

A three-year longitudinal study on the effects of a diet containing genetically modified Bt176 maize on the health status and performance of sheep

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Abstract

This study shows that a diet including insect-resistant Bt176 maize, fed to 53 ewes and their progeny for 3 years, did not have adverse effects on their health or performance and that no horizontal gene transfer to ruminal microorganisms or animal tissues was detected. No differences were observed regarding performance, reproductive traits, haematological parameters, antioxidant defences, lymphocyte proliferative capacity, phagocytosis and intracellular killing of macrophages, and ruminal microbial population characteristics between control and genetically modified (GM) maize-fed animals. Immune response to *Salmonella abortus ovis* vaccination was more efficient in GM maize fed sheep. No modifications of histological features of tissues were found; however, cytochemical analyses of ruminal epithelium by Ki67 staining provided evidence of proliferative activation of basal cells in all GM maize-fed ewes. Preliminary electron microscopy analyses of the liver and pancreas revealed smaller cell nuclei containing increased amounts of heterochromatin and perichromatin granules in GM maize-fed lambs. Meat protein content and water loss by cooking were slightly affected by the dietary treatment. No transgenic DNA was detected in tissues, blood, and ruminal fluid or ruminal bacteria. Longitudinal studies should be included in evaluation of food safety whenever possible and sheep may be a useful animal model for toxicological assessment.

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Keywords: Bt176 maize; Sheep; Health status; Transgene detection

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1. Introduction

Genetically modified (GM) plants, as well as feed and food derived from these plants, have to undergo a risk assessment prior to market authorization in the EU. The particular requirements for risk assessment have been and still are particularly contested issues and only recently attempts have begun to specify requirements in greater detail (Kleter and Kuiper, 2002; König et al., 2004; Jank et al., 2005). A recent review of a number of applications that have been submitted and approved at the European level (Spök et al., 2005), suggests that there are shortcomings in the risk assessment approach, in particular with issues revolving around the concept of substantial equivalence.

The agronomic improvements afforded by genetic modification of crops have resulted in a dramatic increase in their use. Thus, as adoption of GM crops continues to increase, so will their consumption by animals reared for food. However, despite rigorous approval standards for transgenic plants, little is known about the fate of recombinant DNA following ingestion of GM crops by animals and exposure of the plant DNA to microorganisms within the digestive tract (Duggan et al., 2003; Einspanier et al., 2004). Scientific knowledge on horizontal gene transfer from plant cells to mammalian or bacterial cells is still incomplete (van den Eede et al., 2004). The ruminal microbial population is considered one of the most likely targets for natural transformation, when the diet contains GM organisms (GMO). There is a lack of long-term studies, performed on a high number of animals over several generations, aimed at evaluating the effects of genetically modified (GM) feeds on livestock species.

In this context, a 3-year longitudinal study was carried out to evaluate possible effects of a diet containing GMO (Bt176 maize) on a group of meat sheep and their progeny. The study was aimed at assessing: (a) animal welfare and health status through periodic evaluation of metabolic and haemato-chemical profiles as well as immune response following vaccination; (b) performance and reproductive traits; (c) nutritional and organoleptic properties of meat; (d) the presence of transgenes in ruminal microorganisms and animal tissues.

2. Material and methods

2.1. Animals and feeds

One hundred and six Bergamasca × Appenninica ewes, aged approximately 10 months, were divided in 2 groups, treated (T) and control (C), balanced for body

weight (BW: 36.9 (±0.9) vs. 37.1 (±1.2) kg) and body condition score (BCS: 1.9 (±0.03) vs. 1.9 (±0.04)) (Russel et al., 1969). During the first 8 months of the trial, animals were fed a diet based on non-GM mixed hay (crude protein 115.5 (±5.3) g/kg; crude fibre 323.0 (±6.1) g/kg), non-GM maize grains and a non-GM mineral-vitamin supplement (“PRE” period). For the following 36 months of the experiment, starting in May 2002 (“EXP” period), group T had the non-GM maize replaced with the maize hybrid NK COMPA CB (event Bt176). The amount of hay and maize varied according to physiological state (hay: 1700 to 2500 g/day; maize: 100 to 600 g/day, for dry period and lactation, respectively) (NRC, 1981). The animals had free access to water. Compositional analyses were conducted to measure proximate, fibre and mineral content (AOAC, 2000) of the maize (Table 1). Rumen degradability parameters (Ørskov and McDonald, 1979; McDonald, 1981) were assessed using 2 ruminal cannulated ewes (Table 1).

The sheep were subjected to natural mating in June 2002, November 2003 and August 2004 and presented

Table 1
Composition of maize lines (as-fed basis) used in the experiment

Item	Non-GM maize	SEM	GM maize	SEM	<i>P</i> -value
Chemical composition (g/kg)					
Dry matter	877.8	1.6	879.6	1.8	0.50
Ether extract	28.4	4.5	40.7	1.1	0.07
Ash	10.3	1.3	10.9	0.1	6.5
Crude protein	74.3	1.0	75.1	1.1	0.63
Neutral detergent fibre	97.4	0.4	82.7	2.2	<0.01
Acid detergent fibre	18.1	1.4	13.7	0.8	0.06
Acid detergent lignin	4.8	0.5	4.0	0.4	0.31
Starch	630.2	1.8	644.6	3.1	0.06
Minerals (µg/g)					
Calcium	383.80	118.63	157.25	91.05	0.18
Phosphorus	2352.10	208.20	2833.30	185.60	0.04
Magnesium	1088.30	26.83	1056.77	23.43	0.42
Zinc	19.92	1.42	16.89	0.81	0.09
Copper	3.74	0.21	2.69	0.20	<0.01
Iron	24.75	2.40	19.47	0.55	0.06
Manganese	7.80	0.55	7.03	0.62	0.37
Rumen dry matter degradation parameters					
ED (%)	80.50	1.66	81.12	1.66	0.80
Degradation rate (h ⁻¹)	0.06	0.006	0.06	0.006	0.65

ED: effective degradability at $k=0.02\text{ h}^{-1}$ (where k =rate of outflow from the rumen).

normal pregnancies and deliveries. Fertility and twin rate, the lambs' BW at birth, the lambs' mortality and daily weight gain up to weaning (90 days of age) were evaluated.

Sheep BW and BCS were assessed at 180 day intervals. The lambs' diet before weaning was supplemented with a commercial mixture of non-GM steam-rolled cereals and faba bean (crude protein: 210.3 g/kg; crude fibre 57.6 g/kg), which progressively increased from 50 (30 days of age) to 300 g/day (60 days of age).

At weaning, 46 ewes and their lambs (14, 20 and 12 after the first, second and third lambing, respectively), equally distributed between the 2 experimental groups, were slaughtered approximately 12 h after feeding. Samples of ruminal contents, venous blood, gastrointestinal organs and muscles were collected and processed for chemical analyses and recombinant DNA fragment detection.

The present study was carried out in accordance with the guidelines of animal care and experimentation of the Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche.

2.2. Welfare status

Animals were periodically subjected to clinical examinations to assess cardiorespiratory and reproductive functions as well as motory, sensory and/or reflex changes.

Haematological analyses were conducted at 4-month intervals, starting 4 months before the end of the PRE period. Blood samples were taken in K3 EDTA vacutainer tubes to analyse the common haematological profile by a semi-automatic electric impedance analyser (Hemacomp 10, SEAC, Florence, Italy). Leukogram was determined through microscopic observation of blood smears stained using the May-Grunwald Giemsa technique. For the determination of biochemistry parameters, blood was collected in heparinized tubes. Plasma samples were stored at -20°C prior to the following analyses: total protein, aspartate aminotransferase, alanine amino transferase, gamma glutamyltransferase, alkaline phosphatase, albumin, bilirubin, creatin kinase, glucose, calcium, chloride, potassium, sodium, urea nitrogen, creatinine, iron, magnesium, and phosphorus. Analyses were performed using standard enzymatic methods (Boehringer Mannheim GmbH, Mannheim, Germany) with a fully automated Hitachi 704 analyser (Hitachi Ltd., Tokyo, Japan).

Analyses for oxidative stress markers were conducted, as for haematological indexes, at 4-month intervals. Blood samples were taken in EDTA vacutainer tubes. The malondialdehyde content, end product of lipid peroxidation, was assayed in plasma by separating

the malondialdehyde/thiobarbituric acid adduct by reverse-phase HPLC and quantified by using fluorescence detection (Draper et al., 1993). Total scavenger capacity of plasma was evaluated by ABTS (2,2'-azinobis-3-ethyl-benzothiazoline-6-sulphonic acid) test according to Rice-Evans and Miller (1994). Antioxidant defences were assessed in vitro by treating erythrocytes with 2 mmol/l *tert*-butyl-hydroperoxide incubation; BHT (0.1 mmol/l) was used to interrupt the peroxidative chain reaction at 1, 2, 5, and 10 min and the percentage of methaemoglobin, as haemoglobin oxidative product, was calculated (Winterbourn, 1990).

2.3. Immune response

The immunological response to vaccination against *Clostridium* spp. and *Salmonella abortus ovis* was evaluated by an optimized indirect ELISA assay. Two vaccine preparations without adjuvants were used. The first was composed of formaldehyde-inactivated (0.05% v/v) *C. perfringens* Type A, B, C and D, *C. septicum*, *C. chauvoei*, and *C. novy* A, at a total concentration of 3×10^9 CFU/ml with 20 LD₅₀ mouse/ml toxins of *C. perfringens* and 2000 LD₅₀ Guinea pig/ml toxins of *C. septicum*. The second one consisted of *S. abortus ovis* bacteria inactivated with formaldehyde (0.05% v/v) at a concentration of 1.8×10^9 CFU/ml. The time schedule of the experiment is reported in Fig. 1.

Blood samples were collected at 4-month periods from 7 animals per experimental group for cell-mediated immunity evaluation (Fig. 1). Peripheral blood mononuclear cells (PBMC) were obtained from EDTA blood by separating cells on Histopaque (Sigma, St. Louis, MO, USA). Monocytes were separated from lymphocytes by adherence overnight to plastic dishes and distributed into 24-well plates (10×10^6 /well) for killing assessment or in trak tubes (5×10^6 /tube) for phagocytosis study and cultured at 37°C in 5% CO₂ for 10 days, at the end of which the monocytes had matured into macrophages (M/M). Phagocytic activity of M/M was assessed by using both latex fluorescent microparticles (Molecular Probes, Inc., Eugene, USA) and bacterial suspensions of *S. abortus ovis* (Antonelli et al., 1997). Latex beads and bacterial cells were pre-opsonized in RPMI medium, 10%-supplemented with a pool of sheep sera, by incubation at 37°C for 30 min. Bacteria and beads were sonicated to disperse clumps and added to cellular monolayers, maintaining a 1:50 effector/target ratio. After 1 h incubation at 37°C , 5% CO₂, the cultures were washed 3 times with HBSS to remove any extracellular nonphagocytosed microorganism or particle. Phagocytosis and the phagocytic index were

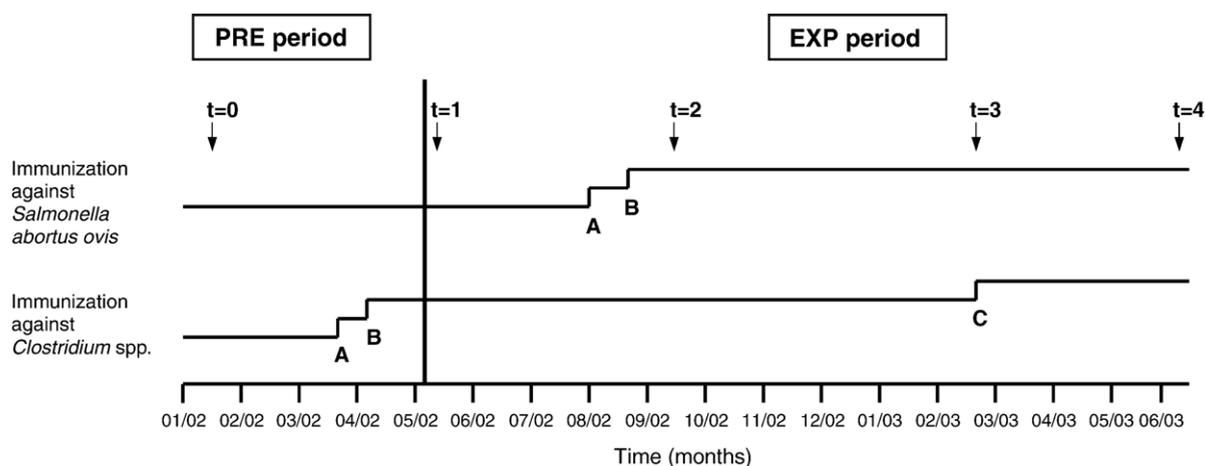


Fig. 1. Time schedule for the accomplishment of the different events of the experiment, referred as months of the year. Vaccination for *Clostridium* spp. and *Salmonella abortus ovis* are indicated with elevated lines, while sera samplings are indicated with arrowheads. PRE period=pre-experimental period, EXP period=experimental period with animals divided in 2 groups (control and GM-maize fed), A=I immunization – first dose, B=I immunization – booster dose, C=II immunization.

evaluated as previously reported (Miliotis, 1991; Roilides et al., 1990).

Intracellular killing was performed with *S. abortus ovis* grown under starvation conditions, preopsonized as described for phagocytosis tests and added to cellular monolayers at a 1/50 ratio. After 30 min incubation, to allow bacterial internalization by macrophages, the cultures were washed three times to eliminate extracellular microorganisms and processed as reported elsewhere (Roilides et al., 1990). The bactericidal activity was calculated as the percentage of killed intracellular bacteria after 60 min, with respect to time zero.

The proliferation capacity of lymphocytes was assessed by monitoring the incorporation of [3 H]thymidine into newly synthesized DNA after concanavalin A, phytohaemagglutinin, and pokeweed stimulation (Chiaradia et al., 2002).

2.4. Ruminal metabolism and microbial population

Samples obtained from the slaughtered ewes ($n=46$) were used. Immediately after slaughter, whole ruminal contents were mixed, the pH was measured and composite samples were collected for microbiological and chemical analyses. A sub-sample of ruminal contents (50 g) was anaerobically processed for total, amylolytic and cellulolytic bacterial numbers evaluation according to the MPN procedure (Dehority, 1969; Dehority et al., 1989). The amylolytic and cellulolytic sub-populations were chosen because adherent to feed particles and more likely to get in close proximity with the plant DNA. A second sub-sample of ruminal contents (50 ml) was

processed for generic counts of protozoa (Dehority, 1984). A further sub-sample of ruminal contents was filtered through 4 layers of cheesecloth and then processed for chemical analyses as follows:

- Volatile fatty acids (VFA)—A gas chromatograph (Carlo Erba GC 6000 Vega Series 2, Milan, Italy), equipped with a flame ionization detector, was used. After filtration and acidic precipitation, samples were centrifuged and the liquid fraction was analysed in a $2\text{ m} \times 2\text{ mm}$ glass column i.d. packed with Carbograph 1 80–120 mesh, AL acid washed +4% Carbowax 20 M (Laboratori Analitici di Ricerca Associati, Rome, Italy).
- $\text{NH}_3\text{-N}$ —After filtration of ruminal contents and precipitation with 1 N cloridric acid, $\text{NH}_3\text{-N}$ content was evaluated in the supernatant fluid using a colorimetric assay (Beecher and Whitten, 1970).
- Lactate—Filtered ruminal contents were mixed with 8% perchloric acid in a 5:1 ratio and centrifuged at $2000 \times g$ for 15 min at 4°C . Analyses on the supernatant fluid were carried out following a colorimetric method (L-Lactate PAP, Randox Laboratories Ltd., Co Antrim, UK).

2.5. Histological analyses

Twenty-six (13 C+13 T) ewes and 32 (16 C+16 T) lambs, distributed between first and second lambing, were used. Samples of liver, spleen, pancreas, duodenum, cecal appendix, mesenteric lymph nodes, rumen and abomasum were fixed in a 10% neutral buffered

formalin solution and routinely embedded in paraffin wax. For histological evaluations, 4 µm-thick sections were stained with haematoxylin–eosin and observed with an Olympus BX51 (Hamburg, Germany) light microscope.

In addition, the ewes' ruminal epithelium from the dorsal sac was submitted to immunohistochemical procedures using a monoclonal antibody against the Ki-67 nuclear protein, a proliferating marker (Gerdes et al., 1984). Four µm-thick tissue sections were deparaffinised, rehydrated, treated with 0.3% H₂O₂–methanol (v/v) and then microwaved in 10 mmol/l citrate buffer. The antibody for Ki-67 protein (Clone MIB1, Dakocytomation, Denmark) was incubated with the sections overnight at 4 °C. Detection of immunoreactive staining was carried out by streptavidin–biotinylated–peroxidase complex using the LSAB kit (Dako, Copenhagen, Denmark). Finally, 3,3-diaminobenzidine was used as the chromogen and Mayer's haematoxylin for counterstaining. Positive controls consisted of normal lymph node tissue, while negative controls were obtained by omitting the primary antibody. Scoring of immuno-reactivity was assessed by counting the total number of positively stained basal cell nuclei (40×) in 5 randomly selected sections per sample and values were expressed as the number of labelled cells/mm².

For transmission electron microscopy, samples of liver and pancreas from the lambs and ewes were processed (Malatesta et al., 2003). Ultrathin sections were stained with the EDTA technique (Bernhard, 1969) to visualize the ribonucleoprotein structural constituents and observed in a Zeiss EM 902 (Thornwood, NY, USA) electron microscope.

2.6. Meat quality

Samples of meat from 20 sheep (10 T and 10 C) and 14 lambs (7 T and 7 C), distributed between first and second lambing, were analysed 24 h following slaughtering. One hundred and fifty gram portions of *Longissimus dorsi* between the 10th and the 15th rib and *Biceps femoris* of each carcass were sampled and chilled for 24 h before analysis. Analyses for moisture, fat, protein and ash content (AOAC, 2000) and for pH, water loss by cooking and tenderness (AMSA, 1995) were carried out for each muscle sample.

2.7. Transgene detection

Transgene detection was performed on: (a) blood collected periodically from 20 ewes, at 4-month intervals, beginning 18 months after the start of the EXP period; (b) blood, liver, spleen, pancreas, jejunum and rumen sampled at slaughter from 12 ewes; (c) ruminal fluid

collected from 24 ewes at slaughter and ruminal bacteria grown in liquid anaerobic media as described above. Samples were equally distributed between the group T and C and between first and second lambing.

- a) *Animal tissues*—Tissue samples and blood with EDTA were quick-frozen and stored at –20 °C until analysis. Total DNA was extracted using a commercial kit for tissue and blood extraction (QIAamp® DNA Mini Kit, QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. The yield and purity of the extracted DNA were determined following measurement of UV absorption at 260 and 280 nm. DNA extraction was shown using primers for amelogenin gene amplification. During each extraction procedure an extraction control (duplicate) was also carried out using only the same buffers/reagents. A pair of primers was used in PCR detection of transgene sequences: Cry03 (5'-CTCTCGCCGTTTCATGTCGGT-3') and Cry04 (5'-GGTCAGGCTCAGGCTGATGT-3') (GenBank accession no. I41419). These primers amplify the last 73 bp region of the CDPK promoter and the first 138 bp of the N-terminus of the CryIA(b) (Hupfer et al., 1998). When assembling each PCR plate, a positive (5 µl of Bt176 maize) and a negative control (5 µl of water) were routinely set up. PCR was performed with a Thermal Mastercycler (Eppendorf) in a 25 µl final volume. Five microliters of each DNA sample were added to 20 µl containing 1× PCR reaction buffer, 1.5 mmol/l MgCl₂, 200 µmol/l dNTP (each), 0.6 µmol/l of each primer and 1 unit of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster, CA, USA). The amplification for transgene sequences was carried out under the following cycling conditions: 30 s at 95 °C, 30 s at 63 °C, and 30 s at 72 °C for 38 cycles and a final extension at 72 °C for 6 min. Amplification products (25 µl) were electrophoresed in 2% (w/v) Tris-borate-EDTA agarose gel stained with ethidium bromide.
- b) *Ruminal fluid and bacteria*—Total DNA was extracted from the ruminal fluid as well as from the microorganisms grown in liquid media for total, amylolytic and cellulolytic bacterial species using Dynabeads® DNA DIRECT™ (DynaL Biotech, Oslo, Norway), following the manufacturer's instruction. PCR analyses were performed to reveal the presence or absence of two different fragments of the delta-endotoxin CryIA(b) using 2 pairs of primers: Cry1 (5'-ACCATCAACAGCCGCTACAACGACC-3') and Cry2 (5'-TGGGGAACAGGCTCACGATGTCAG-3'), designed to amplify a 184 bp fragment of transgene sequence (Hurst et al., 1995), and the

Cry03/Cry04 described above (Hupfer et al., 1998). To exclude false positive results, 2 intrinsic maize genes were assayed as a control for maize DNA contamination in the samples, using the primer pair IVR1/2 (IVR1 5'-CCGCTGTATCACAAGGGCTGGTACC-3'; IVR2 5'-GGAGCCCGTGTAGAGCATGACGATC-3') as primer for the maize invertase gene (Chiter et al., 2000) and the F1/B1 (F1 5'-TACGGCACAAGAAGTTCGAGAC-3'; B1 5'-AACATGGCAGCTTCCACTGG-3') designed to amplify a 200 bp fragment of the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit, which is normally present in plant DNA (Hurst et al., 1995). The universal bacterial primer set 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1525r (5'-AAGGAGGTGWTCARCC-3') were used for the amplification of a 1500 bp of the bacterial 16S rDNA to ensure the quality and suitability of the DNA extracts for PCR.

All PCR were performed with a GeneAmp PCR system 2700 thermocycler (Applied Biosystems, Foster, CA, USA) in a 25 μ l final volume. Four microliters of each DNA sample was added to 21 μ l containing 1 \times PCR reaction buffer, 2.5 mmol/l MgCl₂, 200 μ mol/l (each) dNTP, 400 nmol/l of each primer, bovine serum albumin 200 ng/ μ l, and 0.625 U/reaction of Hot-Rescue DNA Polymerase (DIATHEVA s.r.l, Fano, Italy). The amplifications for transgene sequences and maize endogenous genes were carried out under the same cycling conditions: after 1 cycle at 95 °C for 10 min a 3-step PCR procedure, consisting of 20 s at 95 °C, 15 s at 64 °C and 20 s at 68 °C for 50 cycles and a final extension at 68 °C for 5 min, was used. Finally, 1/5 of the PCR mixture was

subjected to an ulterior 50 PCR cycles. For the bacterial 16S rDNA amplification, the temperature program consisted of denaturation at 95 °C for 10 min, followed by 35 cycles consisting of 95 °C for 20 s, annealing at 60 °C for 15 s, and extension at 68 °C for 80 s and a final extension at 72 °C for 7 min. For each amplification, 10-fold dilutions (1:8 and 1:80 from extracted DNA) were tested to ensure the absence of PCR-inhibiting contaminants in the samples.

When assembling each PCR plate, a positive control (GM maize standard) and a negative control (water) were routinely set up. Amplification products (25 μ l) were electrophoresed in 2% (w/v) Tris-borate-EDTA agarose gel stained with ethidium bromide. All the PCR products were cloned into the pCR 2.1 plasmid vector (Invitrogen, San Diego, CA), following the manufacturer's instructions and directly sequenced. The limit of detection of the PCR assay established, using negative control DNA, spiked with known quantities of plasmids containing the transgene or endogenous maize sequences, was 2 molecules (Fig. 2).

2.8. Statistical analysis

Statistical analyses were performed using the Statistical Analysis Systems statistical software package version 8.2 (SAS, 2001). Performance, reproductive traits and ruminal characteristics were evaluated by ANOVA using the GLM procedure. The main independent factors were dietary treatment (C and T), year of birth and sampling period. Bacterial numbers were log-transformed prior to analysis to satisfy the requirement for constant variance. For BW and BCS data of the adult animals, values obtained during the PRE period were used as a covariate.

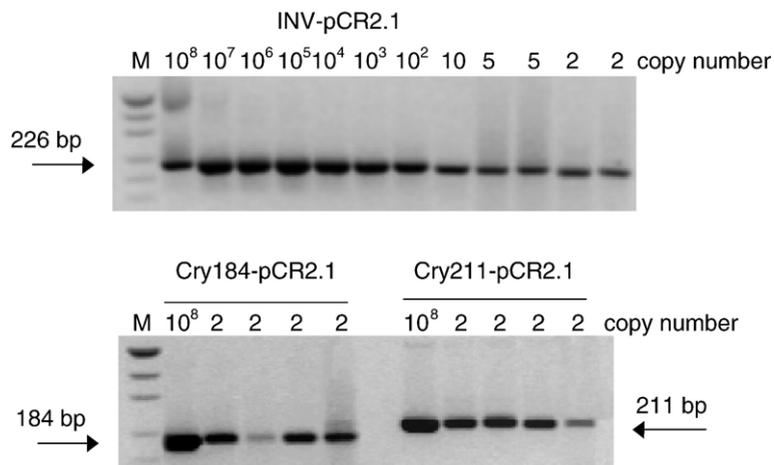


Fig. 2. Sensitivity assay. Negative control DNA was spiked with known quantities of each plasmid and subjected to PCR analyses. INV=maize invertase gene, Cry=Bt176 maize transgene sequences.

The lambs' BW was analysed by taking into consideration sex, dietary treatment (C and T) and year as fixed factors and BW at birth as a covariate. Interactions between factors were included if found significant at ANOVA.

A repeated measures design with sheep within diet as the error term, and diet and period as main effects, was carried out to assess the dietary effect on haematological parameters and oxidative stress markers. Multiple comparisons among treatments were by the Tukey test if significance had been indicated by the analysis of variance. As for the immune response to vaccination, absorbance values obtained for 1:100 serum dilution for *Clostridium* spp. ELISA assay and 1:500 for *S. abortus ovis* assay were used in an ANOVA model for repeated measures. Post hoc comparisons were performed using the Tukey least significant difference test. Cell-mediated immunity data were statistically analysed by the *t* test in case of equal variance assumption.

The histological data were analysed by an ANOVA model which included animal category (ewe and lamb) and dietary treatment (C and T) as main factors.

Factors considered in the ANOVA model for meat quality traits were dietary treatment (C and T), type of muscle (*L. dorsi* and *B. femoris*) and animal category (ewe and lamb). Interactions between factors were not included in the model because they were found not significant at ANOVA.

3. Results

3.1. Animals and feeds

The data in Table 1 indicate that the chemical composition and the ruminal degradability of the 2 maize hybrids were similar. Minor differences between lines were related to neutral detergent fibre, ether extracts,

Table 2
Effect of dietary treatment and sampling period on haematological parameters in ewes

Item	Dietary treatment				P-value		
	GM	SEM	Control	SEM	Dietary treatment	SP	INT
Albumin (mmol/l)	36.53	0.51	35.64	0.54	0.239	<0.001	0.006
Alk phos (U/l)	242.66	10.16	230.64	11.03	0.428	<0.001	0.629
ALT (U/l)	30.10	0.68	28.98	0.75	0.053	<0.001	0.040
AST (U/l)	111.72	2.45	107.05	2.64	0.202	<0.001	0.676
Bilirubin (µmol/l)	3.04	0.16	3.22	0.16	0.431	<0.001	0.177
Glucose (mmol/l)	2.47	0.05	2.42	0.05	0.545	<0.001	0.640
Urea nitrogen (mmol/l)	6.36	0.17	6.43	0.18	0.794	<0.001	<0.001
Creatinine (µmol/l)	90.54	1.24	91.16	1.36	0.735	<0.001	0.973
Total protein (g/l)	59.59	0.60	59.07	0.64	0.557	<0.001	0.211
GGT (U/l)	44.49	0.91	40.19	0.98	0.003	<0.001	0.440
Calcium (mmol/l)	2.46	0.03	2.42	0.03	0.307	<0.001	0.399
Chloride (mmol/l)	104.07	0.66	103.11	0.70	0.324	<0.001	0.028
Potassium (mmol/l)	5.98 ^a	0.18	6.68 ^b	0.20	0.012	<0.001	0.074
Sodium (mmol/l)	154.47	1.48	153.31	1.67	0.605	<0.001	<0.001
Iron (mmol/l)	24.49	0.39	23.79	0.42	0.236	<0.001	0.203
Magnesium (mmol/l)	0.91 ^a	0.03	0.76 ^b	0.03	<0.001	<0.001	<0.001
Phosphorus (mmol/l)	1.73	0.04	1.75	0.05	0.848	<0.001	0.260
Haematocrit (%)	31.38	0.25	31.00	0.26	0.290	<0.001	0.023
Haemoglobin (g/dl)	10.92 ^a	0.11	10.46 ^b	0.11	0.005	<0.001	0.067
MCH (pg)	9.57	0.10	9.47	0.11	0.518	<0.001	0.084
MCHC (%)	30.89	0.19	31.16	0.19	0.323	<0.001	0.179
MCV (fl)	31.54	0.23	30.96	0.24	0.090	<0.001	<0.001
Platelet (10 ³ /µl)	195.44	5.77	179.08	6.18	0.060	<0.001	0.575
Basophil (%)	0.10	0.03	0.10	0.03	0.980	0.119	0.185
Eosinophil (%)	3.95	0.17	4.15	0.17	0.412	<0.001	0.067
Lymphocyte (%)	52.25	0.44	51.60	0.46	0.313	<0.001	0.030
Monocyte (%)	4.74	0.18	4.60	0.19	0.597	<0.001	0.322
Neutrophil (%)	41.95	0.54	40.56	0.57	0.085	<0.001	<0.001
RBC (10 ⁶ /ml)	11.33	0.12	11.22	0.13	0.564	<0.001	0.352
WBC (10 ³ /µl)	12.91	0.35	13.89	0.36	0.056	<0.001	0.002

SP=sampling period; INT=interaction between treatment and SP; Alk phos: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma glutamyltransferase; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular volume; RBC: red blood cells; WBC: white blood cells.

^{a,b}Means values ($n=327$) within a row with unlike superscript letters were significantly different ($P<0.05$).

copper and iron contents. No differences were detected between the two groups of ewes, in all generations studied, as far as BW, BCS, fertility, and twin rate were concerned (62 (± 1.0) vs. 62 (± 1.0) kg, 2.8 (± 0.04) vs. 2.8 (± 0.05), 79 (± 1.6) vs. 72 (± 1.9) %, and 25 (± 1.2) vs. 24 (± 1.1) % for T and C ewes, respectively). The lambs' BW at birth, mortality and daily weight gain up to weaning (5 (± 0.1) vs. 5 (± 0.1) kg, 4 (± 0.4) vs. 4 (± 0.5) %, and 288 (± 5.3) vs. 293 (± 4.8) g, for T and C lambs, respectively) were not affected by dietary treatment.

3.2. Welfare status

Clinical examinations did not detect any alterations of cardiorespiratory and reproductive functions, or changes in motory, sensory and/or reflex behaviour, associated to dietary treatment.

Analysis of variance carried out on haematological findings and least square means of dietary treatments are presented in Table 2. All values, except for the percentage of basophil, were affected by the sampling period. Four (gamma glutamyltransferase, potassium, magnesium, and haemoglobin) of the 30 haematological traits evaluated were affected by diet.

Values recorded for malondialdehyde and in vitro oxidative stress did not show any significant effect due to dietary treatment. The sampling period was able to affect the oxidation of Hb to MetHb by *t*-BuOOH. Values obtained during lactation (second sampling period) were lower than those recorded at the end of the experiment.

Table 3
Statistical analysis of ELISA absorbance values

Phase of analysis*		<i>Clostridium</i> spp.		<i>Salmonella abortus ovis</i>	
		Mean	SEM	Mean	SEM
<i>t</i> =0	C	0.274	0.022	0.07809	0.014
	GM	0.285	0.019	0.07508	0.013
<i>t</i> =1	C	0.955 ^a	0.068	–	–
	GM	1.151 ^b	0.059	–	–
<i>t</i> =2	C	0.938	0.055	1.38	0.053
	GM	1.007	0.048	1.517	0.046
<i>t</i> =3	C	0.714	0.053	0.525 ^a	0.054
	GM	0.839	0.046	0.729 ^b	0.047
<i>t</i> =4	C	1.074	0.066	0.466 ^a	0.045
	GM	1.119	0.058	0.639 ^b	0.039

C: Control ewes; GM: GM maize-fed ewes.

^{a,b}Within a phase of analysis, means values ($n=70$) in a column with unlike superscript letters were significantly different ($P<0.01$).

*Phases of analysis are: *t*=0, 3 months before immunization; *t*=1, first immunization against *Clostridium* spp.; *t*=2, immunization against *Salmonella abortus ovis*; *t*=3, second immunization against *Clostridium* spp.; *t*=4, 4 months after *t*=3.

Table 4
Rumen microbial numbers and metabolism as affected by dietary treatment

	Control	GM	SEM
Bacteria (n/ml, log)			
Total	9.03	9.05	0.08
Amylolytic	8.76	8.96	0.11
Cellulolytic	7.75	8.04	0.11
Protozoa			
Total (n/ml $\times 10^4$)	16.06	14.65	1.70
<i>Entodinium</i> (%)	84.72	84.55	1.64
<i>Diplodiniinae</i> (%)	8.45	8.50	0.82
<i>Isotricha</i> (%)	1.87	2.17	0.40
<i>Dasytricha</i> (%)	3.08	2.58	0.62
<i>Ophryoscolex</i> (%)	1.88	2.20	0.72
pH	6.57	6.54	0.14
NH ₃ -N (mg/dl)	14.96	13.48	1.15
Lactate (mmol/l)	0.32	0.45	0.05
Volatile fatty acids			
Acetate (%)	58	57	0.9
Propionate (%)	23	24	1.0
Butyrate (%)	19	19	1.3

Values are means of 46 measurements. GM: GM maize-fed ewes.

3.3. Immune response

A low titer of anti-*Clostridium* spp. and anti-*S. abortus ovis* antibodies was detected by ELISA assays in the pre-immune sera (*t*=0) of sheep from both groups T and C. The statistical analysis showed that the dietary treatment played a highly significant role in the variations of anti-*S. abortus ovis* titer following vaccination ($P=0.008$). With respect to the anti-*Clostridium* spp. titer, a quasi-significant role of the dietary treatment ($P=0.077$) was detected. For both types of investigations, the mean absorbance values were higher in T animals in every phase of the study. In spite of this, the confidence intervals for differences of means showed significant differences only in *t*=1 sampling for clostridiosis and in *t*=3 and *t*=4 sampling for salmonellosis vaccination (Table 3).

As for cell-mediated immunity, M/M yields obtained at the established periods (4th, 8th, 12th and 16th month) from peripheral blood of both groups C and T were 2.20 (± 0.3) % of the total mononucleated blood cells (Knoll, 2000). The number of phagocytosed bacteria per cell ranged from 3.29 (± 1.68) to 5.67 (± 0.11) for C sheep and from 3.83 (± 2.58) to 6.07 (± 0.56) for T sheep. Phagocytosis of latex beads resulted to be in a range from 5.82 (± 1.78) to 10.9 (± 3.3) particles for C and from 5.79 (± 1.13) to 10.8 (± 5.63) for T samples. Intracellular killing of *S. abortus ovis* resulted to be 18.53% in C sheep vs. 19.6% in T. However, when the data of the phagocytic index and killing obtained from C

and T samples at any sampling time were compared, the differences resulted to be not significant ($P>0.05$).

The response of T and B peripheral lymphocytes to 66 h of stimulation with ConA, PKW, or PHA was unaffected by diet (5 animals per group) and no differences were observed among the periods considered. The percentage of proliferation of the stimulated vs. resting (without mitogen) cells was extremely variable, ranging from 500 to up to 2000.

3.4. Ruminal metabolism and microbial population

Total, amylolytic and cellulolytic bacterial concentrations, as well as protozoal numbers and composition, did not differ between groups (Table 4). The ruminal metabolism indicators taken into consideration (pH, VFA, $\text{NH}_3\text{-N}$, and lactate) were not affected by dietary treatment.

3.5. Histological analyses

Light microscopy observations of liver, spleen, pancreas, duodenum, cecal appendix, mesenteric lymph

nodes, rumen and abomasum sections did not reveal histological differences between groups C and T, for both sheep and lambs.

Immunocytochemical analyses of ruminal epithelium by Ki-67 staining provided evidence of the proliferative activation of basal cells in T ewes: the values of anti-Ki-67 labelled cells/ mm^2 of ruminal epithelium were significantly higher in T animals than in the controls at all ages ($P<0.001$) (Fig. 3). Moreover, preliminary EM analyses of hepatocytes and pancreatic acinar cells revealed smaller, irregularly shaped cell nuclei containing increased amounts of heterochromatin and perichromatin granules (ribonucleoprotein structural components involved in transport and/or storage of already spliced pre-mRNA) (Fakan et al., 1984) in T lambs (Fig. 3).

3.6. Meat quality

Except for protein content (C: 20.3 (± 0.2); T: 19.7 (± 0.2); $P<0.05$) and water loss by cooking (C: 37.9 (± 0.8); T: 40.3 (± 0.7); $P<0.05$), the parameters examined were not affected by the dietary treatment. Other factors

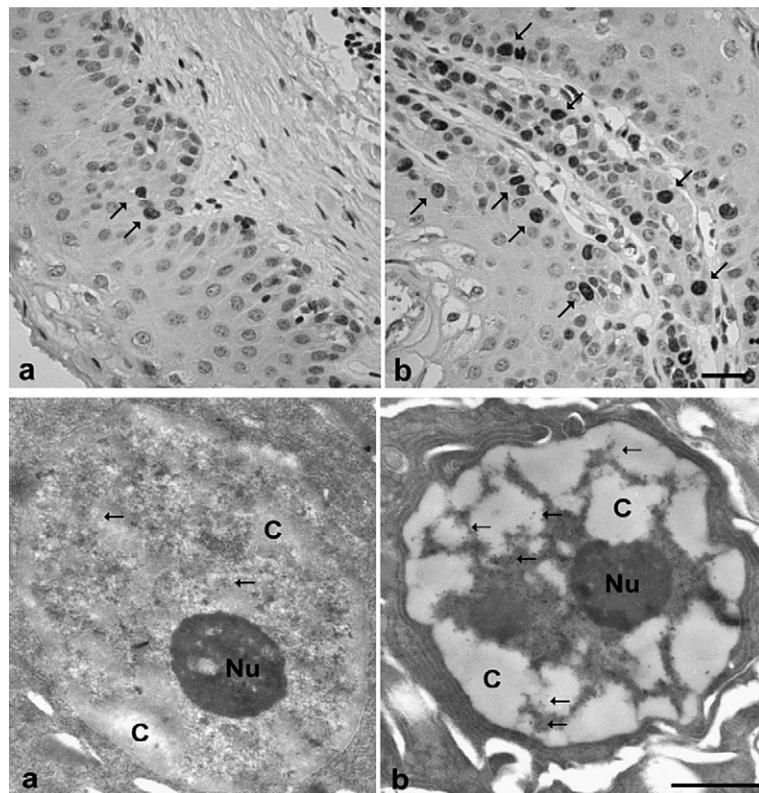


Fig. 3. Top: immunocytochemical detection of Ki-67 protein on control (a) and GM maize-fed (b) ewes. The number of immunolabelled cell nuclei (arrows) is markedly higher in the samples from GM maize-fed animals; bar = 10 μm . Bottom: ultrastructural features of pancreatic acinar cell nuclei from control (a) and GM maize-fed (b) 3 month-old lambs. The cell nuclei from GM-fed animals show smaller size and larger amounts of heterochromatin (C) and perichromatin granules (arrows). Nu = nucleolus, bar = 1 μm .

such as type of muscle (*L. dorsi/B. femoris*) and animal category (ewe/lamb) were found to be significant at ANOVA (data not shown).

3.7. Transgene detection

No transgenic DNA was detected in any of the tissue and blood samples taken from both C and T groups. While bacterial DNA was detected in all ruminal fluid and bacterial culture samples at all time points, neither transgenic maize nor intrinsic maize fragments were detected.

4. Discussion

The differences among the maize hybrids used in the study were so minor that they are unlikely to be of any biological significance. Trace mineral content of the two maize lines was possibly influenced by agronomic traits and geographical origin (Ipharraguerre et al., 2003). The relative lack of chemical composition differences between the Bt and non-Bt hybrid agrees with literature data (Sidhu et al., 2000; Folmer et al., 2002). On the contrary, the crude protein content of the Rh208 maize hybrid was found to be higher than that of its near isogenic line (Rh289) in a study where maize silage composition was examined (Barrière et al., 2001).

In this study, the animals' BW and BCS were not affected by treatment in all generations studied and were similar to that indicated in the literature for animals of the same genotype (Valusso et al., 1998) or physiological state (Bocquier et al., 1988). Other authors have shown the nutritional equivalence of transgenic maize fed to monogastric and polygastric animals, but there is a lack of information concerning the ovine species. Moreover, no multi-generational studies have been published so far. The absence of differences between transgenic maize Bt176 (Rh208Bt) and its near isogenic hybrid (Rh208), fed as silage to Texel ewes, was demonstrated as far as neutral detergent fibre, crude fibre and organic matter digestibility was concerned (Barrière et al., 2001). Similarly, no differences in either digestibility or energy content between Bt-maize and its conventional counterpart, used in a series of trials with poultry, pigs and wethers, were found (Aulrich et al., 2001).

Most of the haematological indexes examined in this study fell within the range normally observed in healthy ewes of similar age (Kaneko, 1989). At present, there is no plausible explanation for the differences found between the groups regarding gamma glutamyltransferase, potassium, magnesium, and haemoglobin. Other authors have reported no differences between the haematologi-

cal and clinical serum parameters of rats fed with GM glyphosate-tolerant soybean meal and controls (Zhu et al., 2004). No effects on haematology and blood biochemical indexes between rats or mice, fed either GM sweet pepper or tomato diets, were found when compared with those fed the non-GM diets (Zhang-Liang et al., 2003). With reference to malondialdehyde and oxidative stress in vitro, results are indicative of a lack of effect by dietary treatment on the antioxidant defences of the animals. No data are available in the literature on this subject. Data from the immune response study indicated that GM maize did not impair two important functions of M/M, such as phagocytosis and intracellular killing. These findings are in agreement with the results obtained regarding the lymphocyte proliferative capacity. The immune response against *S. abortus-ovis* was more efficient in T animals; however, more extensive research is needed in order to reach definite conclusions on this aspect.

Values recorded for ruminal pH, VFA, $\text{NH}_3\text{-N}$, and lactate in samples obtained from both C and T groups were within the physiological ranges and compatible with the diet being fed at slaughter (third month of lactation, characterized by moderate supplementation with maize grain, i.e. 27% of total DM intake). Similar results were obtained with dairy cows fed glyphosate-tolerant soybean (Hammond et al., 1996). The lack of differences observed in protozoal and bacterial populations is in agreement with results indicated in previous studies (Einspanier et al., 2004). In a 4-week feeding experiment, during which Bt176 maize was fed to cows, no significant variations in the microbial population sampled from different organs of the gastrointestinal tract were reported.

The diet containing GM maize did not seem to affect the histological features of the examined tissues. However, cytochemical analyses revealed that functional modifications took place without inducing evident histological alterations. In fact, the basal cells of the ruminal epithelium of T ewes showed a higher expression of Ki-67 in comparison to controls. It is known that sheep rumen is sensitive to age and diet (Lane et al., 2000). The higher expression of Ki-67 in the ruminal cells was possibly induced by some components of the GM maize; it has been reported that Cry1 is able to bind the intestinal mucosal surface, influencing some epithelial cell functions (Vazquez-Padron et al., 2000).

The cell nuclear modifications observed in the pancreatic acinar cells and hepatocytes could also be due to some direct or indirect effect of Bt 176 maize. The changes in chromatin arrangement and ribonucleoprotein constituents observed in T animals are suggestive of functional modifications concerning transcriptional/

post-transcriptional events. However, the significance of this phenomenon is unclear and is presently under investigation. Fine structural modifications of cellular components in relation to GM feed intake have already been described, although without any consequences on organ functions or animal health (Fares and El-Sayed, 1998; Malatesta et al., 2002).

There are no available explanations that could support the effects of diet on the meat chemical traits observed in the present study and no data concerning the effects of GM maize on sheep meat quality traits are available in the scientific literature. Stanford et al. (2003), in a study involving Arcott lambs slaughtered at 45 kg BW, did not observe changes in carcass composition and characteristics except for the moisture content of loin tissue, which was higher in the group fed glyphosate-tolerant (Roundup Ready®) rapeseed compared to the controls. As for studies conducted with monogastrics, Hyun et al. (2004) compared glyphosate-tolerant Roundup Ready® (event NK603) corn with its nontransgenic genetically similar control corn (RX670) in an experiment carried out with barrows and gilts and found no differences in muscle quality traits, composition measurements and *L. dorsi* colour and marbling scores. In a 38-d feeding trial with broiler chickens, in which Bt176 maize was compared with a non-Bt counterpart, minor differences on feed conversion ratio, breast skin, and *Pectoralis minor* yield, not directly associated to dietary treatment, were reported (Brake and Vlachos, 1998).

The present transgene detection study only focused on the presence or absence of the amplicon resulting from the last 73 bp of the CDPK promoter and the first 138 bp of the N-terminus of the CryIA(b) gene. These results are in agreement with most data from the literature, that do not report the presence of transgenic DNA fragments in tissues of either mono- and polygastric animals. In contrast, Mazza et al. (2005) detected a small fragment of the CryIA(b) gene in blood, liver, spleen and kidney of piglets fed for 35 day with a diet containing GM (MON810) maize. Sharma et al. (2006) reported the presence of fragments of the *cp4 epsps* transgene in the gastrointestinal tissues of sheep and pigs fed Roundup Ready® rapeseed. It has been assumed that, when compared with non-GM plant DNA, the exposure to DNA of GMO material is negligible. Most plant DNA is likely to be degraded by DNase activity within the gastrointestinal tract. Furthermore, low pH conditions of the abomasum contribute to denaturize most adenine and guanine bases from naked DNA fragments (Beever and Kemp, 2000).

Although it is not possible to conclusively prove that transgene sequences were not transferred to the ruminal

bacterial species, uptake of transgenic DNA fragments is probably precluded or time-limited by rapid degradation of plant DNA upon plant cell lysis (Sharma et al., 2004). Plasmid DNA exposed to ovine saliva in vitro was able to transform competent *Escherichia coli* cells to ampicillin resistance even after 24 h (Duggan et al., 2000). In contrast, free maize chromosomal DNA was rapidly destroyed within 1 min of incubation in ruminal fluid or silage effluent (Duggan et al., 2000). Stability of DNA fragments in the gastrointestinal tract can be affected by the type of feed being used, with processed feedstuffs being more degradable than the untreated grains (Chiter et al., 2000). Fragments of the Rubisco gene were found in bovine digesta samples obtained from the rumen and duodenum and from faeces and milk, but single-copy genes such as *cp4epsps* and *Cry1A(b)*, from GM-soybean and maize, were only detected in the solid phase of ruminal and duodenal digesta (Phipps et al., 2003). These data are consistent with the results obtained in the current study, where no transgenic DNA was detected in rumen fluid. To our knowledge, the only evidence of gene transfer from GM-soya to gut microorganisms was reported in mixed cultures of human intestinal bacteria (Netherwood et al., 2005).

5. Conclusions

Overall welfare status indexes could confirm substantial equivalence with conventional hybrid as far as nutritional and safety characteristics are concerned. These results were partly expected, considering the genetic transformation involved, and in agreement with previous studies conducted in other monogastric and polygastric species (Aumaitre et al., 2002).

Our findings confirm that transgenes from maize are unlikely to survive in the ruminant prestomachs for 12 h or longer and provide a source of transforming DNA fragments for microorganisms. No transgenic DNA was found in the animals' tissues, which supports the opinion that intact genes from foods are unlikely to be absorbed through the gastrointestinal tract and integrated into the DNA of eukaryotic cells (Schubbert et al., 1997, 1998; Hohlweg and Doerfler, 2001; Einspanier et al., 2001).

However, more extensive research is needed to clarify some of the metabolic aspects under investigation, to improve safety assessments for GM organisms used for feed and food. In particular, the cytochemical modifications of the gastrointestinal organs and the immune response mechanisms that take place in GM-fed animals should deserve special emphasis and priority in future investigations.

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