF413a) certified reference material containing no genetically modified plant ingredients, have been used. Additionally, a ›No template‹ sample was created to confirm the purity of the MasterMix, in which water was used instead of DNA.

Table 2

Characteristics of primers for PCR analysis (Lipp et al. 2001, Querci et al. 2006)

Symbol	Primer sequences	Melting point G/C
<i>p35S-cf3</i>	CCACGTCTTCAAAGCAAGTGG	57.4
<i>p35S-cr4</i>	TCCTCTCCAAATGAAATGAACTTCC	56.3
<i>HA-nos188f</i>	GCATGACGTTATTTATGAGATGGG	56.2
<i>HA-nos118r</i>	GACACCGCGCGGATAATTTATCC	61.2

Statistical analysis

The analysis of variance (GLM procedure of SAS/STAT 2010, SAS Institute Inc., Cary, NC, USA) was performed to evaluate the differences in the level of the traits between three feeding groups. The analysis was made on data referring to the generations I, II, III and IV. The significance of differences between the values of the traits was estimated by Duncan's test. The models for analysis of variance included fixed effects as follows: for the body weight in the brooding period – the feeding group, subsequent generation, age, feeding group-by-age interaction was used; for the body weight in the laying period – the effects of the feeding group, subsequent generation, sex and age of birds were taken into consideration. Additionally, the interaction of feeding group-by-age-by-sex was used. For the laying rate the effects of the feeding group, subsequent generation, age and feeding group-by-generation, group-by-age and generation-by-age interactions were considered. For the egg weight the effects of the feeding group, subsequent generation, age and feeding group-by-generation, group-by-age interactions were considered.

All procedures were approved by the Local Ethics Commission and performed in accordance with the Guiding Principles for the Care and Use of Research Animals.

Results

Performance of birds

Throughout the four generations, the birds' growth was of a similar pattern. The effect of age and generation were significant. The body weight in the brooding period was significantly higher in group B compared to A (Table 3), but only in week 4. At the end of the laying period (week 16), females from group C (non-GMO) were significantly heavier compared to the ones from groups A and B (Table 4). However, these differences were not observed in each of the generations. For the observed time of ten production weeks, the laying rate was at the same level in all three feeding groups, also the average egg weight was the same regardless of the feed used (Table 5).

Molecular analyses

A fragment of the quail-specific *IGF1* gene (813 bp) was amplified to confirm the integrity of the isolated DNA. A 123 bp DNA fragment from the *CaMV* 35S promoter sequence and 118 bp DNA fragment from the *nos* terminator sequence in reference material were amplified by PCR and detected after separation with agarose gel electrophoresis. In the negative control, no amplification was observed. Typical examples for the amplification results are given in Figures 1 and 2.

Reference material showed the presence of characteristic bands (410gk, 410dk, 413f), whereas the tested samples (1-16 and 1-30, respectively) and reference material with no GMO (410a, 413a) showed no presence of the evaluated gene constructs. There were no *CaMV* 35S promoter sequence or *nos* terminator sequence amplification in none of the 1 332 examined samples derived from examined tissues and germinal shields from each of the feeding groups (A, B and C).

Table 3
Quails body weight changes in the brooding period, average of four generations*

Feeding Group	Day 0	Week 1	Week 2	Week 3	Week 4
A	7.46 ± 1.91	26.23 ± 1.91	41.73 ± 1.84	82.96 ± 1.72	116.67 ± 1.52 ^a
B	7.38 ± 1.91	27.15 ± 1.91	43.33 ± 1.84	86.30 ± 1.72	121.32 ± 1.52 ^b
C	7.23 ± 1.91	26.64 ± 1.91	43.33 ± 1.84	84.40 ± 1.72	119.23 ± 1.52

*g; LSM ± standard error, ^{a,b}LSM values in the columns with different letters differ significantly at *P* < 0.05

Table 4
Quails body weight changes in the laying period, average of four generations*

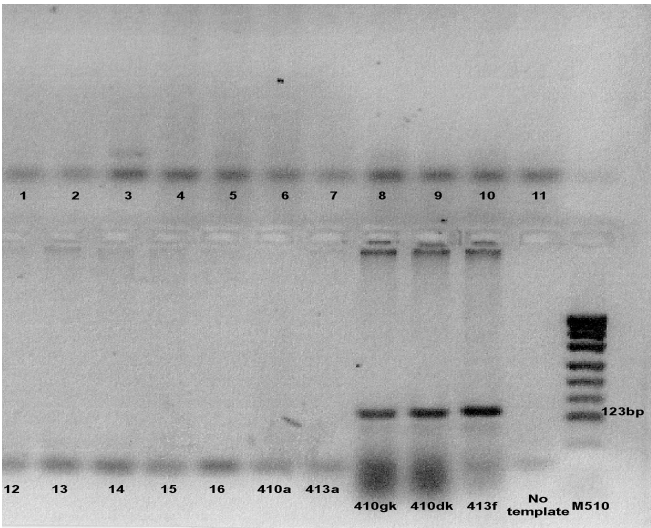
Feeding group*		Week 7	Week 11	Week 15
Females	A	208.243	224.409	232.806 ^a
	B	210.159	226.063	232.212 ^A
	C	207.442	223.084	240.673 ^{B,b}
Males	A	169.216	182.626	189.134
	B	170.744	183.112	187.251
	C	170.229	181.669	187.248

*g; LSM ± standard error (=2.197), ^{a,b}LSM values in the columns with different letters differ significantly at *P* < 0.05, ^{A,B}LSM values in the columns with different letters differ significantly at *P* ≤ 0.01

Table 5
Laying rate and egg mass in three feeding groups, average of four generations*

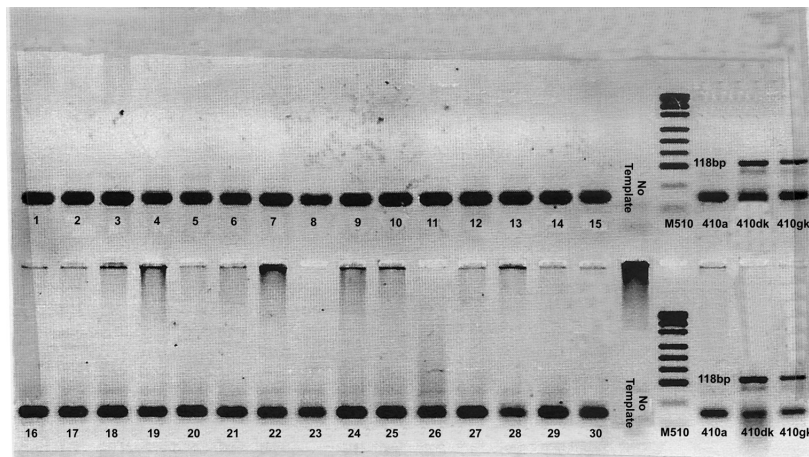
Feeding group	A n=240**	B n=240**	C n=240**
Laying rate, %	79.11 ± 0.4473	79.16 ± 0.4473	78.73 ± 0.4473
Egg mass, g	11.49 ± 0.0337	11.53 ± 0.0334	11.53 ± 0.0336

*LSM ± standard error, **n=6 cages × 10 weeks of laying period × 4 generations



1-16: investigated samples (1-8 breast muscle and 9-16 heart sample, group A, 3rd generation); 410a, 413a: negative control; 410gk, 410dk, 413f: positive control; No template: negative control of the MasterMix, in which water was used instead of DNA; M510: pxUC19 DNA/ MspI – size marker; 123bp: DNA fragment from the *CaMV* 35S promoter sequence

Figure 1
Screening of animal tissues for *CaMV* 35S component of genetically modified corn (exemplary electrophoretic image)



1-30: investigated samples (1-15 breast muscle and 16-30 heart sample, group B, 5th generation); 410a: negative control; 410dk, 410gk: positive controls; No template: negative control of the MasterMix, in which water was used instead of DNA; M510: pUC19 DNA/MspI – size marker; 118 bp: DNA fragment from the *nos* terminator sequence

Figure 2

Screening of animal tissues for *nos* terminator component of genetically modified soya (exemplary electrophoretic image)

Discussion

So far, there have been performed many studies to demonstrate the effects of genetically modified crops in animal feed on their productivity. The research assessed also whether there is a possibility of retention of the transgenic DNA and the modified protein as the product of its expression in animal tissues (Alexander *et al.* 2004, Aeschbacher *et al.* 2005). Poultry such as broiler chicken, laying hen or quail represent a good model species used for this type of research. Generally, these are the animals of low body weight, hence the low cost of living, while having a short reproductive cycle, which allows several generations in a year. Poultry, especially broilers, are particularly sensitive to the quality and nutritive value of feed and the possible content of harmful substances in it. It is also the animal material which is usually available in large quantities. Initial studies around the world indicate a lack of a negative effect of genetically modified feed material on animal organisms (Rossi *et al.* 2005). Most of these experiments are one-generation research on poultry. Similar results like the absence of modified DNA in poultry products were obtained by other authors (Kan & Hartnell 2004, McNaughton *et al.* 2007, Taylor *et al.* 2007, Świątkiewicz *et al.* 2010a). The *Bacillus thuringiensis*-gene specific construct from Bt-corn was not detected in any of the poultry samples, neither in organs, meat nor eggs (Einspanier *et al.* 2001, Jennings *et al.* 2003, Aeschbacher *et al.* 2005, Świątkiewicz *et al.* 2010b). Also in studies on laying hens, it was found that the modified protein was digested in the gastrointestinal tract of hens and therefore excrement, internal organs (liver) and the eggs were free from transgenic DNA (Ash *et al.* 2003, Świątkiewicz *et al.* 2011). Interesting results have also been received by Flachowsky *et al.* (2005). The diet used in this experiment containing GM maize has been fed

to quails. Then the birds were observed during the next 10 generations. This is one of a few studies done on many generations of animals. No negative effect was observed in the case of lay, the health of birds, feed efficiency and quality of meat and eggs. On average, male and female quails fed with transgenic maize showed a lower body weight compared to the control receiving genetically modified free feed, but this difference was not significant. There was also no transfer of DNA to the examined tissue samples. The results of our study are in line with results presented in discussed publications. No differences were noted regarding production traits and DNA transfer to tissues. A significant influence of generation number on the evaluated traits was found. However, this could be explained by the different season of the year in different generations. Slight differences between birds' body weight at the end of the brooding period and in the group of females at the end of the laying period, require further analysis in the subsequent generations.

In conclusion, the results of molecular analysis of samples obtained from four generations in this study indicate that the experimental diets containing GMOs (soya bean meal and maize grain) did not affect the results with the presence of modified DNA in examined muscle, internal organs and eggs derived from Japanese quail. No negative effect of the use of genetically modified soya bean or maize was found in the course of the trial with regard to most of the birds' performance traits. These are preliminary results, which do not allow definitive conclusions so far. Further research, evaluating more generations, will be carried out in the future.

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