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Genetically modified soya bean in rabbit feeding: detection of DNA fragments and evaluation of metabolic effects by enzymatic analysis

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Abstract

The presence of DNA fragments in tissues from rabbits given genetically modified (GM) soya-bean meal (solvent extracted) was investigated by using the polymerase chain reaction (PCR) approach. Moreover, the possible effects on cell metabolism were evaluated by determination of several specific enzymes in serum, heart, skeletal muscle, liver and kidney. The chloroplast sequence for tRNA Leu by using the Clor1/Clor2 primers designed on chloroplast trnL sequence was clearly detected. On the contrary, two couples of species specific primers for conventional (Le1-5/Le 1-3 which amplifies the soya bean lectin gene) and genetically modified (35S1/35S2 which amplifies the 35S CMV promoter that is present in the genomic structure of GM soya bean) soya bean were not found in all samples. No differences in enzyme levels were detected in serum, but a significant increase of lactic dehydrogenase, mainly concerning the LDH1 isoenzyme was found in particular in kidney and heart but not in the muscle, thus suggesting a potential alteration in the local production of the enzyme. Finally, no significant differences were detected concerning body weight, fresh organ weights and no sexual differences were detected.

Keywords: *genetic modification, polymerase chain reaction, rabbits, soya-bean oil meal.*

Introduction

Several genetically modified (GM) plants have been produced and approved by regulatory agencies worldwide for cultivation and commercialization. The insertion of new genes or the repression of endogenous gene expression can be in fact an useful tool to obtain specific characteristic which can lead to an improvement of agronomically relevant traits or food quality. Resistance to insects and tolerance to herbicides are the most recurrent agronomic traits modified in GM crops approved for feeding.

Nowadays, a number of GM plants have been approved for animal and human consumption but concerns over safety persist in the public. Allergenicity and toxicity, which can be related to novel foods are a major concern (HINO, 2002). Potential toxicological risks of a GM plant as whole food are evaluated on laboratory and target animals according to the classical methods used for drugs: blood and urine chemistry, organ weight and gross histo-pathological examination (Food and Agriculture Organisation-World Health Organisation, 2000; Organisation for Economic Co-operation and Development, 2003, novel foods OECD no. 9; European Food Safety Authority, 2004). It has also suggested finding specific biomarkers of early effects in order to increase diagnostic value and sensitivity of toxicity tests on food.

Animal nutritionists have evaluated in several studies the nutritional equivalence and the efficacy of the new feeds, in comparison with near isogenic or conventional varieties of plants (Aumaitre *et al.*, 2002; Cromwell *et al.*, 2002) and no direct evidence that GM plants may represent a possible danger for animal health has been reported so far (for a review, see Aumaitre (2004)). Another aspects that has been studied is the fate and integrity of forage plant DNA in the gastro-intestinal tract (GIT) of various animal models. Some authors have shown that highly fragmented plant DNA can be isolated from animal organs and tissues, thus suggesting that plant DNA is not completely degraded during animal digestion (Chowdhury *et al.*, 2003; Duggan *et al.*, 2003; Einspanier *et al.*, 2004).

The aims of this research have been the evaluation, by the polymerase chain reaction (PCR) approach, of the presence of plant DNA fragments in rabbit tissues to follow the fate of plant fed and the possible health effects of a GM diet by studying the activity of organ specific enzymes in rabbits.

Material and methods

Animal and diets

Twenty weaned 30-day-old New Zealand rabbits (10 males and 10 females) individually caged were equally assigned to control (C) and treated (T) groups. The animals were given (130 g/day) a diet constituted per kg of 800 g pelleted concentrate (165 g crude protein and 155 g crude fibre, as fed; dehydrated lucerne meal, sunflower meal, wheat, carob, soft wheat middlings, sugar-beet pulp, barley) and 200 g soya-bean meal (solved extracted) (SBM) which was from conventional or genetically modified (Roundup Ready®) beans, for group C and T, respectively. Roundup Ready® (RR) is tolerant to the glyphosate family of herbicides by expressing transgenic DNA from the CP4 strain of *Agrobacterium tumefaciens*, encoding 5-enolpyruvilshikimate-3-phosphate synthase protein (cp4 epsps).

The proximate composition of conventional and GM SBM was determined according to Association of Official Analytical Chemists (1990); the fibre fraction was analysed as suggested by Van Soest *et al.* (1991).

Water was given *ad libitum*. The rabbits were slaughtered at 70 ± 5 days of age (2 ± 0·2 kg live weight), food was available until 12 h before slaughtering. Body weights were taken before the onset of the experiment and immediately before slaughtering, organ weights were taken soon after slaughtering.

Sampling

Blood was withdrawn just before slaughtering and put in two different plastic tubes, with or without sodium citrate 9:1. Small pieces of liver, muscle, kidney and heart were washed in saline. All samples were stored at −20°C. As controls, conventional and transgenic SBM were used.

DNA extraction

Plant samples were extracted according to the Wizard extraction method (Promega, Madison, Wisconsin). One hundred milligrams of SBM were resuspended by careful vortexing in 860 μl of extraction buffer (10 mmol/l Tris HCl (pH 8·0), 150 mmol/l NaCl, 2 mmol/l EDTA, 1% (w/v) SDS), 100 μl guanidine hydrochloride (5 mol/l) and 40 μl of proteinase K (20 mg/ml). Samples were then incubated at 58°C for at least 3 h on a shaking incubator and then centrifuged at 20 000 **g** for 10 min. Five hundred microlitres of the supernatant were incubated with 5 μl RNase (10 mg/ml) at 37°C for 10 min. One millilitre of Wizard DNA Purification Resin (Promega) was added to the supernatant and mixed by gently inversion. A 2-ml syringe was mounted on the column and the mixture was pushed with the plunger through the column. The DNA-resin mixture was washed with 2 ml 80% (v/v) isopropyl alcohol following by centrifugation at 20 000 **g** for 1 min. After drying at 70°C for 10 min, the DNA was eluted with 50 μl of 70°C elution buffer (10 mmol/l tris HCl (pH 9·0), 0·1 mmol/l EDTA) and centrifuged at 20 000 **g** for 1 min.

Tissue (25 mg) and blood (200 μl) samples were extracted by using the 'nucleo-spin tissue' and 'blood-spin tissue' (Macherey-Nagel, Duren, Germany), respectively, according to users' manual. Briefly, 25 mg of ground tissue were incubated with 180 μl buffer T1 and 25 μl proteinase K solution at 56°C for at least 3 h on a shaking incubator. After digestion, the lysates were again incubated with 200 μl buffer B3 at 70°C for 10 min. About the blood samples, they were slowly defrosted (in ice-water bath) and then 200 μl of whole blood were incubated with 25 μl proteinase K solution and 200 μl lysis buffer B3 at 70°C for 15 min on a shaking incubator. To both samples (tissue and blood) were added 210 μl ethanol (96 to 100%), and all of the precipitate was loaded on the column placing into a 2 ml collecting tube and then centrifuged at 11 000 **g** for 1 min. The silica membrane was washed with 500 μl buffer BW and 600 μl buffer B5 following by centrifugation at 11 000 **g** for 1 min. After drying by centrifugation at 11 000 **g** for 1 min, the DNA was eluted with 100 μl pre-warmed elution buffer BE (70°C), incubating for 1 min, and centrifuged the column at 11 000 **g** for 1 min.

The DNA concentration was determined by measuring the UV absorption at 260 nm, then its quality was checked from 260/280 nm UV absorption ratios. All extracted DNA was stored at −20°C until used.

Primers

The quality of DNAs extracted has been checked in a PCR reaction with UNIV P/UNIV Q primers to amplify a conserved portion of animal mtDNA 16S rRNA gene (Sawyer *et al.*, 2003). Therefore, samples have been monitored for the presence of the chloroplast sequence for tRNA Leu by using the Clor1/Clor2 primers designed on chloroplast trnL sequence (Terzi *et al.*, 2004). Finally, two couples of species specific primers for conventional and GM soya bean were used: Le1-5/Le 1-3 which amplifies the soya-bean lectin gene (Kuribara *et al.*, 2002) and 35S1/35S2 which amplifies the 35S CMV promoter that is present in the genomic structure of GM soya bean (Lipp *et al.*, 1999). The sequence of all the primers is shown in Table 1.

Table 1 *Sequence, annealing temperature and amplicon size (bp) of primer pairs used in the study*

Primers	Sequence (5′ to 3′)	Annealing temperature(°C)	Amplicon size (bp)	Reference
Clor1	TTCCAGGGTTTCTCTGAATTTG	60	100	Terzi *et al.* (2004)
Clor2	TATGGCGAAATCGGTAGACG			
UNIV P	GGTTTACGACCTCGATGTT	55	104	Sawyer *et al.* (2004)
UNIV Q	CCGGTCTGAACTCAGATCAC			
Le1-5	GCCCTCTACTCCACCCCCA	59	118	Kuribara *et al.* (2002)
Le1-3	GCCCATCTGCAAGCCTTTTT			
35S-1	GCTCCTACAAATGCCATCA	54	195	Lipp *et al.* (1999)
35S-2	GATAGTGGGATTGTGCGTCA			

The primer pairs have been selected among those reported in literature (Jennings *et al.*, 2003) with the aim of obtaining short amplicons (118 bp), compatible with highly fragmented DNA samples.

PCR analysis

PCR reactions were performed in 20 μl reaction volumes containing 20 mmol/l Tris HCl pH 8·4, 1·5 mmol/l $MgCl_2$, 50 mmol/l KCl, 100 mmol/l of each dNTPs, 900 nmol/l forward and reverse primers (see Table 1), 100 ng of genomic DNA templates, and 1U of Taq polymerase (Invitrogen, Carlsbad, CA). Amplifications were performed using an Applied Biosystems Gene Amp PCR System 2400 programmed as follows: one step of 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at annealing temperature (see Table 1), 1 min at 72°C; and one step of 3 min at 72°C. The PCR products were separated on 2·5% agarose gels in TBE buffer.

Enzyme assay

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), lactic dehydrogenase (LDH), gamma glutamyltransferase (GGT) and alkaline phosphatase (ALP) were assayed in serum and in homogenates from liver, kidney, heart and skeletal muscle. Briefly, one gram of tissue was put into an ice-cold homogenization buffer (in mmol/l): 280 mannitol, 10 KCl 1 $MgCl_2$, 0·2 pefabloc SC, 10 hepes, pH 7·0 adjusted with tris. Samples were homogenized by an Ultra-Turrax homogenizer and then centrifuged in a Beckman L7 ultracentrifuge at 10 000 **g** for 10 min, the upper layer was used for analysis.

Enzyme activity was determined spectrophotometrically by using reagents from Spinreact SA, Sant Esteve de Bas, Spain. Since significant differences were found for LDH between control and treated groups, in order to assess the isoenzymatic distribution of LDH, electrophoretic separation was performed on each sample. Briefly, 20 μl of sample were applied on cellulose acetate membranes and electrophoresis was performed under undenaturing conditions at 200 V for 50 min in barbital buffer. Following electrophoresis, the membranes were stained to reveal the LDH isoenzymes by using the ISO-LAD commercial kits (Chemetron Chimica S.p.A., Milan, Italy). Quantification of isoenzymes fractions was done by using a densitometer (CGA, Florence, Italy). The relative distribution of the isoenzymes in the samples was expressed as a percentage of total enzymatic activity.

Statistics

Results were expressed as mean ± standard deviation. Differences in enzyme levels between groups were analysed by the Student *t* test (Statistical Packages for the Social Sciences (SPSS), 1999). Diet and sexual differences within groups were analysed by analysis of variance (ANOVA) using the model: $y_{ijk} = \mu + D_i + S_j + D \times S_{ij} + \varepsilon_{ijk}$, where $y =$ single observation; $\mu =$ general mean; $D =$ diet effect ($i =$ control or treated); $S =$ sex effect ($j =$ male or female); $D \times S =$ interaction between diet and sex effects; $\varepsilon =$ error (SPSS, 1999).

Results

Detection of DNA fragments

The chemical compositions of conventional and GM SBM were superimposable (Table 2), in agreement with the results summarized by Aumaitre (2004).

Figure 1 shows the DNA amplification in one rabbit from each group by using the UNIV P/UNIV Q primers; as seen, a 104 base pair (bp) band was detected in all the samples thus showing the good quality of extracted DNA.

A similar representative example is reported in Figure 2 for Clor1/Clor2 primers, where a 100 bp band was found in many tissues thus showing the presence of chloroplast DNA in tissues and blood from both control and treated groups. The Clor1/Clor2 primers were not detected in all samples, in particular, percentages of positive samples were: 50% (blood), 70% (muscle), 80% (heart), 70% (liver) and 80% (kidney).

In Figure 3 a representative example obtained by using Le1-5/Le1-3 soya-bean specific primers shows how the signal could not be detected in all samples and the band (118 bp) was seen only in the plant sample.

A similar aspect can be seen in Figure 4 where also the 35S1/35S2 primers gave undetectable results (195 bp) in all samples except for the GM soya bean.

Table 3 shows body weight before and after the end of the experiment and the organ weights in control and treated

Table 2 *Composition (g/kg dry matter) of conventional and genetically modified (GM) soya-bean meal (SBM)*

	Component†						
	CP	EE	CF	Ash	NDF	ADF	ADL
Conventional SBM	544	250	41	71	157	140	20
GM SBM	536	248	43	69	152	125	38

† CP: crude protein; EE: ether extract; CF: crude fibre; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin.

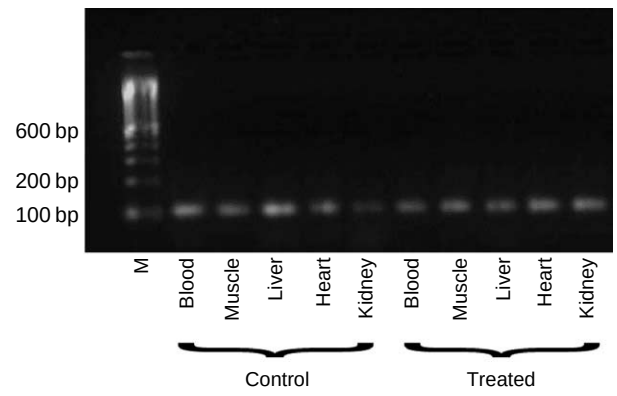

Figure 1 Electrophoretic analysis of DNA amplification in one rabbit from each group by using the UNIV P/UNIV Q primers. M = 100 bp DNA ladder.

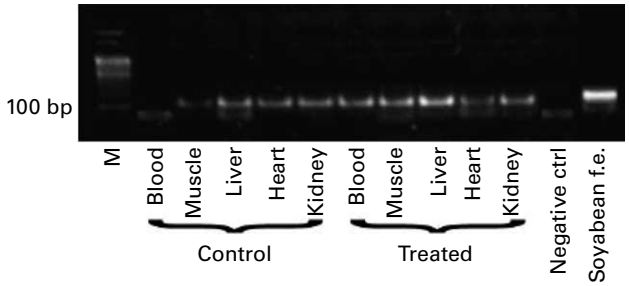

Figure 2 Electrophoretic analysis of DNA amplification in one rabbit from each group by using the Clor1/Clor2 primers. M = 100 bp DNA ladder.

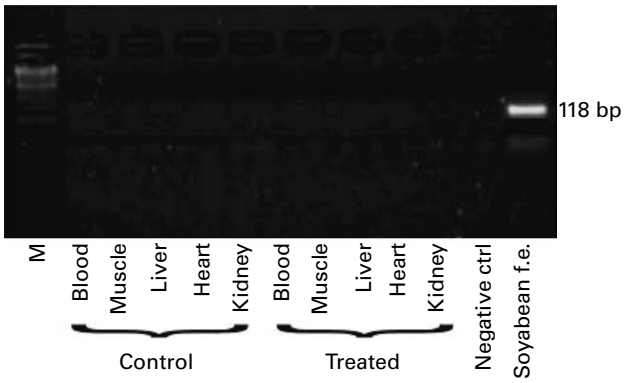

Figure 3 Electrophoretic analysis of DNA amplification in one rabbit from each group by using the Le1-5/Le1-3 conventional soya bean specific primers. M = 100 bp DNA ladder.

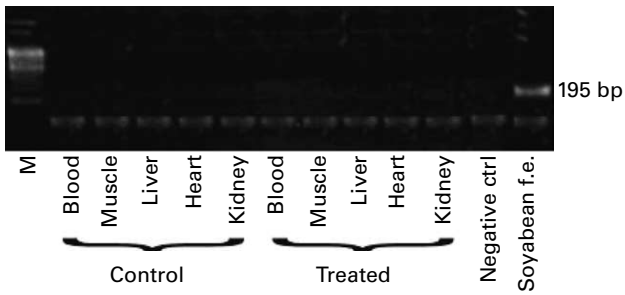

Figure 4 Electrophoretic analysis of DNA amplification in one rabbit from each group by using the 35S-1/35S-2 GM soya bean specific primers. M = 100 bp DNA ladder.

animals, no statistical difference was detected. Finally, no statistical differences were found between sexes.

Enzyme activity

Figure 5 shows the differences in enzyme activity in heart, skeletal muscle, kidney, liver and serum. Statistical differences ($P < 0{\cdot}05$) were detected in kidney for ALT, GGT and LDH whereas in the heart such result was seen only for LDH. No statistical differences were found for serum, liver and skeletal muscle. No statistical differences were found between sexes (data not shown).

Table 4 shows the relative distribution of LDH isoenzymes in serum and in tissues. Significant differences ($P < 0{\cdot}05$) between control and treated animals were detected for heart LDH1 and LDH2 and for kidney LDH1, thus confirming the significant increase of the enzyme in these tissues. Moreover, despite no significant differences were found for LDH total activity in liver, a significant increase (LDH1) and decrease (LDH4) were found also in this organ.

Discussion

DNA

Our findings confirm that, despite chloroplastic plant DNA can be fragmented by technological processes for food preparation and by digestion, multicopy gene can be found in rabbit tissues by using the Clor1/Clor2 primers.

The persistence of short DNA sequences from plant tissues offered has been shown in the GIT of ruminants, from the oral cavity of sheep to rumen and abomasum ingesta of cattle, differing in the case of maize silage and grain (Duggan *et al.*, 2003; Einspanier *et al.*, 2004). The high level of degradation of ubiquitous plant chloroplast DNA in the last section of cattle GIT (jejunum and colon) has been demonstrated by Einspanier *et al.* (2004). In the GIT of monogastrics, plant DNA is detectable in pigs (Klotz *et al.*, 2002; Chowdury et al., 2003; Reuter and Aulrich, 2003), in chickens (Chambers *et al.*, 2002) and in humans (Martìn-Orùe *et al.*, 2002; Netherwood *et al.*, 2004).

In blood, muscular tissues and organs the presence of residual plant DNA has been demonstrated in poultry but not in pig (Klotz *et al.*, 2002; Jennings *et al.*, 2003; Reuter and Aulrich, 2003). Contrasting results have been reported also for ruminants, such as cattle and sheep (Einspanier *et al.*, 2001; Duggan *et al.*, 2003; Phipps *et al.*, 2003).

Also concerning the research of specific DNA fragments the data in the literature are controversial. Indeed, the fragment of invertase gene (ivr, 226 bp) from corn was found in the liver, spleen and muscle of poultry by Aeschbacher *et al.* (2002) but not by Tony *et al.* (2003). In the pigs, while Chowdhury *et al.* (2003) detected fragments of zeina (242 bp), ivr (226 bp) and cry1A(b) (110–437 bp) gene from conventional and GM maize in the gastric and intestinal contents, Jennings *et al.* (2003) did not find fragments (198 bp) of le1 gene for soybean lectin in the muscle. In the present trial, by using the same gene sequence (gi170005/gbK00821·1) of Jennings *et al.* (2003) in the same sample where we found the chloroplast fragment it was not possible to detect neither a shorter fragment (118 bp) of the lectin gene nor the 35S promoter thus confirming that plant low-abundance genes are not detectable in animal tissues.

This finding agrees with the results obtained by Phipps *et al.* (2003) in cow blood and milk, confirming that single copy

Table 3 *Organ fresh weights and body weights in control and treated animals*

		Weight (g)†									
		Liver		Kidney		Muscle		Heart		Body	
	No.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d	Mean	s.d.
Diet											
Control	10	89·0	5·2	14·7	1·2	127·0	12·6	8·7	0·7	2063·1	114·9
Treated	10	86·9	7·2	14·5	1·5	125·5	10·0	8·8	0·7	2040·0	203·5
Sex											
Males	10	87·0	7·2	14·2	1·6	124·2	11·7	8·5	0·7	2070·3	135·0
Females	10	88·9	5·2	15·0	0·9	128·3	10·7	9·0	0·7	2030·4	191·0

† Two-way ANOVA: main effects diet, sex and interaction between diet and sex, revealed no significant differences ($P > 0{\cdot}05$).

gene are difficult to identify (Artim *et al.*, 2001). However, in a previous trial we found the single copy gene of barley (data not yet published) in the tissues and organs of rabbits.

Our negative results in detecting single copy gene from SBM could be affected by its processing. Indeed, according to Forbes *et al.* (1998) and Chiter *et al.* (2000), the oil extraction or the heat treatment can cause fragmentation of food DNA.

The persistence of transgenic proteins in the GIT and tissues of animal models have been evaluated in monogastrics.

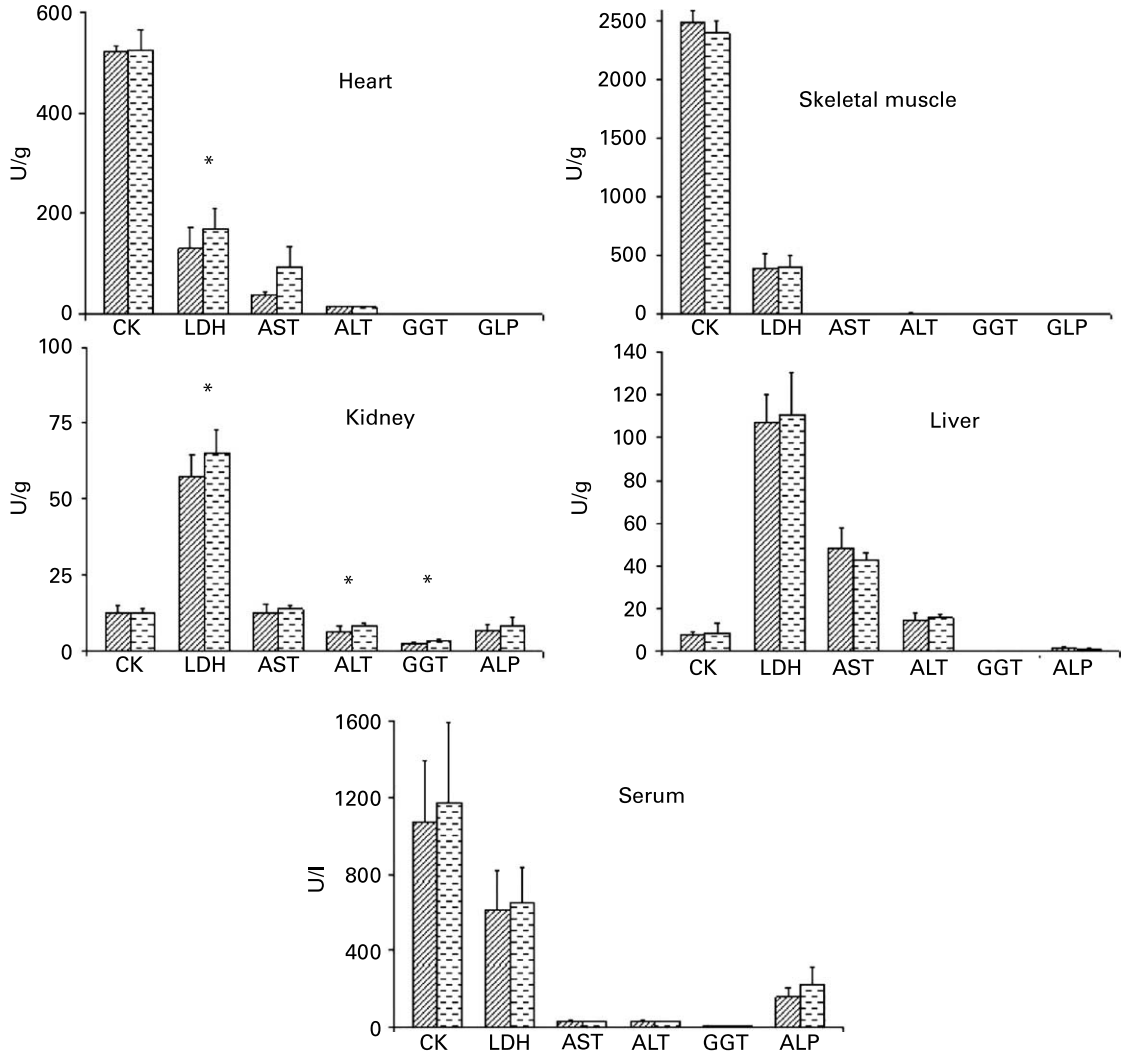

Figure 5 Levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), lactic dehydrogenase (LDH), gamma glutamyltransferase (GGT) and alkaline phosphatase (ALP) in serum and in homogenates from heart, skeletal muscle, kidney and liver from control (▨) and treated (▩) rabbits.

Table 4 *Relative distribution of lactic dehydrogenase (LDH) isoenzymes in serum and in homogenates from heart, skeletal muscle, kidney and liver from control and treated rabbits*

				Relative distribution of LDH isoenzyme (U/g)									
		Total LDH activity		LDH-1		LDH-2		LDH-3		LDH-4		LDH-5	
Tissue	Group†	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
Serum (U/l)	C	614·8	204·2	15·6	6·4	31·8	10·3	37·8	10·8	139·0	33·2	390·6	146·4
	T	646·8	189·6	27·3	4·3	34·9	12·6	40·2	11·0	154·9	42·6	399·5	122·8
Heart (U/g)	C	129·9	34·6	122·5	31·9	3·9	2·1	1·5	1·1	0·9	0·5	1·1	0·5
	T	168·8*	19·2	153·5*	18·0	6·6*	1·4	2·7	1·6	1·9	2·2	1·9	1·7
Skeletal muscle (U/g)	C	385·3	127·3										
	T	398·9	107·1										
Kidney (U/g)	C	57·5	6·8	34·4	4·0	13·4	1·2	6·1	0·9	1·6	0·9	1·6	0·9
	T	65·2*	7·3	41·4*	4·6	15·1	2·3	5·0	1·6	1·3	0·5	1·3	0·5
Liver (U/g)	C	107·5	12·6	54·9	5·9	20·4	2·5	13·1	3·5	9·9	3·9	9·3	3·9
	T	110·5	19·8	63·7*	10·3	19·6	4·6	12·5	3·3	5·3*	3·5	7·4	4·1

† C = control; T = treated. *Significant differences are indicated for T *v.* C groups ($P < 0·05$).

Yonemochi *et al.* (2002) for example evaluated transgenic event CBH 351 (StarLink) corn in broiler chicks feeding, finding that both cry9C gene and cry9C protein were not detected in blood, liver and muscle.

Enzymes

Another interesting aspect of our results concerns the enzyme activity in serum and organs. The levels of the enzyme tested in serum did not show significant differences thus suggesting that no adverse effects were induced by GM soya bean in treated animals. Such result is in agreement with Yonemochi *et al.* (2003), who found no effects of maize GM on serum LDH levels in dairy cows. By contrast, the analysis of enzyme relative activities in tissues gave a different picture. As depicted in Figure 5, significant differences in enzyme levels concerned mainly the kidney, showing higher levels of LDH, ALT and GGT in treated animals. Such a result seems to indicate that some alteration occurred in kidney even if serum levels were not affected. Moreover, LDH was significantly increased also in the heart thus showing that the local production of LDH altered in two of the most important organs of the body. The relative distribution of LDH isoenzymes confirms this hypothesis showing significant differences for heart LDH1 and LDH2 and for kidney LDH1. LDH1 was the dominant isoenzyme in both organs and, additionally, a significant increase of this isoenzyme and a decrease of LDH4 were also shown in the liver, despite no significant differences were found for total LDH activity in this organ. Such a result means that, since LDH is a tetrameric enzyme made up of M and H subunits, a different combination of H and M subunits occurred in the liver ($LDH\text{-}H_4 > LDH\text{-}H_1M_3$). The reason for that shift is not clear, substrate specificity is greater for pyruvate than for alpha-hydroxybutyrate, but a higher amount of H subunit is thought to mean a higher specificity for the reduction of alpha-hydroxybutyrate to alpha-oxobutyrate. Anyway, such shift supports the hypothesis that some metabolic changes occurred in the liver. Therefore, an increased activity of LDH1 occurred in three organs from GM-fed rabbits. The reason for such increase is not fully understood and confirmed but such result suggests that even slight modifications occurred in the local production of the LDH1 isoenzyme in GM-fed rabbits. Moreover, it is known that serum enzyme activities are a reflection of the relative contribution of each tissue to the serum pool. From the lactate dehydrogenase isoenzymatic pattern of the serum and in comparison to the various tissue patterns, it seems that most lactate dehydrogenase activity in the rabbit serum originates from the skeletal muscle which is the major mass component of the body. It is also known that the dominant isoenzyme in muscle is the LDH5 and, for these reasons, it is possible that the LDH1 increase was not detected in serum. Moreover, since LDH1 is known to be involved in cell metabolism by favouring the reaction of lactate to pyruvate (Van Hall, 2000), our results should indicate a general increase of cell metabolism. Such hypothesis is in agreement with other authors who showed significant modifications of some nuclear features in GM-fed mice suggesting a high metabolic rate and an intense molecular trafficking (Malatesta *et al.*, 2002). Anyway, since no diseases were detected in treated animals and serum activities of all the enzymes showed similar levels between the groups, it should be overspeculative to assess that the GM diet is responsible for that but it is a fact that the synthesis of LDH changed in more than one organ and such results should be taken into account for future research.

In any event, our results suggest that an accurate enzymatic analysis can be useful to detect the effects of the diet on cell metabolism even in absence of clinical and biochemical signs. Since the techniques for enzyme assay are well established, enzymes can represent an additional tool to evaluate the risks of GM feeding for animal and human health.

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