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Original article

Assessing the possibility of genetically modified DNA transfer from GM feed to broiler, laying hen, pig and calf tissues

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Abstract

The aim of this study was to assess the possibility of genetically modified DNA transfer from feed containing RR soybean or/and MON810 maize to animal tissues, gut bacterial flora, food of animal origin, and the fate of GM DNA in the animal digestive tract. The experiment was carried out on broilers, laying hens, pigs and calves. All animals were divided into four groups: I – control group (non-modified feed), II – GM soybean group (non-modified maize, RR soybean), III – GM maize group (MON810 maize, non-modified soybean), and IV – GM maize and soybean group (MON810 maize, RR soybean). Samples of blood, organs, tissues, digesta from the gastrointestinal tract, and eggs were analysed for the presence of plant species specific genes, and transgenic sequences of CaMV 35S promoter and NOS terminator. PCR amplifications of these GM sequences were conducted to investigate the GM DNA transfer from feed to animal tissues and bacterial gut flora. In none of the analysed samples of blood, organs, tissues, eggs, excreta and bacterial DNA were plant reference genes or GM DNA found. A GM crop diet did not affect bacterial gut flora as regards diversity of bacteria species, quantity of particular bacteria species in the animal gut, or incorporation of transgenic DNA to the bacteria genome. It can be concluded that MON810 maize and RR soybean used for animal feeding are substantially equivalent to their conventional counterparts. Genetically modified DNA from MON810 maize and RR soybean is digested in the same way as plant DNA, with no probability of its transfer to animal tissues or gut bacterial flora.

Key words: GMO, maize, soybean, feed, transfer

Introduction

The first generation of genetically modified (GM) plants is widely used across the world as food and feed. Following commercial release in 1996, the proportion of GM plants has grown rapidly, and in 2012 170.3 mln hectares of genetically modified crops were grown globally (James 2012). GM soybean has continued to be the principal GM crop since 1996, occupying, in 2012, more than 80 million hectares,

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followed by GM maize (around 55 million hectares), cotton and canola. GM maize MON810 is one of the transgenic maize varieties that can be grown in the EU. This maize has been modified by integration of a gene isolated from the *Bacillus thuringensis* (Bt) soil bacterium, in order to express the insecticidal Cry 1 A(b) protein, which confers insect resistance to the European corn borer (*Ostrinia numbilatis*), the primary corn pest in Europe. Genetically modified soybean is imported to EU countries as the main protein source for animal feeding. Roundup Ready™ (RR) soybean (in EU registered as event GTS 40-3-2) is the dominant GM soybean variety grown globally. RR soybean is herbicide tolerant and has been developed by introducing a gene isolated from the *Agrobacterium* sp. CP4 strain of soil bacterium, expressing 5-enolpyruvylshikimate-3-phosphate synthase in plants. This enzyme confers glyphosate tolerance upon plants, glyphosate being the active substance in herbicides. These two transgenic crops are mostly used from among all GM crop varieties in animal feeding in EU countries.

To date, many articles about animal feeding studies with GM crops, including RR soybean and MON810 maize, have been published (Aulrich et al. 2001, Aulrich et al. 2002, Aumaitre et al. 2002, Reuter et al. 2003, Sanden et al. 2004, Deaville et al. 2005, Flachowsky et al. 2005, McCann et al. 2005, Rossi et al. 2005, Flachowsky et al. 2007, Sissener et al. 2009). All of these trials indicated that GM crops are safe for animals, including poultry, pigs, cattle and fish. Moreover, in all of these experiments there were no significant statistical differences in production indices, quality of carcasses and food products of animal origin, when the animals were fed diets containing conventional and GM crop varieties. Despite the number of studies with GM crops, the problem of GM feed raises many doubts and concerns, especially when interpretations of the test results contain suggestions about the negative impact of GM feed on the health and safety of animals (Seralini et al. 2012). One of the problems still raised by GM food and feed opponents is the potential possibility of GM DNA transfer, originating from animals fed diets containing genetically modified crops, to animal gut bacterial flora and animal tissues, then to animal products such as eggs, meat and milk.

Therefore, the aim of this study was to assess the transfer of genetically modified DNA from diets containing RR soybean or/and MON810 maize to animal tissues and food of animal origin in a trial involving a large number of animals of different species. Moreover, much attention was paid to the aspect of GM DNA fate in the digestive tracts of poultry, pigs and calves, with assessment of GM diet impact on bacteria species diversity and incorporation of GM DNA sequences to the bacteria genome.

Materials and Methods

Experiment design

The experiment was carried out on broilers, laying hens, pigs and calves. Animals from each species were divided into four groups: I – control group (non-modified maize and soybean), II – GM soybean group (non-modified maize and genetically modified RR soybean), III – GM maize group (genetically modified MON810 maize and non-modified soybean), and IV – GM maize and soybean group (genetically modified maize MON810 and RR soybean). Maize grains consisted of GM maize plants expressing Cry 1A(b) protein (MON810, YieldGard) and its non-modified isogenic parental line (DKC 3420). The GM soybean Roundup Ready™ (GM event GTS 40-3-2) variety was used with its non-modified counterpart used as control. In the case of control, the environmental conditions for growth of maize and soybean plants were the same for both varieties.

Broilers

Six hundred and forty sexed Ross 308, one-day-old animals were obtained from a commercial hatchery. Food and water were available *ad libitum*. Chickens were fed a mash, maize-soybean starter diet for the first 21 days and grower-finisher diet for the next 21 days. All diets were formulated to meet nutrient requirements of growing broilers. Each treatment (experimental group) was divided into four replicates (pens) of 40 birds (20 male and 20 female). At the end of the experiment, at day 43, six birds from each group were decapitated and bled at 43 days of age. Samples of tissues (blood, whole liver and spleen, and breast muscle) and digesta samples from different parts of the gastrointestinal tract (gizzard, duodenum, jejunum, ileum, caecum, and cloaca) were taken, collected and frozen at -20°C in sterile plastic bags.

Laying hens

The study was carried out on 96 Bovans Brown hens aged 18 weeks, obtained from a commercial source. Before the study (up to 25 weeks of age) the animals were fed a commercial laying hen diet offered *ad libitum*. At 25 weeks of age the hens were randomly assigned to one of four treatments, each comprising

24 individually caged layers. During the experiment, from 25 to 54 weeks of age, the layers were fed a mash, maize-soybean meal based diet, formulated to meet the nutrient requirements of laying hens. At 48 weeks of age, one egg was collected from each hen (24 eggs from each treatment). At the end of the experiment (54 weeks of age) six birds from each group were decapitated and bled. Samples of tissues (blood, liver, spleen, and lungs) and digesta samples from different parts of the gastrointestinal tract (gizzard, duodenum, jejunum, ileum, caecum, and cloaca) were taken, collected and frozen at -20°C in sterile plastic bags.

Fatteners

Forty eight fatteners originated from (Polish Landrace x Large White Polish) sows mated with (Duroc x Pietrain) boar were chosen. The animals were fed isonitrogenous and isoenergetic feed mixtures according to the requirements of growing (30-60 kg BW) and finishing (60-110 kg BW) pigs. All fatteners were fed individually restricted feed amounts according to body weight. At the start of the experiment the animal's weight was about 30 kg, and finally the fatteners reached about 110 kg body weight. At the end of fattening, all pigs were slaughtered and samples of digesta from the stomach, duodenum, jejunum, caecum, and colon, as well as samples of tissues: liver, spleen, lung, *longissimus* muscle, and blood were taken from six pigs (three barrows and three gilts) from each group. Samples were frozen and kept at -20°C in sterile plastic bags.

Calves

The experiment was carried out on 40 young (7-10 days of age) bulls of Black-White race. At the end of the experiment the animals were 90 days old. Until day 56 of life the calves were fed milk replacers, and then had free access to water and compound feed, to ensure the whole nutrition requirements for calves. Ten calves were randomly selected for each experimental group. At the end of the experiment all calves were slaughtered and samples of digesta from the stomach, duodenum, jejunum, and colon, as well as sample of tissues: liver, spleen, lung, kidney, pancreas, muscle, and blood were taken from five cattle from each group. Samples were frozen and kept at -20°C in sterile plastic bags.

DNA isolation and PCR reactions

DNA was extracted from homogenised samples of feed and gastrointestinal tract digesta with CTAB methods (PN-EN ISO/IEC 21571:2007). DNA isolation from heparinised blood, tissues, and stool was conducted with commercial extraction kits (Blood Genomic AX Kit, Genomic Tissue AX Kit, Genomic Stool AX Kit all by DNA, Poland), according to the kit's manuals. Methods used for GMO detection and identification were based on EN ISO norm (PN-EN ISO/IEC 21569:2005), which included PCR for CaMV 35S promoter and NOS terminator (regulatory sequences used for transformation of both GM crops; for MON810: CaMV 35; for RR soybean: CaMV 35S and NOS), and species specific PCR – invertase gene from maize and lectin gene from soybean. Analyses of feed for all trial groups and animal species were done to check if the GMO composition was consistent with the study assumptions. Tissue and blood analyses were done to assess the possibility of GM DNA transfer from GM feed to animal tissue. Digesta from the gastrointestinal tract and stool were also analysed to assess the degree of digestion of feed DNA. The Limit of Detection of all PCR methods was equal to 5 copies of proper DNA fragment per reaction. The PCR products were analysed by electrophoresis on 2.0% agarose gels containing ethidium bromide intercaling dye.

Gastrointestinal microflora

Additionally to PCR reaction of digesta from different parts of the gastrointestinal tract, samples of ileum and/or colon content were also taken for microbiological analysis. Some species of common gut bacteria such as *Escherichia coli*, *Enterococcus faecalis* and *Enterococcus faecium* were isolated and their relative quantity in the gut was measured, their DNA was also investigated for the presence of transgenic DNA. Cultivation of bacteria was done on Brain Hearth Infusion (BHI) medium. For detection and enumeration of *Escherichia coli* selective, chromogenic medium Tryptone Bile X-Glucuronide medium (TBX) was used. *Enterococcus* was isolated on Slanetz and Bartley medium.

The obtained data were subjected to analysis of variance and the differences between mean values were estimated using the Duncan test (Statistica 5.1). A p-value of 0.05 was considered as significant.

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Table 1. Results of PCR reactions for the presence of CaMV 35S promoter, NOS terminator, species specific genes – invertase (maize) and lectin (soybean) DNA sequences in animal tissues, digesta from gastrointestinal tract and food of animal origin.

Sample	Group I				Group II				Group III				Group IV			
	CaMV 35S	NOS	Invetrase	Lectin.	CaMV 35S	NOS	Invetrase	Lectin.	CaMV 35S	NOS	Invetrase	Lectin.	CaMV 35S	NOS	Invetrase	Lectin.
Blood	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Liver	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Spleen	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Lung[1]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Kidney[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pancreas[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Muscle**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Gizzard[3]	–	–	+	+	+	+	–	+	+	–	+	–	+	+	+	+
Stomach[4]	–	–	+	+	+	+	–	+	+	–	+	–	+	+	+	+
Duodenum*	–/–	–/–	–/+	–/+	–/+	–/+	–/–	–/+	–/+	–/–	–/+	–/–	–/+	–/+	–/+	–/+
Jejunum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ileum[3]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Caecum[5]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Cloaca[3]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Colon[6]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Excreta	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Eggs[7]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[1] except broilers, [2] only calves, [3] only broilers and laying hens, [4] except broilers and laying hens, [5] except calves, [6] only pigs and calves, [7] only laying hens, * positive results only for pigs, ** broilers – breast muscle, fatteners – *longissimus* muscle, calves – muscle tissue.

Results

In this study all animal species and feeding groups achieved satisfactory performance indices, with no significant statistical differences in any of the parameters across dietary treatment. All feed used during the experiment for different groups and species of animals were analysed for chemical composition, including proximate analysis, amino acid content, NDF (neutral detergent fiber), ADF (acid detergent fiber), phosphorus and calcium level. Taking into account the quality of feed and nutrient requirements of the animals, proper diets were formulated. The chemical composition of the feed indicates that there is no significant difference between non-modified and genetically modified maize and soybean. Such parameters as average daily weight gain, feed utilisation, carcass measurement, laying performance indices, egg quality indices *etc*. were similar in all analysed dietary groups for all animal species. In this study we focused on assessing the possibility of GM DNA transfer from GM feed to animal tissues and food of animal origin, to bacterial gut flora and changes in diversity of bacteria species when using GM feed. Feed samples were analysed for the presence of GM crops to ch eck if all group feed were properly composed according to the trial scheme. The results indicated that feed mixtures for all species and feeding groups were prepared according to the scheme of the experiment. GM maize MON810 was present only in feed for groups III and IV, GM soybean RR variety in feed for groups II and IV, and feed used for feeding animals from group I was totally GMO free.

Analysis of digesta samples from different parts of the gastrointestinal tract indicated that relatively small fragments of reference genes (226 bp – invertase, 118 bp – lectin) and/or transgenic sequences (123 bp – CaMV 35S, 118 bp – NOS) were detected in all samples of bird gizzard and stomach content of all species, but also in the pig duodenum (Table 1). The presence of particular DNA sequences depended on the feed used for feeding of animals from one of the four different groups. There were no PCR products of plant reference genes and transgene sequences in digesta of the jejunum, ileum, caecum, cloaca, colon, and excreta taken from all animal species.

Similar negative results of plant and transgenic DNA transfer were observed in animal tissues and eggs. In the samples of blood, liver, spleen, pancreas, kidney, lungs, eggs and muscles no copy of plant reference genes, CaMV 35S or NOS sequences were found.

We did not find any differences between all experiment groups in quantity and diversity of bacterial gut flora for investigated bacterial species. Moreover, DNA samples of particular *Enterobacteriaceae* species

isolated from all four feeding groups of broilers, laying hens, pigs and calves did not contain any detectable copy of reference plant genes, CaMV 35S or NOS sequences.

Discussion

The substantial equivalence of GM crops and its conventional counterparts has been indicated in earlier publications of other authors (Padgette et al. 1996, Gaines et al. 2001, Aulrich et al. 2001, 2002, Aumaitre et al. 2002, Flachowsky et al. 2005, 2005a, Rossi et al. 2005, Flachowsky et al. 2007). Although the chemical composition of GM crops is the same as non-modified plants, besides new expressed proteins coded by inserted transgenes, substantial equivalence was strongly criticized as not satisfactory for the assessment of the safety of GM crops, food and feed. Much more precise and careful studies are necessary to estimate the safety of genetically modified organisms. One such parameter is the possibility of GM DNA transfer from GM plants to microorganisms living in animal gut and to animal blood, tissues, and then to food of animal origin. In feed the animals take DNA from plants, that DNA is quickly digested by enzymes in the digestive tract. Our results showed that DNA fragments of 118 bp or longer were not present in hundreds of samples of blood, tissues and eggs analysed during the experiment. All PCR reactions conducted on DNA isolated from such matrixes did not give positive results. There were no PCR products in the case of maize and soybean reference genes and in the case of transgenic sequences of CaMV 35S and NOS. Comparable results have been presented in many articles describing GM feed studies carried out on various species of animals (Beever and Kemp 2000, Aulrich et al. 2001, Aulrich et al. 2002, Aumaitre et al. 2002, Reuter and Aulrich 2003, Deaville and Maddison 2005). Jennings et al. (2003) did not detect the transgene of CP4 EPSPS enzyme in samples of pig muscle in an RR soybean feeding trial. The lack of detectable GM crop transgenes in the blood and milk of cows was reported by Klotz et al. (2002), as well as in the meat, liver, spleen, stomach, kidneys, heart, and eggs of quails by Flachowsky et al. (2005). Comparison of many GM crop feeding studies conducted on different species animals was done by Flachowsky et al. (2007). These experiments were done using Bt-corn, Bt-potato and GM soybean as feed components for broilers, layers, cattle, dairy cows, pigs, and quails. Authors of all the compared experiments reported that transgenes from GM crops were not detectable by PCR in animal tissues, blood or food of animal origin. But results have also shown that plant DNA fragments, other than transgenes from GM crops, were detectable in samples of blood, organs and animal tissues. Aulrich et al. (2002) found short DNA fragments (shorter than 200 bp) of plant chloroplasts in cow white blood cells and very little positive signal in milk. Other cows tissues did not give positive results for the presence of plant DNA, nor for the presence of transgenic sequences. Mazza et al. (2005) reported that in some cases plant DNA fragments were present in animal tissues and cells, mostly in organs rich in blood vessels and involved in filtration, such as the liver and kidney. The authors concluded that blood is the main tissue involved in the uptake of short DNA fragments since it collects macromolecules directly absorbed by the intestinal epithelium and the cells of the immune system. Sanden et al. (2004) stated in their article that an explanation of DNA transfer to animal tissues and cells could be the fact that DNA makes complexes with protein, in which protein is like a protective coat for the DNA. On the other hand, DNA sensitivity to inactivation and degradation is very high. As regards feed, deoxyribonuclease I produced by animal salivary glands, pancreas and small intestine is a potent degradative enzyme, and the low pH of the stomach or ruminant abomasal acts to remove adenine and guanine residues, thereby eliminating biological activity (Beever and Kemp 2000, Beever and Phipps 2001). In the present study, fragments of transgenic DNA and plant DNA were detectable in the content of the bird gizzard and stomach, and pig duodenum. Results of an experiment with pigs conducted by Klotz et al. (2002) indicated that fragments of chloroplast DNA from maize could be detected in intestinal digest contents up to 12 h, or, as other authors suggested, after 72 h after last feeding (Jansen 1989). Deaville and Maddison (2005) reported that transgenic DNA from RR soybean meal or Bt maize was detectable in the broiler gizzard, but not in intestinal digesta. An experiment with broilers has shown that the *bla* gene (part of the Bt construct from Bt176 GM maize) was present in the gizzard content, but not in small intestine, caecum and rectum digesta (Chambers et al. 2002). Such a high degradation and digestion of plant DNA in the alimentary tract of animals makes the possibility of GM DNA transfer to gut bacterial very improbable. Most experiments focusing on the possibility of gene transfer from GM crops to microorganisms conclude that a such probability is extremely low. Beever and Phipps (2001) estimated that dairy cows fed with Bt maize ingest 57 g of total plant DNA per day, of which only 54 ,g is transgenic Bt DNA (less than 0.00094% of total DNA intake). De Vries et al.

(2001) calculated in their experiment, in which *nptII* gene transfer to bacteria cells was studied, that a such probability in optimal conditions is less than 1x10^{-13}. A similar study conducted by Schluter et al. (1995), showed that GM potato transgene transfer to *Erwinia chrysanthemi* is possible, but in natural conditions the probability of this is 2x10^{-17}. Clearly, gene transfer is very significant for bacterial evolution, but occurs mainly between two bacterial cells, rather than between plant cell and bacteria. Moreover, in the digestive tract, where DNA is quickly degraded for low fragments or single nucleotides and absorbed in the intestine, such possibility of transgene transfer from GM feed to bacteria cells is almost impossible.

In summary, it can be concluded that the results of this study with a wide range of animals, broilers, layers, pigs and calves, showed efficient digestion of GM DNA and plant DNA in the gastrointestinal tract of all animals. The absence of detectable fragments of DNA in blood, organs, tissues, excreta and food of animal origin of animals fed diets containing Bt maize MON810 and/or RR soybean was also confirmed. This trial has shown that the transfer of GM DNA to animal tissues and then to food of animal origin is not possible.

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