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Fate of transgenic deoxyribonucleic acid fragments in digesta and tissues of rabbits fed genetically modified soybean meal[1]

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ABSTRACT: Numerous animal feeding studies have investigated the presence of DNA from transgenic plants in tissues from different animal species, but the data reported are sometimes controversial. The aim of this study was to investigate the presence of transgenic DNA (tDNA) in the digesta and tissues of a meat rabbit breed fed genetically modified (GM) soybean meal. Fifteen male New Zealand White rabbits were used for the experimental trial. Ten rabbits (treated group [TG]) were fed a mixed feed containing 10% GM soybean meal and 5 rabbits (control group [CG]) received a mixed feed containing conventional soybean meal, both from weaning (28 d of age) to slaughter (80 ± 3 d). Samples of blood, liver, kidney, heart, stomach, intestine (jejunum), lateral quadricep muscle, longissimus muscle, and perirenal adipose tissue were collected to assess the possible DNA transfer from GM feed to animal tissues. Samples of stomach contents and feces were also taken to study the degradability of ingested tDNA from feed in the digestive tract of rabbit. Moreover, samples of hair were collected to determine the possible environmental contamination from feed powders present on the farm. The DNA extraction was performed using specific genomic DNA kits. All samples were monitored, by using real-time PCR, for oligonucleotide primers and probes specific for the transgenic Roundup Ready soybean 40-3-2 and for the endogenous *lectin* (*LE1*) gene. As an internal control of rabbit tissues, the presence of the *β-actin* (*ACTB*) gene was used. In this study, no fragments of tDNA were detectable in tissue DNA samples of rabbits except in the extracted DNA from stomach digesta, feces, and hair of rabbits fed with GM soybean. Similar results were found for the reference *LE1* gene, whereas the presence of the *ACTB* gene was detected in all rabbit tissues. The lack of tDNA of soybean in rabbit tissues represents an important result, which demonstrates that meat from rabbits fed a diet containing GM feed is as that derived from rabbits fed conventional crops. The recombinant DNA recovered in the stomach digesta and in feces indicates an incomplete digestion of the soybean DNA in the gastrointestinal tract of the rabbit, whereas the presence of trace soybean transgene in the hair of the TG rabbits is suggestive of an environmental contamination.

Key words: digesta, genetically modified soybean, rabbit, real-time polymerase chain reaction, tissues, transgenic deoxyribonucleic acid

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INTRODUCTION

Over recent years, demand for genetically modified (**GM**) plants has grown rapidly so that the cultivation area for transgenic crops has dramatically increased, reaching over 181.5 million ha worldwide in 2014. Most of the GM plants are widely used as food and feed (James, 2014). Numerous animal feeding studies, with GM crops, have established the substantial equivalence between the current GM crops and conventional varieties (García-Villalba et al., 2008; Herman and Price, 2013) and some have revealed no significant differences in feed digestibility, performance, or health in animals fed GM feeds compared with animals fed non-GM feeds (Flachowsky, 2013; Van Eenennaam and Young, 2014). Furthermore, no biologically relevant differences have been observed in the nutritional value of products derived from animals consuming GM feeds compared

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with animals consuming non-GM feeds (Guertler et al., 2010; Tufarelli et al., 2015). Additional studies (CAST, 2006; Flachowsky et al., 2007) have followed the fate of the transgenic DNA (**tDNA**) of the GM crops within the gastrointestinal (**GI**) tract of farm animals to verify the potential for tDNA to transfer into animal tissues and derived products intended for human consumption. In these studies, no traces of tDNA have been detected in any fluid, tissue, or organ samples obtained from animals fed GM crops, indicating that their edible meat is comparable with that derived from animals fed conventional crops. Surprisingly, however, some studies have traced very small DNA fragments from transgenic plants in tissues from pigs, sheep, goats (Mazza et al., 2005; Sharma et al., 2006; Tudisco et al., 2010), and fish (Chainark et al., 2008). These conflicting results may generate public concerns regarding animal and human health. In order to provide further knowledge on this topic, the aim of this study was to assess the fate of recombinant DNA in the GI tract and tissues of meat rabbits fed a mixed feed containing GM soybean meal.

MATERIALS AND METHODS

Animals, Housing, and Experimental Design

Fifteen male New Zealand White rabbits were used in the experimental trial. Ten rabbits (treated group [**TG**]) came from a herd that used GM feeds for over 15 yr and were fed a diet of mixed feed containing 10% Roundup Ready Soybean 40-3-2 (Monsanto, Saint Louis, MO) meal from weaning (28 d age) to slaughter (80 ± 3 d of age). Chemical characteristics (on a DM basis) of the mixed feed were 88% DM, 18% CP, 3.6% ether extract, 13.8% crude fiber, 9.4% ash, 1.0% Ca, 0.7% P, and 0.3% Na. The other 5 rabbits (control group [**CG**]) came from an organic herd that does not use GM feeds and were fed a diet with mixed feed containing 8% conventional nontransgenic soybean meal from weaning (28 d of age) to slaughtering (80 ± 3 d of age). Chemical characteristics (on a DM basis) of the mixed feed were 88.3% DM, 17.7% CP, 2.9% ether extract, 16.8% crude fiber, 9.6% ash, 1.1% Ca, 0.75% P, and 0.3% Na. The number of rabbits in the TG group was double that in the CG group. The TG group had a greater number of rabbits with respect to the CG group to provide a greater chance of detecting the transgene.

During the fattening phase, the TG rabbits were housed indoors in individual cages (12:12 h light:dark schedule, 18 ± 2°C ambient temperature, 70 ± 5% relative humidity, and mechanical ventilation), whereas the CG rabbits were raised outdoors in individual cages, according to the different farming systems. Access to diets and water was ad libitum.

Sample Collection

Representative samples of the 2 diets were collected and stored at –20°C until analyzed to confirm the presence (in the transgenic diet) or absence (in the control diet) of the GM soybean. As positive and negative controls, samples of GM and conventional soybean meal were used.

To avoid any accidental cross-contamination, at the end of the fattening (3.00 ± 0.23 kg BW) period, the 2 groups of rabbits were slaughtered under the control of the veterinary inspector on 2 different days and in 2 different municipal (public) slaughter houses, following European rules (council regulation [European Commission] numbers 853/2004 (EC853, 2004a), 854/2004 [EC854, 2004b], 1/2005 [EC1, 2005], 1099/2009 [EC1099, 2005]).

During samples collection, all the surgical instruments were cleaned with 90% ethanol solution and new disposable gloves were used for each sample. Just before slaughter, 1 mL of blood was withdrawn from the central vein of the ear of each animal and put into a sterile tube containing 10 μL EDTA as an anticoagulant. Soon after slaughter, samples of liver, kidney, heart, stomach, intestine (jejunum), lateral quadricep muscle, longissimus muscle, and perirenal adipose tissue were collected. Samples of stomach contents and feces (directly from the rectum) and hair samples from each rabbit were also taken. Before tissue collection, to avoid contact with the hair, all animals were peeled in a separate room. Before DNA extraction, stomach and intestine samples were washed with a sterile physiological solution to avoid the contamination with their content. All tissues, GI contents, and hair samples were placed on ice, vacuum packaged, and stored at –20°C until DNA extraction.

Deoxyribonucleic Acid Extraction

To prevent contamination during DNA extraction, all samples derived from the CG were processed before the samples derived from the TG were processed. Genomic DNA from mixed feed and soybean meal samples, transgenic and not, and DNA from stomach content and hair samples were isolated by using the Plant/Fungi DNA Isolation Kit (Norgen Biotek Corporation, Thorold, ON, Canada) according to the manufacturer's protocol. Deoxyribonucleic acid from blood was extracted by using the WIZARD Genomic DNA Purification Kit (Promega Corporation, Madison, WI) according to the manufacturer's protocol. The Isolate II Genomic DNA Kit (Bioline USA Inc., Taunton, MA) was used to isolate the DNA from rabbit tissues according to the manufacturer's protocol. Deoxyribonucleic acid from feces was extracted

Table 1. Primers, probes, and real-time PCR conditions used for the detection of target genes in the feeds, tissues, stomach digesta, feces, and hair samples of rabbit

A. Target: junction region between the insert and the plant genome (GenBank accession number AJ308514)			
Gene[1]	Sequence 5′–3′	Amplicon size, bp	Temperature of annealing, °C
40-3-2 For	TTCATTCAAAATAAGATCATACATACAGGTT	84	55
40-3-2 Rev	GGCATTTGTAGGAGCCACCTT		
40-3-2 Pr	FAM - CCTTTTCCATTTGGG - MGBNFQ		
B. Target: soybean lectin gene (GenBank accession number K00821)			
Gene	Sequence 5′–3′	Amplicon size, bp	Temperature of annealing, °C
Lectin For	CCAGCTTCGCCGCTTCCTTC	74	60
Lectin Rev	GAAGGCAAGCCCATCTGCAAGCC		
Lectin Pr	FAM - CTTCACCTTCTATGCCCCTGACAC - TAMRA		
C. Target: β-actin gene (GenBank accession number NM001101683)			
Gene	Sequence 5′–3′	Amplicon size, bp	Temperature of annealing, °C
β-actin For	CTGGAACGGGTGAAGGTGACA	73	60
β-actin Rev	CGGCCACATTGCAGAACTTT		

[1]For = Forward; Rev = Reverse; Pr = Probe.

by using the Stool DNA Isolation Kit (Norgen Biotek Corporation) according to the manufacturer's protocol. The extracted double-stranded DNA (**dsDNA**) was analyzed by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining. A Quant-iT PicoGreen dsDNA Assay (Invitrogen, Eugene, OR) was used to quantify the dsDNA following purification.

Real-Time PCR Analysis

Detection of specific DNA sequences was performed by real-time PCR (**rtPCR**) assay, using the LightCycler instrument 2.0 (Roche Molecular Diagnostics, Mannheim, Germany). All samples were monitored for oligonucleotide primers and probes specific for the recombinant event 40-3-2 of Roundup Ready Soybean, to generate amplicon sizes of 84 bp. Oligonucleotide primers pairs and probes specific for the lectin (***LE1***) gene, as an internal control to identify soybean DNA from feed, were used to generate an amplicon size of 74 bp (CRL-GMFF, 2009). To ensure the quality and suitability of the extracted DNA from tissues for rtPCR analysis, the presence of amplicons of 73 bp, generated from oligonucleotide primers specific for the rabbit *β-actin* (***ACTB***) gene, were investigated. All samples were analyzed in duplicates. The specific primers, probes, and rtPCR conditions used for the detection of the target genes in all test materials are given in Table 1. Each rtPCR product was analyzed by electrophoresis on 2.5% agarose gel and visualized by ethidium bromide staining.

The limit of detection for 40-3-2 Roundup Ready Soybean gene fragments by rtPCR was 13 copies/reaction volume for DNA from mixed feed, stomach content, feces, and hair (Armbruster and Pry, 2008; Bustin et al., 2009).

RESULTS

Deoxyribonucleic Acid Detection in Feed

The results of rtPCR analysis confirmed the presence of the reference lectin gene in the GM and non-GM feed samples as well as in the transgenic and conventional soybean meal samples used as positive and negative controls (Fig. 1a), whereas the transgenic soybean 40-3-2 was detected only in the mixed feed containing GM soybean meal and in the transgenic soybean meal samples (Fig. 1b). These results confirmed the correct provenance of the samples.

Deoxyribonucleic Acid Detection in Stomach Content and in Feces

Results of rtPCR analysis showed the presence of soybean 40-3-2 DNA in the stomach contents (100% of recovery samples) and feces (100% of recovery samples) of rabbits offered the GM feed (Fig. 2a) but not in CG digesta (Fig. 2b). In contrast, the *LE1* gene was detected in the stomach contents (100% of recovery samples) and feces (100% of recovery samples) taken from both the TG (Fig. 3a) and the CG (Fig. 3b) rabbits.

Deoxyribonucleic Acid Detection in Tissues

No transgenic soybean 40-3-2 DNA fragments and reference *LE1* gene were detected in any of the tissues analyzed (i.e., blood, liver, kidney, heart, stomach, intestine [jejunum], lateral quadricep muscle, longissimus muscle, and perirenal adipose tissues) of the TG (Fig. 2a and 3a) and CG rabbits (Fig. 2b and 3b). As expected, all of the tissue samples were positive for

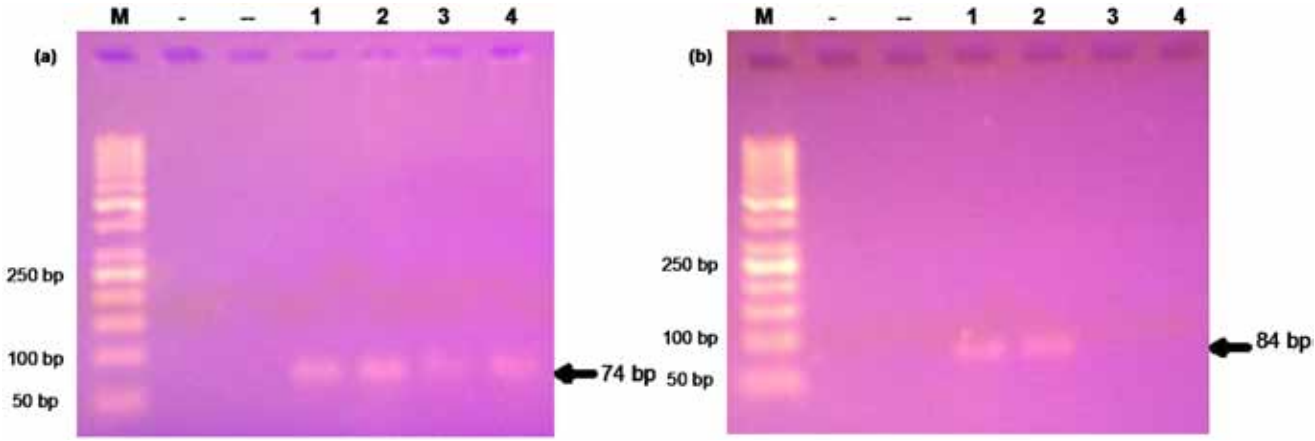

Figure 1. The real-time PCR (rtPCR) products from the genetically modified (GM) soybean meal (Lane 1), mixed feed containing GM soybean meal (Lane 2), conventional soybean meal (Lane 3), and mixed feed containing conventional soybean meal (Lane 4). Panel (a) shows the endogenous *lectin* (*LE1*) gene of soybean (74 bp) and panel (b) shows the exogenous soybean 40-3-2 gene (84 bp). M = marker; – = negative control (no DNA); -- = negative control (DNA from rabbit tissue, not containing *LE1* gene). Arrows indicate the expected length of rtPCR products.

the presence of the endogenous *ACTB* gene in the TG (Fig. 4a) and CG (Fig. 4b) rabbits.

Deoxyribonucleic Acid Detection in Hair

The results of rtPCR analysis confirmed the presence of tDNA fragments of soybean 40-3-2 and the reference *LE1* gene in the hair samples of rabbits fed GM feed (Fig. 2a and 3a), whereas neither gene was detected in the hair samples of rabbits fed conventional feed (Fig. 2b and 3b).

DISCUSSION

Farm animals and humans are constantly exposed to different sources of exogenous DNA as part of their diet. The DNA from GM plants, introduced into feed or food, is equivalent to exogenous DNA from existing food organisms that have always been consumed with diets (Jonas et al., 2001; CAST, 2006). After ingestion, all plant DNA, transgenic or not, is broken down rapidly within the GI tract of both animals and humans by digestive enzymes and microbial activities into small fragments and nucleotides (Duggan et al., 2000; Einspanier et al., 2004; Sharma et al., 2006; Alexander et al., 2007). In ruminants, McAllen (1982) estimated that more than 85% of plant DNA consumed is degraded to nucleotides within 4 h and absorbed into rumen microbes or passed into the duodenum.

This study was conducted to investigate the fate of recombinant DNA in the digestive tract of rabbits fed transgenic soybean. Fragments of digested tDNA were detected only in the stomach contents and feces of rabbits fed GM feed whereas the endogenous *LE1* gene was found in the stomach contents and feces of both TG and CG rabbits. Our findings strongly indicate that feed-ingested DNA, recombinant or not, is resistant to mechanical, chemical, and enzymatic activities and also to microbial processes of the digestive tract of rabbits and is not completely degraded. Moreover, our data also indicated that tDNA undergoes the same fate as DNA from conventional feed. These findings agree with results of feeding studies with quail, pigs, and rabbits (Chowdhury et al., 2004; Flachowsky et al., 2005; Wiedemann et al., 2006; Tudisco et al., 2010), in which DNA, transgenic or not, was detected at all sites of the digestive tract examined.

Many studies investigated the fate of recombinant plant DNA in different species, demonstrating that the survival of plant DNA in the animal's GI tract is different. Experimental trials with laying hens, broilers, dairy cows, fallow deer (*Dama dama*), and rodents fed GM diets showed that tDNA was not detected in any contents of the GI tract or in feces but the presence of transgenic genes decreases during their passage through the digestive tract of these animals, to become completely undetectable in the distal tract of intestine (Chambers et al., 2002; Phipps et al., 2003; Ma et al., 2013). In human ileostomists, although the soybean transgene survived the passage through the small bowel, it was completely degraded in the large intestine (Netherwood et al., 2004).

One of the main issues raised from consumer concerns is the risk of a possible GM DNA transfer from GM plants to farm animal tissues and then to food of animal origin consumed by humans. Wiedemann et al. (2009) reported that small plant DNA fragments can survive after digestion because of their interaction with some dietary compound in the upper digestive tract and that some could enter the intestinal epithelium and be absorbed by the host organism, even if biologically relevant functional activity of that DNA is highly unlikely. Because the tDNA is not different from other sources of

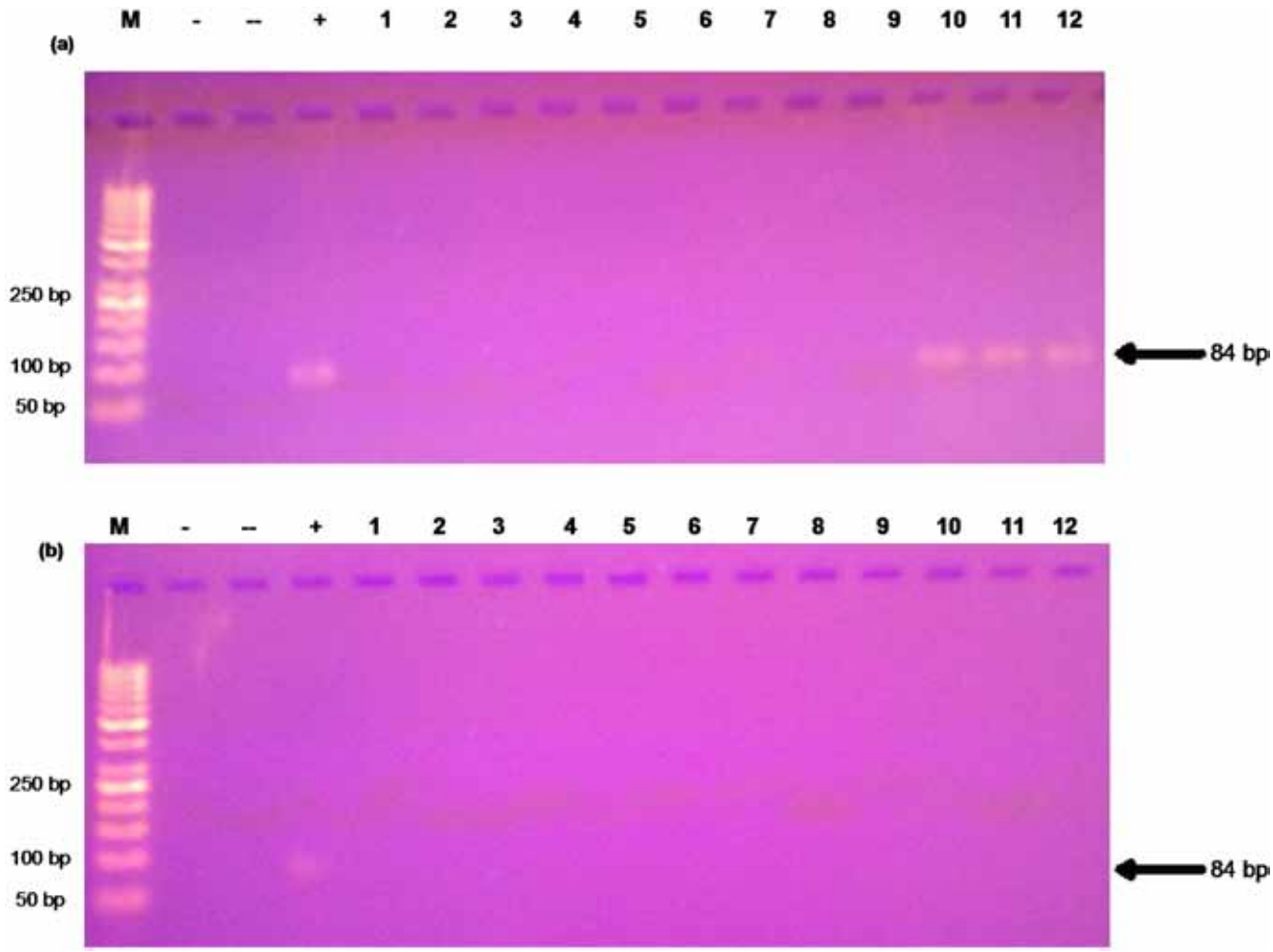

Figure 2. The real-time PCR (rtPCR) products of the exogenous soybean 40-3-2 gene from tissues (lanes 1 to 9: blood, liver, lateral quadricep muscle, longissimus muscle, kidney, stomach, intestine [jejunum], perirenal adipose tissue, and heart, respectively), stomach digesta (lane 10), feces (lane 11), and hair (lane 12) of the treated group (panel a) or control group (panel b) rabbits. M = marker; – = negative control (no DNA); -- = negative control (DNA from conventional soybean meal); + = positive control (DNA from genetically modified soybean meal). Arrows indicate the expected length of rtPCR products.

DNA in the diet, if plant DNA is absorbed, it could be that tDNA may also be absorbed (Phipps et al., 2003).

In this study, the potential transfer of recombinant DNA from GM feed to rabbit tissues, such as blood, liver, kidney, heart, stomach, intestine (jejunum), lateral quadriceps muscle, longissimus muscle, and perirenal adipose tissue, was investigated. No fragments of tDNA from single-copy soybean transgenes were detected in the blood and in other tissue samples from rabbits fed GM soybean meal. Similarly, the fragments from a single-copy endogenous *LE1* gene were undetectable in the blood and in other rabbit tissues of both TG and CG treatments. Endogenous *ACTB* gene was recovered in all tissues examined, assuring the suitability of the extraction and amplification of extracted DNA. Results of this study clearly indicated that, even with the incomplete digestion of the DNA, transgenic or not, in the GI tract of the rabbit, there was no evidence of any soybean transgene translocation to blood and other tissues of rabbits fed GM feed. This lack represents an important result, which demonstrates and confirms that meat from rabbits fed a diet containing GM feed is comparable with that derived from rabbits fed conventional crops. Comparable results have been reported in studies, summarized in recent reviews (Deb et al., 2013; Ma et al., 2013; Van Eenennaam and Young, 2014; Tufarelli et al., 2015), undertaken to investigate the fate of tDNA in tissues, organs, and products of farm animals such as broiler chickens, quail, laying hens, pigs, calves, and dairy cows or in mice and rats fed diets containing GM crops. In those studies, no fragments of recombinant or endogenous DNA were found in samples of blood, tissues, meat, milk, or eggs obtained from these animals (Phipps et al., 2003; Chowdhury et al., 2004; Wiedemann et al., 2009; Ma et al., 2013).

Other researchers, however, have traced small fragments of naturally occurring multicopy plant genes (e.g., chloroplast gene) in the digestive tracts and in certain tissues and fluids of animals including broilers, layer hens, pigs, calves, rabbits, and rodents (Schubbert

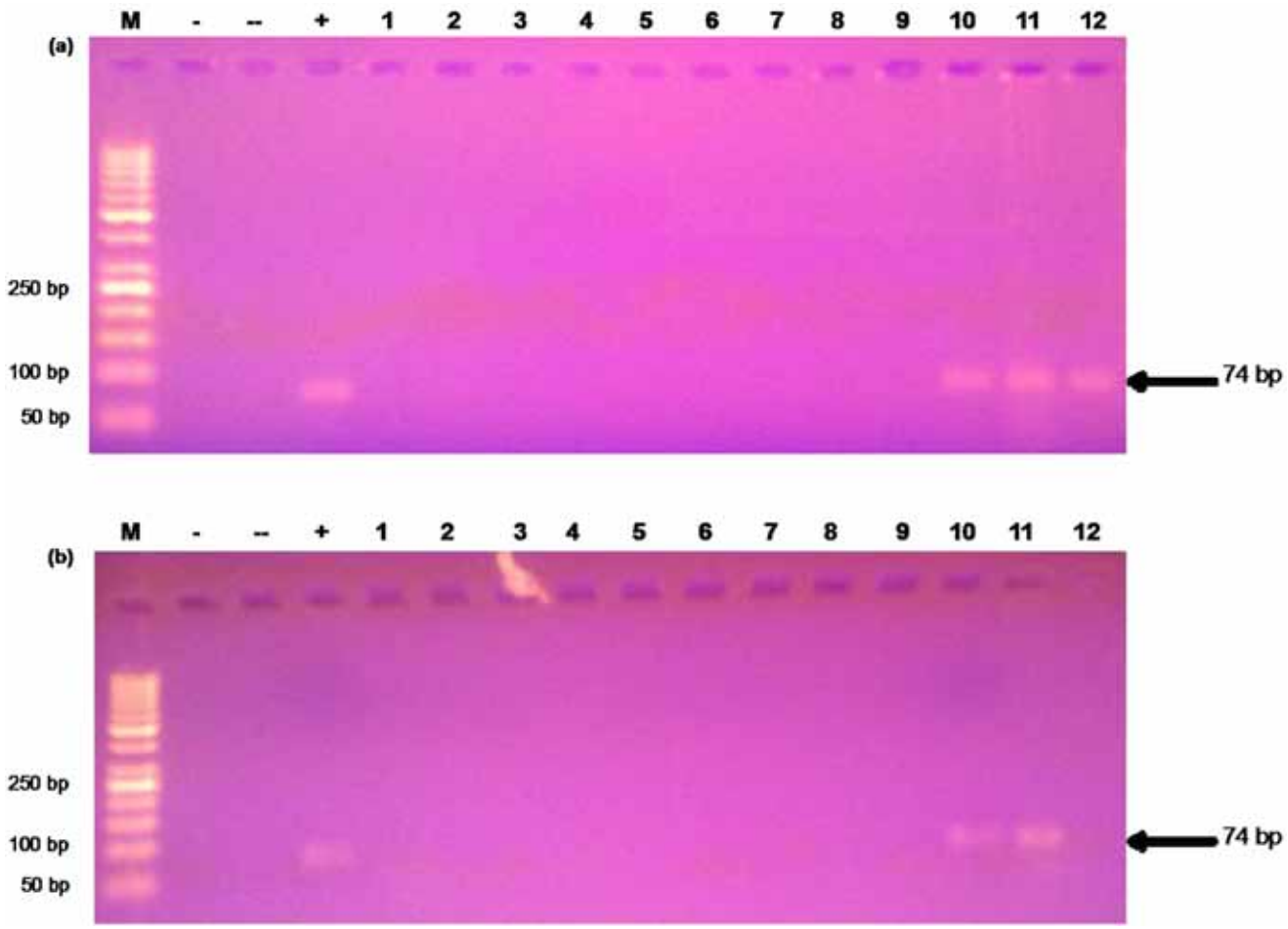

Figure 3. The real-time PCR (rtPCR) products of the endogenous *lectin* (*LE1*) gene from tissues (lanes 1 to 9: blood, liver, lateral quadricep muscle, longissimus muscle, kidney, stomach, intestine [jejunum], perirenal adipose tissue, and heart, respectively), stomach digesta (lane 10), feces (lane 11), and hair (lane 12) of the treated group (panel a) or control group (panel b) rabbits. M = marker; – = negative control (no DNA); -- = negative control (DNA from tissues, not containing *LE1* gene); + = positive control (DNA from genetically modified soybean meal). Arrows indicate the expected length of rtPCR products.

et al., 1997; Reuter and Aulrich, 2003; Chowdhury et al., 2004; Tudisco et al., 2006) as well as in the milk of dairy cows (Einspanier et al., 2001; Phipps et al., 2003). The authors suggested that the transfer of these small DNA fragments in animal tissues and, concomitantly, their recovery is a function of the fragment size and the number of copies of the gene, usually greater than single-copy tDNA (Flachowsky et al., 2005; Alexander et al., 2007). Therefore, the detection of DNA in samples seems to be related to its abundance.

In the present experiment, it is possible that even if the presence of soybean 40-3-2 was traced at the 2 sites of the rabbit's GI tract analyzed, the amount of the single-copy numbers of the soybean transgene was so small compared with the amount of the multicopy genes as to make the potential absorption of tDNA fragments a rare event. In addition, it has been observed in mammals that cells of the gut and blood immune system may phagocytize any DNA fragment that may enter the body and blood, making the DNA detection much more difficult.

During the last few years, a few studies have, surprisingly, showed that small transgenic single-copy DNA fragments from plants can be absorbed through the intestinal mucosa and detected in trace amounts in blood, tissues, and organs of animals, such as broiler chickens (Rehout et al., 2008), pigs (Mazza et al., 2005; Sharma et al., 2006), and fish (Chainark et al., 2008). Netherwood et al. (2004) reported that intestinal microflora of the small bowel of some human ileostomists was capable of acquiring and harboring DNA sequences from GM soybean even if no gene transfer from the bacteria to the mammalian cells occurred. In those studies, no detrimental effects have been identified for farm animals or humans; however, these findings continued to fuel public concerns about using GM feeds because of the possibility of horizontal transfer of tDNA from a GM diet to farm animals and derived products intended for human consumption.

Despite the controversial results of this debate, scientific evidence indicates that the possible presence of plant DNA fragments, transgenic or not, in animal tis-

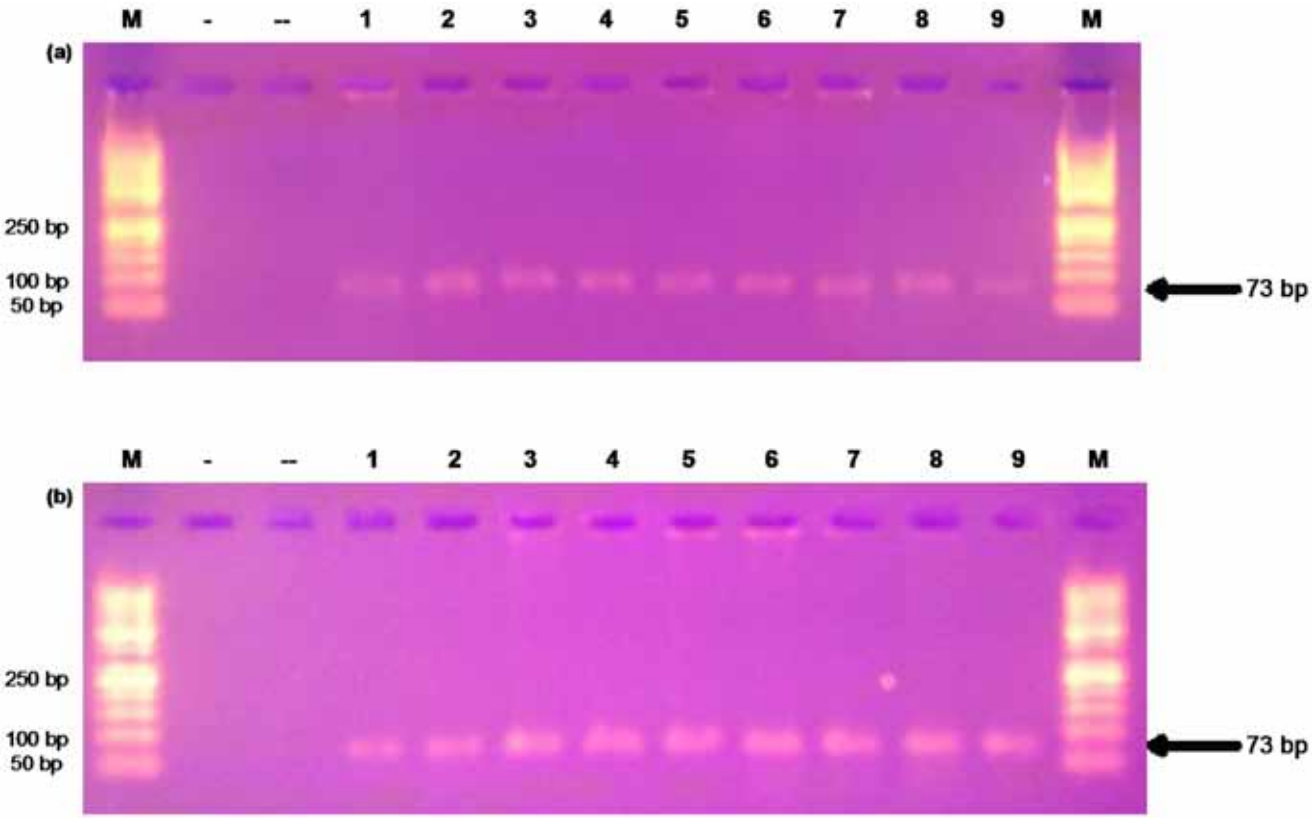

Figure 4. The real-time PCR (rtPCR) products of the endogenous *β-actin* (*ACTB*) gene from tissues (lanes 1 to 9: blood, liver, lateral quadricep muscle, longissimus muscle, kidney, stomach, intestine [jejunum], perirenal adipose tissue, and heart, respectively) of rabbits fed diet containing genetically modified soybean meal (panel a) or conventional soybean meal (panel b). M = marker; – = negative control (no DNA); -- = negative control (DNA from conventional soybean meal, not containing *ACTB* gene). Arrows indicate the expected length of rtPCR products.

sues and fluids presents no health risks for animals and human. This is because of the size of individual nucleotides or the sequences of the fragments absorbed is so small compared with that of the intact transgenes that they are unlikely to transfer genetic information (Rossi et al., 2005). In any case, the likelihood of transfer and functional integration of DNA from ingested feed or food is rare (Mazza et al., 2005). Furthermore, no gene or DNA fragments absorbed by vertebrates via intestinal mucosa have ever been detected in the genome of animals and humans (Beever and Kemp, 2000).

In the present study, hair samples from each rabbit were taken to determine the possible environmental contamination with dust of the feed present on the herd. Results of the rtPCR analysis showed traces of soybean 40-3-2 and *LE1* gene only in the hair of rabbits fed GM feed. These findings confirm the hypothesis of a possible environmental contamination of rabbit hair with the GM feed dust present in the herd of TG rabbits. Similar results were not found on the hair of CG rabbits raised from a biological herd in which no GM feedstuffs were used and the animals were raised outdoor. This result indicates that, during sampling, it is very important to put in place all the necessary procedures to avoid the contact of the material test with GM contaminated sources. Otherwise, it is possible that contaminated samples (e.g., tissues) return positive results when analyzed for tDNA.

Summary and Conclusions

In conclusion, in this study, the detection of soybean 40-3-2 in the stomach digesta and feces indicated an incomplete degradation of tDNA in the GI tract of rabbit fed with GM soybean meal. There was also no evidence of a transfer of recombinant DNA from feed into the tissues of rabbits consuming a diet containing GM soybean. This represents an important result that demonstrates and confirms that meat from rabbits fed diets containing GM feed is as safe as that derived from rabbits fed conventional crops. The presence of traces of soybean transgene in rabbit hair is due to environmental contamination, which might lead to false positive results.

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