

## Transgenic maize in the presence of ampicillin modifies the metabolic profile and microbial population structure of bovine rumen fluid *in vitro*

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Recently, transgenic crops have been considered as possible donors of transgenes that could be taken up by micro-organisms under appropriate conditions. In an *in vitro* rumen simulation system, effects of ampicillin on microbial communities growing either on rumen contents with transgenic maize carrying a gene that confers resistance to ampicillin or its isogenic counterpart as substrates were examined continuously over 13 d. Rate of production of SCFA was measured to determine functional changes in the rumen model and single-strand conformational polymorphism was used to detect alterations in structure of the microbial community. Rumen contents treated with ampicillin displayed a marked decrease in the rate of production of SCFA and diversity of the microbial community was reduced severely. In the presence of transgenic maize, however, the patterns of change of rumen micro-organisms and their metabolic profiles were different from that of rumen fluid incorporating maize bred conventionally. Recovery of propionate production was observed both in the rumen fluid fed transgenic and conventional maize after a delay of several days but recovery occurred earlier in fermenters fed transgenic maize. Alterations in the microbial population structures resulting from the ampicillin challenge were not reversed during the experimental run although there was evidence of adaptation of the microbial communities over time in the presence of the antibiotic, showing that populations with different microbial structures could resume a pre-challenge metabolic profile following the introduction of ampicillin, irrespective of the source of the plant material in the growth medium.

**RUSITEC: Single-strand conformation polymorphism: *Bt* maize: Transgenic crops: Rumen microbial community: Ampicillin**

Nutrients and drugs are both known to influence the functionality and structure of the microbial communities of the forestomachs and hindgut of ruminants; the interaction between nutrients and drugs has rarely been studied. Recently, transgenic crops have been discussed as possible donors of transgenes (foreign genes) that could be taken up by micro-organisms under favourable conditions. The following arguments have contributed to the debate. First, transgenic constructs in plants are often of bacterial origin, suggesting that homologous DNA sequences could facilitate the incorporation of plant transgenes into the bacterial genome after transformation (Gebhard & Smalla, 1998). Second, there is increasing evidence that transformable, competent bacteria exist in the gastrointestinal tracts of man and animals, including in the forestomachs of ruminants (Gebhard & Smalla, 1998; Mercer *et al.* 1999; Duggan *et al.* 2003). Third, free, undegraded, transformable plant DNA is available in the mouth and is also found in the rumen and the lower intestines of animals and man, respectively (Chiter *et al.* 2000; Phipps *et al.* 2003; Einspanier *et al.* 2004; Netherwood *et al.* 2004). Degradation of plant DNA in the rumen is normally rapid, due to the high concentration of nucleases in the rumen

contents, but particle-associated bacteria could be capable of incorporating plant DNA directly, before significant degradation of DNA occurs. In consequence, a general discussion of the risk assessment of transgenic plants for feed or food uses has arisen.

Antibiotic-resistance genes are often used to facilitate the assembly of transgene cassettes in bacteria before they are inserted into plants and some may be used to facilitate the selection of transgenic cells that otherwise lack a positive selection after the plant transformation process. The selection of transgenic plant cells is often accomplished using *npt* II, encoding resistance to kanamycin and neomycin and engineered to be expressed in plant cells. Many bacterial plasmids used to assemble transgenic cassettes carry *bla*<sub>TEM</sub>, encoding resistance to  $\beta$ -lactam antibiotics including ampicillin. Although many commercial transgenic crop lines have been developed without the inclusion of *bla*<sub>TEM</sub>, this gene may be found in some lines of *Bt* maize. A possible gene transfer in the gastrointestinal tract would be conceivable if facilitated by a selective pressure from the environment.

Because of the considerations outlined, the effects of an antibiotic challenge in feeding experiments using transgenic

*Bt* maize (Bt-176) were evaluated. Micro-organisms from bovine rumen fluid were cultured in an *in vitro* rumen simulation system (RUSITEC; Czerkawski & Breckenridge, 1977). Either transgenic *Bt* maize or its conventional, isogenic counterpart was introduced into the model system and the metabolic profile was examined by analysis of the rate of production of SCFA. The single-strand conformation polymorphism (SSCP) technique was used for genetic profiling of microbial populations using PCR-amplified fragments of 16S rRNA genes. Thus, changes can be examined in the entire microbial ecosystem and not just in those microbes that can be recovered in artificial culture (Schwieger & Tebbe, 1998; Schmalenberger *et al.* 2001). To date, however, studies on microbial population structures linked with observations on functionality of the populations are important but are relatively neglected (Weinbauer *et al.* 2002). The present study combines metabolic profiling with SSCP studies on microbial population structures to examine the effects of antibiotic challenge on rumen samples containing either transgenic maize carrying a gene that confers resistance to ampicillin or its conventional counterpart. Rumen micro-organisms were not checked for the presence of either endogenous occurring  $\beta$ -lactamase genes or  $\beta$ -lactamase genes originating from transgenic maize that may have transferred into the rumen micro-organisms.

### Material and methods

Rumen contents were collected from one Hinterwolder cow (4 years old; 350 kg body weight, dry) fistulated in the dorsal rumen sac. For 3 weeks before sampling, the cow was fed with maize that had been bred conventionally (300 g/d) and hay *ad libitum*. Ground maize seeds of isogenic and transgenic maize plants were used for feeding the microbial community incubated in the RUSITEC system. *Bt*-176 maize (type Valmont) containing  $\beta$ -lactamase gene *bla*<sub>TEM</sub> was used in the present study, which was cultivated in Sachsen-Anhalt (Germany), side-by-side with its isogenic counterpart (type Prelude). Storage and handling of maize seeds was identical for both maize types. Reuter *et al.* (2002) confirmed the equality of chemical composition and substantial equivalence of the transgenic maize and its conventional counterpart.

#### *Detection of bla*<sub>TEM</sub> $\beta$ -lactamase gene in transgenic maize by polymerase chain reaction

Maize DNA isolation was performed using a NucleoSpin Plant Kit (Macherey & Nagel, Duren, Germany). A 350 bp fragment of  $\beta$ -lactamase gene was amplified using the following specific primers: sense 5'-CCCCGAAGAACGTTTTTC-3' and antisense 5'-TCGTCGTTTGGTATGGC-3'. Each PCR reaction contained in final concentrations 1  $\times$  PCR buffer, 1.5 mmol MgCl<sub>2</sub>/l, 0.2 mmol/l of each dNTP, 50 pmol of each primer, 1  $\mu$ l isolated maize DNA and 0.625 U HotStar Taq polymerase (Qiagen, Hilden, Germany) in a total volume of 25  $\mu$ l. PCR amplification conditions included an initial denaturing at 95°C for 15 min and thirty cycles with 30 s denaturing at 90°C, annealing for 2 min at 42°C and elongation for 45 s at 72°C. Final extension time was 5 min at 72°C. Plasmid pUC18 was used as a positive control

since pUC18 contains a nearly identical *bla*<sub>TEM</sub> gene. A negative control reaction was performed with water instead of maize DNA.

#### RUSITEC experiments

RUSITEC incubations were carried out as described previously by Czerkawski & Beckenridge (1977). Eight incubation vessels (1 litre volume) were used simultaneously in each experiment. The inner vessel was filled with two nylon bags (pore size 150  $\mu$ m) containing 2 g of a commercial concentrate and 6 g hay cut into 1 cm lengths. In addition, either 2 g transgenic maize or of its conventional, isogenic counterpart were added to the bags. On day 1, one bag was filled with solid rumen contents to inoculate particle-associated micro-organisms into the system. The incubation vessel was filled with rumen fluid to inoculate fluid-associated micro-organisms. On each day of the experiment, one bag was replaced by another containing fresh substrate, so that each bag had a 48 h retention time in the system. Bags were exchanged under anaerobic conditions using N<sub>2</sub> to flush the incubation vessels. To maintain conditions as close to those of the *in vivo* rumen as possible, the incubation temperature was 39°C and rumen fluid turnover was simulated by a continuous buffer perfusion at a rate of 750 ml/d. Perfusion buffer comprised NaHCO<sub>3</sub>, 97.9 mmol/l; Na<sub>2</sub>HPO<sub>4</sub>, 10 mmol/l; NaH<sub>2</sub>PO<sub>4</sub>, 10 mmol/l; NH<sub>4</sub>Cl, 5 mmol/l; NaCl, 28 mmol/l; KCl, 7.7 mmol/l; MgCl<sub>2</sub>, 0.63 mmol/l; CaCl<sub>2</sub>, 0.22 mmol/l. The pH was 8.2 and the osmolality was 300 mosmol/l. By moving the inner vessel up and down continuously rumen motility was simulated and exchange between the fluid and particle phases was facilitated. Rumen gas was collected in gas-tight collecting sacs to ensure a closed system; the fluid outflow was collected in ice-cooled Erlenmeyer flasks.

#### Experimental time schedule and sampling

Each RUSITEC experiment was subdivided into three periods; 7 d for equilibrating the system, 3 d for the control period under steady-state conditions and 10 d of the experimental period during which ampicillin was added at a rate of 1.25 g/d. This is equivalent to a concentration of 1.67 g/l. Ampicillin was given in a therapeutic dose, but in ruminants ampicillin is not delivered orally for therapeutic purposes. Ampicillin was given to create a selective pressure for rumen microbes for a potential transfer of  $\beta$ -lactamase gene(s). During the total experimental period the redox potentials and pH were measured daily at the same time point to check the anaerobic status of the incubation system and to ensure adequate environmental conditions for microbial survival. Samples of outflow were collected each day to assay SCFA levels. Additionally, outflow samples (about 100 ml) were collected once in the control period (day 2) and twice during the experimental period (days 8 and 13) for the isolation of fluid-associated bacteria for SSCP profiling.

The incubation vessels were divided into four groups with two incubation vessels in each group. Vessels A, B, C and D were supplied with maize bred using conventional technology while vessels E, F, G and H were supplied with transgenic

maize. During the experimental period, vessels A and B, incorporating conventional maize, and vessels E and F, incorporating transgenic maize, were perfused with standard perfusion buffer without ampicillin; vessels C and D, incorporating conventional maize, and vessels G and H, incorporating transgenic maize, were perfused with standard perfusion buffer containing ampicillin (1.67 g/l). RUSITEC experiments, each lasting 13 d, were performed in duplicate.

#### Short-chain fatty acids analyses

Fluid outflow samples were centrifuged at 40 000 *g* for 20 min at 4°C. From each supernatant fraction, 1 ml of fluid was acidified by adding 0.1 ml 98 % formic acid and centrifuged again at 40 000 *g* for 10 min at 4°C in order to remove precipitated substrates. SCFA analyses were performed by GC (model 5890 II; Hewlett Packard, Boeblingen, Germany) using Chromosorb WAW (mesh 80/100) with 20 % neopentyl glycol succinate and 2 % *o*-phosphoric acid as the column matrix and He as the carrier gas (von Engelhardt & Sallmann, 1972). Graphs were performed using GraphPad Prism software version 3 (GraphPad Software, Inc., San Diego, CA, USA). Values are given as means and SD (four incubation vessels). Statistical analyses of SCFA production data were performed by two-way ANOVA with maize and ampicillin as factors. The factor 'experiment' was included by analysing the data as randomised-block data. The level of significance was set at  $P < 0.05$ . Bonferroni post-test was used to adjust the  $P$  values (done for four fermenters). Linear regression was used to calculate the velocity of recovery by comparing the slopes of the two regression lines, focused on the results of days 7 to 10.

#### Single-strand conformation polymorphism analyses

Isolation of fluid-associated bacteria was achieved by differential centrifugation, washing bacteria with ice-cold physiological saline (Brandt & Rohr, 1981). Re-suspended bacterial pellets were frozen in liquid N<sub>2</sub> and stored at -80°C before being analysed. The SSCP procedure was carried out as described by Schwieger & Tebbe (1998). Briefly, DNA was isolated from 120 mg of each bacterial pellet using the Fast DNA Kit (chemical no. 654-400; Q Biogene, Heidelberg, Germany) according to the manufacturer's protocol. DNA was diluted 1:10 in Tris/EDTA buffer (tri(hydroxymethyl)-aminomethane-HCl (10 mmol/l), EDTA (1 mmol/l); pH 8)

and 1 µl was used as a template for PCR amplification in a total volume of 50 µl.

#### Primers and polymerase chain reaction conditions

The primers which were used for the amplification of the gene encoding 16S rRNA to characterise the total microbial community and domain- and family-specific structures are given in Table 1. PCR amplification was performed in a total volume of 50 µl containing a final concentration of 1 × PCR buffer; MgCl<sub>2</sub>, 1.5 mmol/l; each dNTP, 0.2 mmol/l; forward and reverse primers, 0.5 mmol/l; Hot-Star-Taq polymerase, 1.25 U (Qiagen). Amplification was performed in a Thermocycler gradient (Biometra, Göttingen, Germany) with an initial denaturation of 15 min at 95°C, followed by thirty cycles of denaturation at 94°C for 60 s, primer annealing for 60 s and elongation at 72°C for 60 s. The temperature of annealing varied, depending on the primer pair being used. For amplification of the total microbial population and for bacteria, annealing was at 50°C and for archaea, 52°C. A final elongation step was carried out at 72°C for 10 min. Each domain-specific PCR was followed by a second PCR with primers Com 1 and Com 2Ph to amplify an internal fragment for verification purposes.

#### Single strand preparation

PCR products were purified using a Qiaquick PCR Purification Kit (chemical no. 28 106; Qiagen) according to the manufacturer's protocol. Eluted dsDNA was quantified fluorimetrically using a PicoGreen-dsDNA Quantification Kit (chemical no. P-7581; Molecular Probes Inc., Eugene, OR, USA). Single-stranded DNA was obtained from the PCR product by removing the 5'-phosphorylated strand by λ-exonuclease digestion. A total of 800 ng dsDNA was exposed to exonuclease digestion at 37°C for 45 min in a total volume of 40 µl containing 1 × exonuclease buffer and 2.5 U λ-exonuclease (chemical no. M0262S; New England Biolabs, Frankfurt am Main, Germany). Single-strand DNA was purified using a MiniElute PCR Purification Kit (chemical no. 28 006; Qiagen).

#### Gel electrophoresis and staining

Separation of ssDNA due to conformational polymorphism was performed in a 0.625 % Mutation Detection Enhancement

**Table 1.** Single-strand conformation polymorphism primers for amplification of 16S rRNA fragments of the total microbial community, eubacteria, and archaea

Target	Name of primer	Position of primer	Primer sequence	Reference
16S rRNA total community	Com 1(f)	519–536	5'-CAGCAGCCGCGTAATAC-3'	Schwieger & Tebbe (1998)
	Com 2Ph(r)	907–926 in <i>Escherichia coli</i>	5'-CCGTCAATTCCTTGAGTTT-3'	Brosius <i>et al.</i> (1978)
16S rRNA bacteria	F27	8–27	5'-AGAGTTTGATC(C/A)TGGCTCAG-3'	Weisburg <i>et al.</i> (1991)
	R1492	1492–1513	5'-TACGG(C/T)TACCTTGTTACGACTT-3'	
16S rRNA archaea	A109f	109–125	5'-AC(G/T)GCTCAGTAACACGT-3'	Grosskopf <i>et al.</i> (1998)
	A934	915–934	5'-GTGCTCCCCGCCAATTCCT-3'	

gel; chemical no. 50620 (Cambrex, Rockland, ME, USA) in  $1 \times$  Tris/Boric acid/EDTA buffer; Tris base (89 mmol/l), boric acid (89 mmol/l), EDTA (2 mmol/l) (pH 8.3) (Sambrook *et al.* 1989) using a MacroPhor sequencing system (Amersham Pharmacia Biotech, Freiburg, Germany). Before loading, ssDNA was denatured in loading buffer (950  $\mu$ l 95% formamide; 10  $\mu$ l NaOH (1 mmol/l); 0.025% bromophenol blue) at 95°C for 2 min and subsequently cooled on ice for 3 min. Electrophoresis took place under the following conditions: 17 h, 400 V, 8 mA at 20°C. Following electrophoretic separation of the DNA, gels were silver stained, fixed in 10% acetic acid and air-dried according to the manufacturer's protocol. Gel images were recorded using a ScanJet4c/T scanner (Hewlett Packard). Comparative analyses of SSCP band patterns were achieved using GelCompar II version 3.0 (Applied Maths, Kortrijk, Belgium). SSCP was performed from samples of one RUSITEC experiment.

## Results

### Polymerase chain reaction-based verification of transgenicity of *Bt* maize

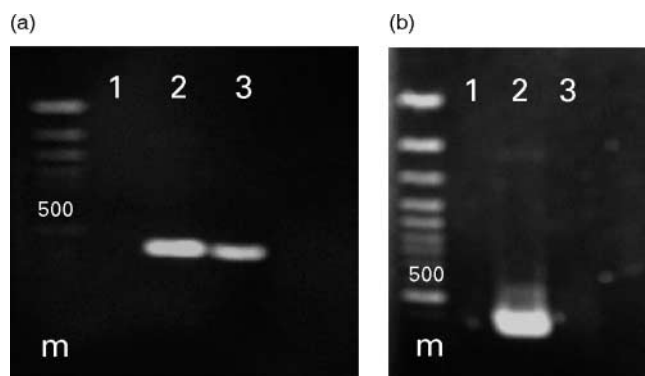
A fragment of *bla*<sub>TEM</sub>, part of the gene coding for  $\beta$ -lactamase which was used as a marker gene in *Bt*-176 maize, could be detected in DNA isolated from *Bt*-176 maize (Fig. 1(a)). Amplification of *bla*<sub>TEM</sub> in DNA from the counterpart isogenic maize failed (Fig. 1(b)). PCR validity was confirmed by a negative control with water and a positive control with the plasmid pUC18, which carries *bla*<sub>TEM</sub>.

### Effects of ampicillin on the metabolic profile of rumen fluid incorporating either transgenic maize or its conventional, isogenic counterpart

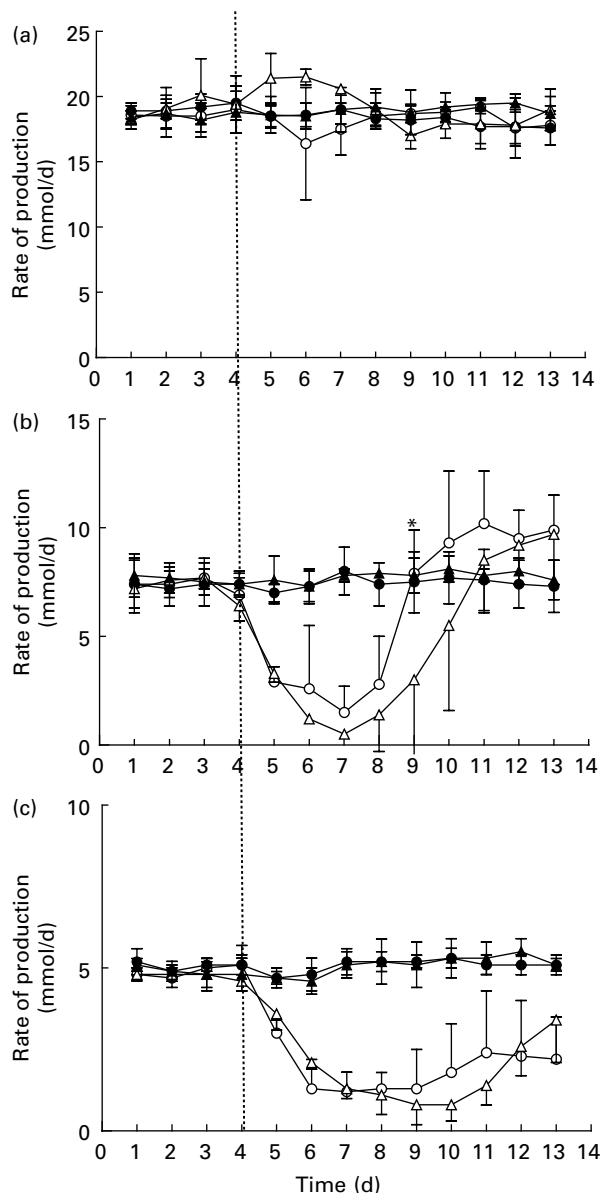
To obtain a metabolic profile of the rumen fluid samples, the rate of production of SFCA was measured daily for 2 weeks. In the first 4 d of the experiment, the production of acetate, propionate and butyrate was constant, and similar for rumen fluid with conventional or transgenic maize (Fig. 2). On day

4, ampicillin (1.67 g/l) was introduced into half of the test vessels. This had no effect on the rate of production of acetate in the rumen fluid but the production of both propionate and butyrate was markedly reduced following the introduction of ampicillin, irrespective of the nature of the maize in the reaction vessels (Fig. 2).

On day 7, the rate of production of propionate was lowest and began to rise to pre-challenge levels in rumen fluid



**Fig. 1.** PCR-based detection of *bla*<sub>TEM</sub> gene coding  $\beta$ -lactamase in transgenic maize. (a) Amplification of *bla*<sub>TEM</sub> gene by PCR resulted in a 350 bp fragment in DNA isolated from transgenic maize *Bt*-176 (lane 3); lane 2 detection of *bla*<sub>TEM</sub> gene in plasmid pUC18; lane 1 negative control with water. (b) Amplification of *bla*<sub>TEM</sub> gene by PCR in DNA of isogenic maize failed (lane 3); lane 2 detection of *bla*<sub>TEM</sub> gene in pUC18; lane 1 negative control with water. m, Marker DNA.



**Fig. 2.** The effect of maize type and antibiotic presence on SCFA production rates of rumen fluids incorporating conventional or transgenic maize and in the absence or presence of ampicillin. The rates of production of (a) acetate, (b) propionate and (c) butyrate are given in mmol/d. Where used, ampicillin was introduced on day 4, indicated by the dotted lines. Each point represents the mean value derived from four incubation vessels, with standard deviations represented by vertical bars. Propionate production rate challenged by ampicillin was significantly higher at day 9 in transgenic compared to isogenic fed fermenters ( $P < 0.05$ ; two-way ANOVA with Bonferroni post-test-effect of maize). ( $\blacktriangle$ ) Conventional maize in the absence of ampicillin; ( $\bullet$ ) transgenic maize in the absence of ampicillin; ( $\triangle$ ) conventional maize in the presence of ampicillin; ( $\circ$ ) transgenic maize in the presence of ampicillin. \* $P < 0.05$ .

incorporating transgenic maize and ampicillin within the following days. By day 9 there was complete recovery of propionate to a pre-challenge rate in rumen fluid incorporating transgenic maize. In rumen fluid incorporating conventional maize, the recovery of propionate production to a pre-challenge rate was complete on day 11 (Fig. 2(b)). Focusing on days 7 to 10 of recovery, linear regression was used to compare propionate production in vessels fed isogenic and transgenic maize and subjected to ampicillin challenge. Recovery of propionate production occurred faster in vessels fed transgenic maize than in those fed isogenic maize. The difference in the rate of recovery could be confirmed by different mean slope values (transgenic, 2.85; isogenic, 1.66), while correlation coefficients confirmed the quality of calculation ( $r^2$  0.93 for fermenters fed with transgenic maize;  $r^2$  0.96 for fermenters fed with isogenic maize). Analysing the data with ANOVA, propionate production rates were not influenced by the type of maize at days 1 to 4 (one-way ANOVA) (Table 2). Propionate production at days 5 to 8 was strongly influenced by ampicillin (two-way ANOVA;  $P < 0.001$ ) irrespective of the type of maize used. At day 9, ampicillin only affected propionate production in vessels fed isogenic maize, as calculated by the Bonferroni post-test. Therefore, an overall influence of ampicillin was lacking, but type of maize significantly affected propionate production, resulting in an interaction between maize type and ampicillin. At days 10 to 13 neither the type of maize nor ampicillin influenced propionate production (Table 2). Butyrate production recovery was only slightly expressed and did not reach control levels within the experimental period (Fig. 2(c)).

#### *Effects of ampicillin on the microbial population structures of rumen fluid incorporating either transgenic maize or its conventional counterpart*

Changes in the metabolic profiles of rumen fluid samples following ampicillin challenge were qualitatively mirrored by changes in the microbial population structures as shown by SSCP band patterns. On day 2 of the experiment, 2 d before ampicillin was introduced and during a period where SCFA production was constant, similar SSCP band patterns

derived from the total microbial populations of all treatments indicated no differences in community structures. Comparing lanes by GelCompar resulted in an analogy between isogenic and transgenic fed vessels of  $\geq 90\%$  which was set to be equal. Variability within the two similar vessels of each incubation was also low ( $\geq 90\%$  analogy) (Fig. 3; bacteria sampling 1; Table 3).

On day 8, in the absence of ampicillin, no change in SSCP band pattern was apparent whether the rumen fluid contained transgenic maize or its conventional counterpart. In samples containing ampicillin, however, there was a reduction in the number of SSCP bands, reflecting a potential reduction in diversity of micro-organisms in the presence of the antibiotic. Furthermore, the band patterns derived from samples incorporating transgenic maize were different from those derived from samples incorporating conventional maize. Although there was an overall reduction in the number of bands derived from rumen bacteria in the presence of ampicillin, some bands seemed to be much more prominent in these samples. At day 8, analogy between isogenic- and transgenic-fed vessels without ampicillin was  $\geq 90\%$  with a low inter-vessel variability ( $\geq 90\%$  analogy), while with ampicillin challenge, analogy was reduced to 32%, indicating differences in ampicillin effect on the microbial population structure fed with isogenic or with transgenic maize. Analogy within the two similar vessels of each incubation was 72% (Fig. 3, bacteria sampling 2; Table 3). The ampicillin effect at day 8 was strong, resulting in only 20% analogy irrespective of the type of maize fed (Table 3).

By day 13, the SSCP band patterns from samples grown in the absence of ampicillin were indistinguishable from those derived from samples on day 2 and day 8. Analogy was nearly  $\geq 90\%$  (89%) in vessels without ampicillin challenge irrespective of type of maize. Inter-vessel variability was low ( $\geq 90\%$ ). Despite a restoration in the metabolic profile by day 13 in samples challenged with ampicillin, the reduction in microbial diversity seen at day 8 was still apparent. Comparing patterns derived on day 8 with those derived on day 13 demonstrated further changes in the microbial population structures in samples challenged with ampicillin. Analogy between vessels challenged with ampicillin was low due to the type of maize fed (60%). Variability within the two similar vessels of each incubation was nearly equal (isogenic, 88%; transgenic,  $\geq 90\%$ ) (Fig. 3, bacterial sampling 3; Table 3). The effect of ampicillin at day 13 was lower than at day 8; analogy was 36% irrespective of type of maize (Table 3).

Using a different primer set amplifying bacterial genes encoding 16S rRNA showed similar patterns of change (Fig. 4). SSCP band patterns compared within each day were indistinguishable on days 2, 8 and 13 grown in the absence of ampicillin, irrespective of the nature of the maize present in the sample (Table 4). In samples challenged with ampicillin, however, differences in band patterns were apparent. At day 8, analogy was only 20% between vessels fed either isogenic or transgenic maize with a low variability between vessels of each incubation (Table 4). At day 13, influence of maize type resulted in 55% analogy while variability between vessels of each incubation was constantly low (Table 4). The effect of ampicillin at day 13 was lower than at day 8; analogy was 22 and 10% irrespective of type of maize (Table 4).

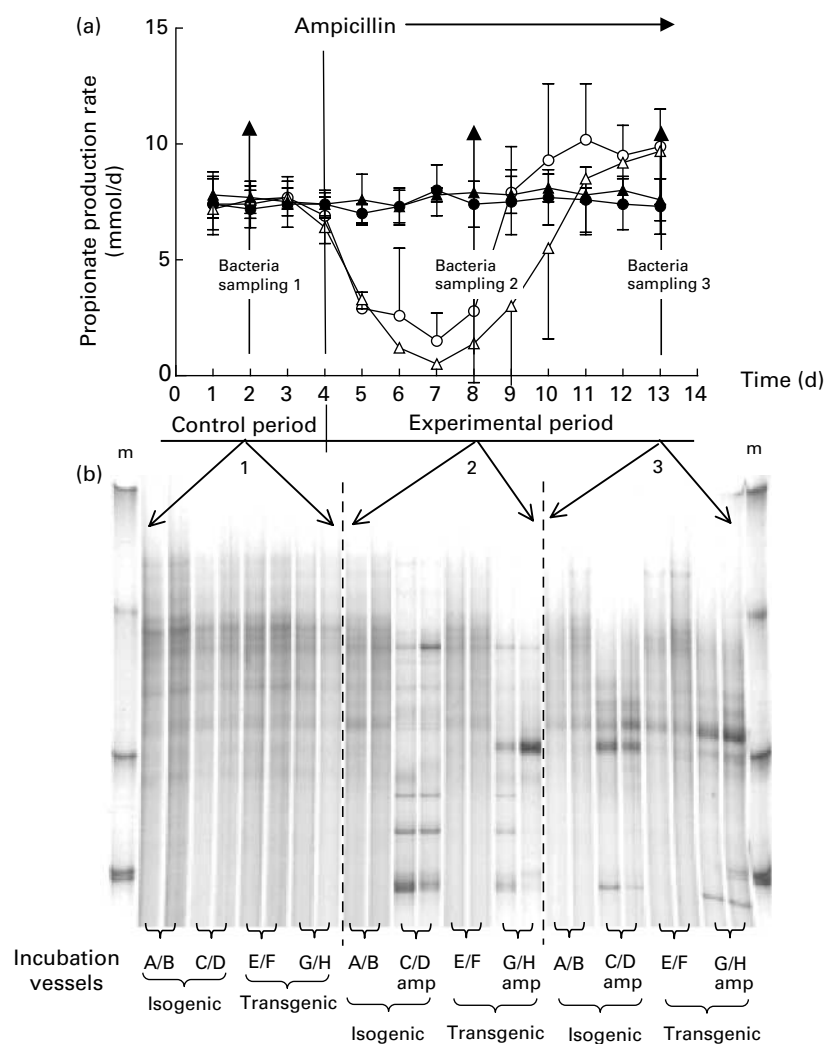
**Table 2.** Influence of maize, ampicillin and interaction between maize and ampicillin on propionate production rates (two Rusitec experiments; four fermenters)

Day	Maize <i>P</i>	Ampicillin <i>P</i>	Maize × ampicillin <i>P</i>
Days 1–4*	0.6095		
Day 5†	0.1959	0.0001	0.7780
Day 6†	0.4364	0.0001	0.4017
Day 7†	0.1833	0.0001	0.3369
Day 8†	0.5616	0.0001	0.2491
Day 9†	0.0479	0.0591‡	0.0340
Day 10†	0.2332	0.7241	0.1493
Days 11–13†	0.3149	0.1012	0.4505

\* Analysed by one-way ANOVA.

† Analysed by two-way ANOVA. Data were analysed as a randomised block to include the factor 'experiment'.

‡ Ampicillin affected only propionate production rates in isogenic-fed incubation vessels ( $P < 0.05$ ; Bonferroni post-test), not in transgenic-fed fermenters.



**Fig. 3.** The effect of maize type and antibiotic presence on single-strand conformation polymorphism (SSCP) profiles of the total microbial populations. (a) Propionate production rates (mmol/d) are depicted to show the points of bacteria sampling. (▲) Conventional maize in the absence of ampicillin; (●) transgenic maize in the absence of ampicillin; (Δ) conventional maize in the presence of ampicillin; (○) transgenic maize in the presence of ampicillin. (b) SSCP patterns were derived from samples taken on day 2, during the control period, and days 8 and 13 when, if appropriate, ampicillin (amp) was present in the reaction vessels. Each lane represents the structural profile of the microbial community of one incubation vessel. Brackets and capital letters indicate the vessels that belong together because of the type of maize used and the presence or absence of ampicillin. Lanes marked with 'm' contain marker DNA, which was generated from partial rRNA genes amplified by PCR with Com 1 and Com 2-Ph primers, and single-strand digested (see p. 822) from (top to bottom) *Bacillus licheniformis*, *Rhizobium trifolii*, *Flavobacterium johnsoniae* and *R. radiobacter* (double band).

When primers specific for archaea were used to generate SSCP profiles, only slight differences were induced by ampicillin at day 13 (Fig. 5; Table 5). The type of maize did not influence SSCP patterns of archaea.

### Discussion

The results presented demonstrate that, while the equality of chemical composition and substantial equivalence of the transgenic maize and its conventional counterpart have been established previously (Reuter *et al.* 2002), there are qualitative differences between *Bt*-176 and its conventional counterpart. These only become apparent, however, when the transgenic maize is placed in a model rumen environment in which ampicillin is present.

The experimental approach of an ampicillin challenge to the rumen microbial community was not performed due to therapeutic use of ampicillin in ruminants but, rather, serves as an artificial challenge of the microbial system due to the possibility of creating a selective pressure for the transfer of a gene encoding antibiotic resistance. Thus, these observations are only presented as descriptive data. The insect-resistant maize *Bt*-176 contains three bacterial genes: a modified *cry* 1A(b) gene from *Bacillus thuringiensis*, a streptomycete *bar* gene and a *bla*<sub>TEM</sub> variant derived from the pUC18 cloning vector (Koziel *et al.* 1993). The *cry* 1A(b) gene from *Bacillus thuringiensis* encodes the parasporal crystal toxin that targets insects, particularly the European maize borer, *Ostrinia nubilalis*. The streptomycete *bar* gene encodes resistance to the herbicide glufosinate ammonium, which acts by interfering with glutamine metabolism. As well as acting as a herbicide, glufosinate

**Table 3.** Results of comparison of band pattern of Corn single-strand conformation polymorphism (SSCP) (see Fig. 3) with Gelcompar II version 3.0 (Applied Maths, Kortrijk, Belgium)

Day of SSCP	Vessels	Analogy (%)*	Variability within two vessels	Analogy (%)*
Day 2†	Isogenic compared with transgenic without ampicillin	≥ 90		≥ 90
Day 8†	Isogenic compared with transgenic without ampicillin	≥ 90		≥ 90
	Isogenic compared with transgenic with ampicillin	32	Isogenic with ampicillin Transgenic with ampicillin	72 72
Day 13†	Isogenic compared with transgenic without ampicillin	(≥ 90) 89		≥ 90
	Isogenic compared with transgenic with ampicillin	60	Isogenic with ampicillin Transgenic with ampicillin	88 ≥ 90
Day 8‡	Isogenic + transgenic with and without ampicillin	20		
Day 13‡	Isogenic + transgenic with and without ampicillin	36		

\* Analogy of band patterns of ≥90% were set to be equal.

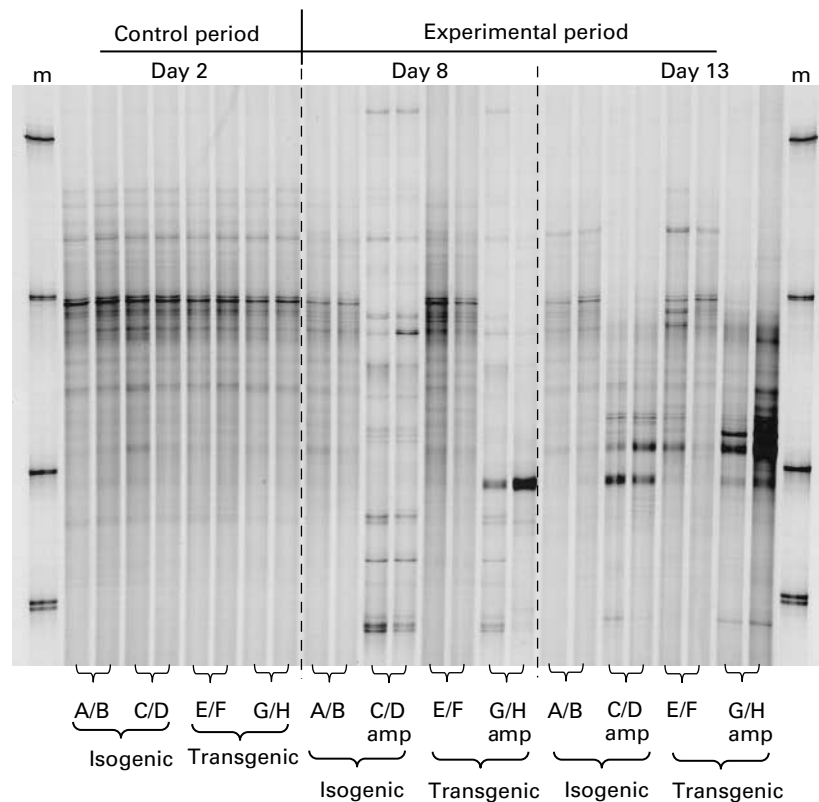
† On days 2, 8 and 13, isogenic- and transgenic-fed vessels were compared in each case.

‡ On days 8 and day 13, incubation vessels were compared for ampicillin effect irrespective of isogenic or transgenic feeding.

ammonium has notable antimicrobial properties. The *bla*<sub>TEM</sub> variant present in *Bt* maize 176 encodes a TEM-type β-lactamase, which confers resistance to ampicillin and other β-lactam antibiotics (Yanisch-Perron *et al.* 1985). The observed changes in microbial metabolism and community structure could be based on these properties of *Bt* maize. Detection of *bla*<sub>TEM</sub> gene transfer by PCR is lacking. It is considered very unlikely that the results described result from a secondary effect of the herbicide tolerance trait engineered into the maize.

Acetate production remained unaffected by the presence of ampicillin, although production of propionate and butyrate

was depressed initially when rumen fluid was challenged with ampicillin. One explanation for this different reactivity to antibiotic challenge may be that acetate is a common bacterial metabolic endproduct that is generated by a wide diversity of bacteria, whereas propionate and butyrate are specialist metabolic endproducts that are each produced by a limited range of bacteria. Thus relatively small changes in the populations of bacteria responsible for the production of propionate and butyrate will have a disproportionately large effect on their production compared with small changes in the bacterial communities responsible for acetate production.



**Fig. 4.** The effect of maize type and antibiotic presence on single-strand conformation polymorphism (SSCP) profiles of the bacterial populations. SSCP patterns were derived from samples taken on day 2, during the control period, and days 8 and 13 when, if appropriate, ampicillin (amp) was present in the reaction vessels. Each lane represents the structural profile of the microbial community of one incubation vessel. Brackets and capital letters indicate the vessels that belong together because of the type of maize used and the presence or absence of ampicillin. Lanes marked with 'm' contain marker DNA (see Fig. 3).

**Table 4.** Results of comparison of band pattern of bacteria single-strand conformation polymorphism (SSCP) (see Fig. 4) with Gelcompar II version 3.0 (Applied Maths, Kortrijk, Belgium)

Day of SSCP	Vessels	Analogy (%)*	Variability within two vessels	Analogy (%)*
Day 2†	Isogenic compared with transgenic without ampicillin	≥ 90		≥ 90
Day 8‡	Isogenic compared with transgenic without ampicillin	≥ 90		≥ 90
	Isogenic compared with transgenic with ampicillin	20	Isogenic with ampicillin Transgenic with ampicillin	63 84
Day 13‡	Isogenic compared with transgenic without ampicillin	≥ 90		≥ 90
	Isogenic compared with transgenic with ampicillin	55	Isogenic with ampicillin Transgenic with ampicillin	80 76
Day 8‡	Isogenic + transgenic with and without ampicillin	10		
Day 13‡	Isogenic + transgenic with and without ampicillin	22		

\* Analogy of band patterns of ≥90% were set to be equal.

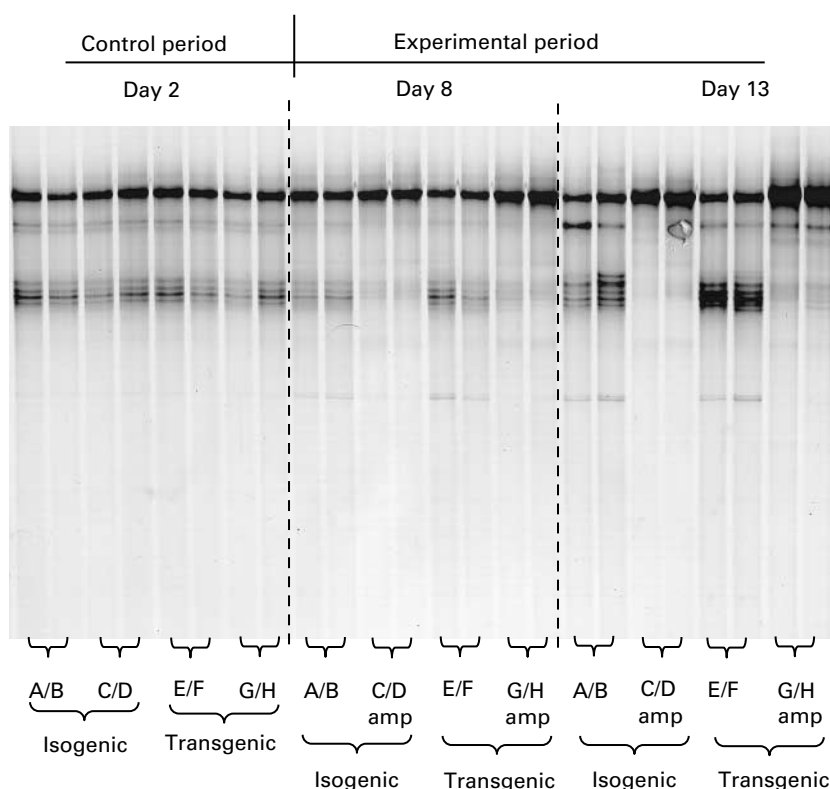
† On days 2, 8 and 13, isogenic- and transgenic-fed vessels were compared in each case.

‡ On days 8 and day 13, incubation vessels were compared for ampicillin effect irrespective of isogenic or transgenic feeding.

Adaptation to the presence of ampicillin took several days, irrespective of which type of maize was incorporated. Recovery to pre-challenge levels of propionate seemed to be faster in fermenters incorporating transgenic maize than in fermenters containing conventional maize. Changes in SCFA production occurred simultaneously with changes in the various microbial population structures. In all cases, introduction of ampicillin into the fermenters led to a reduction in biodiversity. Over time in the presence of ampicillin, microbial populations adapted and the population structures could be seen to change. As expected, these did not return to a pre-challenge state, since the antibiotic selective pressure was maintained, and this

would exclude bacteria susceptible to ampicillin. The change of population structures over time in the presence of ampicillin reflects the adaptation of the microbial communities permitting a restoration of the production of SCFA to pre-challenge levels.

When the effects of ampicillin challenge on fermenters incorporating transgenic maize are compared with those produced by ampicillin in fermenters incorporating conventional maize, another important difference is discovered. Recovery of propionate production started between 3 and 4 d following ampicillin challenge when transgenic maize was incorporated into the fermenters. This was 2 d before recovery from the ampicillin challenge started in fermenters incorporating conventional maize.



**Fig. 5.** The effect of maize type and antibiotic presence on single-strand conformation polymorphism (SSCP) profiles of the populations of archaea. SSCP patterns were derived from samples taken on day 2, during the control period, and days 8 and 13 when, if appropriate, ampicillin (amp) was present in the reaction vessels. Each lane represents the structural profile of the microbial community of one incubation vessel. Brackets and capital letters indicate the vessels that belong together because of the type of maize used and the presence or absence of ampicillin.



**Table 5.** Results of comparison of band pattern of archaea single-strand conformation polymorphism (SSCP) (see Fig. 5) with Gelcompar II version 3.0 (Applied Maths)

Day of SSCP	Vessels	Analogy (%)*	Variability within two vessels	Analogy (%)*
Day 2†	Isogenic compared with transgenic without ampicillin	≥ 90		≥ 90
Day 8†	Isogenic compared with transgenic without ampicillin	≥ 90		≥ 90
	Isogenic compared with transgenic with ampicillin	≥ 90 (97.4)	Isogenic with ampicillin	≥ 90 (99.8)
			Transgenic with ampicillin	≥ 90 (98.8)
Day 13†	Isogenic compared with transgenic without ampicillin	≥ 90		≥ 90
	Isogenic compared with transgenic with ampicillin	(90.5)	Isogenic with ampicillin	≥ 90 (96)
			Transgenic with ampicillin	≥ 90 (99)
Day 8‡	Isogenic + transgenic with and without ampicillin	≥ 90 (91.4)		
Day 13‡	Isogenic + transgenic with and without ampicillin	65		

\* Analogy of band patterns of ≥90% were set to be equal. At days 8 and 13 the absolute values were given since differences were small between isogenic- and transgenic-fed vessels with ampicillin challenge.

† On days 2, 8 and 13, isogenic- and transgenic-fed incubation vessels were compared in each case.

‡ On days 8 and day 13, incubation vessels were compared for ampicillin effect irrespective of isogenic or transgenic feeding. Overall ampicillin effects were much lower in archaea than in other micro-organisms examined. Numbers in brackets indicate the absolute values of analogy.

It can only be speculated by which mechanisms the faster recovery was mediated. It is conceivable that transfer of *bla*<sub>TEM</sub> from plant to microbes occurred under the *in vitro* conditions used in this experiment, although this has not been investigated by PCR-based detection of this gene in microbial DNA so far. It may be assumed, however, that prerequisites for gene transfer from plant to micro-organisms are to be found under rumen conditions. If single transfer events occur, these transformed micro-organisms have a greater chance of survival. Additionally, they have the potential to transfer their newly acquired resistance genes to other micro-organisms. This trans-kingdom gene flow is also likely to be a rare event (Heritage, 2004). Nevertheless, it has been reported that gene transfer occurs between micro-organisms in the rumen environment and that bacterial genes have been incorporated into fungi (Nicholson *et al.* 2005). It is conceivable that under the selective pressure of an ampicillin challenge, both processes result in an increasing number of micro-organisms that express plant-derived β-lactamase additional to endogenous existent resistance mechanisms. Resistance to ampicillin resulted in the recovery of propionate production and in changes of the microbial structure evident from the SSCP studies, but is more pronounced in micro-organisms fed with transgenic maize.

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