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Influence of Transgenic Corn on the *In vitro* Rumen Microbial Fermentation

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ABSTRACT : In this study, the comparative effects of transgenic corn (Mon 810 and Event 176) and isogenic corn (DK729) were investigated for their influence on *in vitro* rumen fermentation. This study consisted of three treatments with 0.25 g rice straw, 0.25 g of corn (Mon810/Event176/DK 729) mixed with 30 ml rumen fluid-basal medium in a serum bottle. They were prepared in oxygen free conditions and incubated at 39°C in a shaking incubator. The influence of transgenic corn on the number of bacterial population, *F. succinogenes* (cellulolytic) and *S. bovis* (amylolytic), was quantified using RT-PCR. Fermentative parameters were measured at 0, 2, 4, 8, 12 and 24 h and substrate digestibility was measured at 12 and 24 h. No significant differences were observed in digestibility of dry matter, NDF, ADF at 12 and 24 h for both transgenic and isogenic form of corns ($p>0.05$) as well as in fermentative parameters. Fluid pH remained unaffected by hybrid trait and decreased with VFA accumulation as incubation time progressed. No influence of corn trait itself was seen on concentration of total VFA, acetic, propionic, butyric and valeric acids. There were no significant differences ($p<0.05$) in total gas production, composition of gas (methane and hydrogen) at all times of sampling, as well as in NH_3-N production. Bacterial quantification using RT-PCR showed that the population number was not affected by transgenic corn. From this study it is concluded that transgenic corn (Mon810 and Event 176) had no adverse effects on rumen fermentation and digestibility compared to isogenic corn. However, regular monitoring of these transgenic feeds is needed by present day researchers to enable consumers with the option to select their preferred food source for animal or human consumption. (**Key Words :** Transgenic Corns, GMO, Mon810, Event176, Rumen)

INTRODUCTION

Gene modification techniques for plants, animals, and microorganisms that are of economic importance have been greatly improved worldwide, since attempts to make genetically modified organisms (GMO) originated in the 1970s. In particular, the development and use of biotechnology/GM crops is considered by many countries as an important tool to meet their food/feed crop requirements and also to meet demands of their increasing population with a decreasing area of land available for crop production. (Phipps et al., 2002; James, 2004). The global area of commercialized biotechnology/GM crops increased more than 50 fold during the nine-year period from 1996 to 2005, from 1.7 million hectares in 6 countries in 1996 to 90.0 million hectares in 21 countries in 2005 (James, 2005).

Whilst crops have been genetically modified for a range of different traits, herbicide tolerance and insect protection are the two most common traits, and corn and soybeans are the most widely grown biotechnology/GM crops (Phipps et al., 2002). Among them, *Bt* corn was the second most dominant biotechnology crop in 2005, occupying 11.3 million hectares, equivalent to 13% of the global biotechnology crop area (James, 2005). It is grown commercially in the USA, Argentina, Canada, South Africa, the Philippines, Spain, Uruguay, Honduras, Portugal, Germany, France, and the Czech Republic. Also, the other transgenic corns are herbicide tolerant corn, and *Bt*/herbicide tolerant corn. The *Bt* corn hybrids are enhanced to express proteins that are negative to *Bacillus thuringiensis* (*Bt*) bacterium and are resistant to damage

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caused by European corn borer (*Ostrinia nubilalis*), a common pest in corn fields (Koziel et al., 1993). Corn borers reduce the quality and yield of corn and damage the plant tissue, resulting in increased opportunity for fungal growth (Clark and Ipharraguerre, 2001). Herbicide-tolerant corns are generally produced by the stable insertion of a gene that expresses glyphosate tolerance (LeBrun et al., 1997). Glyphosate is a commercial herbicide that inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, which is involved in the synthesis of essential aromatic amino acids required for plant growth (Steinrücken and Amrhein, 1980).

At present there are rising public and scientific concerns about the safety of transgenic feeds for animals and consequently for human beings as they consume animal products. The early studies (Brake and Vlachos, 1998; Sidhu et al., 2000) have not found significant differences in general nutritional assessment and feed quality from isogenic and transgenic corn. Reviews carried out by Clark and Ipharraguerre (2001) and Beever et al. (2003) showed that transgenic feedstuffs on the market had similar feeding value to that of normal feeds and have not adversely affected animal performance. However, as use of GM crops extends worldwide, there is a need for rigorous evaluation of transgenic feeds and continuous monitoring on their long-term influence on animal production and health. Therefore, the objectives of these experiments were to determine the influence of isogenic and transgenic corns on *in vitro* rumen fermentation.

MATERIALS AND METHODS

Preparation of feed corns and *in vitro* substrates

Five hundred grams of corn was randomly collected from a bulk corn stock and ground to 500 μm size by Rotor-Speed Mill P14 (Fritsch GembH, Germany) for detection of transgenic DNA from corn.

As *in vitro* substrate, non-GM corn DK729 from the National Livestock Research Institute of Korea was used, and GM corns were Mon810, insect protection, and Event176, insect protection and herbicide tolerance. Each corn grain was identified for transgenic DNA and the grains showing positive reaction in PCR were separately collected into Mon810 and Event176. The corns of DK729, Mon810, and Event176 were ground for use in *in vitro* rumen fermentation.

Rumen fermentation *in vitro*

The rumen microorganism and *in vitro* culture were prepared by Kim et al. (2005) and Wang et al. (2005) with some modification. Rumen contents were obtained from a rumen fistulated Holstein steer (550 kg) which was fed a diet of 40% concentrate and 60% timothy on a DM basis twice a day. The collected rumen fluid was used as a source of seed microorganisms after straining through four layers of cheesecloth. The *in vitro* rumen fermentation was completed in 60 ml serum bottles which contained 0.25 g of ground corn together with 0.25 g of ground rice straw as a source of fiber and filled with rumen fluid-basal medium mixture (30 ml). The rumen fluid-basal medium mixture was prepared by mixing one volume of rumen fluid and two volumes of McDougall (1948) buffer solution. These preparations were done under O_2-free CO_2 conditions at all times. The prepared serum bottles were incubated at 39°C in a 120 rpm shaking incubator. The samples were collected from triplicate bottles at 0, 2, 4, 8, 12 and 24 h for the determination of fermentative parameters and at 12 and 24 h for substrate digestibility and bacterial population.

Extraction of total DNA

For detection of transgenic corn, genomic DNA was extracted from feed corn according to the manual for DNA isolation from plant tissue by using the DNeasy™ Plant Maxi kit (QIAGEN GmbH, Hilden, Germany), with the following small modification: All centrifugation was performed at 3,000×g, and genomic DNA was eluted by distilled water. Eluted fraction was treated with isopropanol precipitation. Precipitant was rinsed with 70% ethanol dried, and resuspended with 100 μl of TE buffer.

For rumen bacterial analysis, the undigested large particles were removed from culture fluids by centrifugation at 160×g for 5 min and then total DNA was extracted by a method using glass beads as described by Purdy et al. (1996). Culture fluid (0.8 ml) was centrifuged at 16,100×g (centrifuge 5415 D, Eppendorf, Germany) for 10 min and then the pellet was extracted for total DNA after washing one time with 0.9% saline solution. The pellet was mixed with 0.35 ml of TE buffer (pH 8.0) and 0.5 ml of Tri-buffered phenol (pH 8.0) in a 2 ml tube containing 0.25 g of glass beads (Ø 0.5 mm, BioSpec Products Inc. USA). The tubes were shaken three times for 2 min with 2 min incubation on ice between shakings, after adding 40 μl of 10% SDS. Thereafter, the tubes were centrifuged at 13,000×g for 2 min and supernatant was collected in a new tube. The supernatant was purified by hydroxyapatite chromatography (Hydroxyapatite Bio-Gel HTP Gel, Bio-Rad Laboratories, Inc, CA) and then applied to gel filtration (MicroSpin S-200 HR Columns, Amersham Biosciences, UK) after treatment with DNase-free RNase. The purified total DNA was used for Real-Time PCR after measuring DNA quality by spectrophotometer (Biomate 5, Thermo Spectronic, USA).

Determination of transgenic corns

The feed corn was analyzed to quantify the introduced transgenic traits (Mon810, Bt11, Event176, T25, GA21,

Table 1. List of line-specific primers for analysis of transgenic corn in Real-Time PCR systems

Primer name	Specificity		Length	Reference
M810 2-5	Hsp-70/sense	MON810	113 bp	Luribara et al. (2002)
M810 2-3	cryA(b)/anti-sense			
Bt11 3-5	Adh1-1S/sense	Bt11	127 bp	Luribara et al. (2002)
Bt11 3-3	cryA(b)/anti-sense			
E176 2-5	cryA(b)/sense	Event176	100 bp	Luribara et al. (2002)
E176 2-3	PEPC#9 intron/anti-sense			
T25 1-5	pat/sense	T25	149 bp	Luribara et al. (2002)
T25 1-3	t35S/anti-sense			
GA21 3-5	OPT/sense	GA21	133 bp	Luribara et al. (2002)
GA21 3-3	m-epsps/anti-sense			
TC-F195	ubiquitin/sense	TC1507	251 bp	Lee et al. (2004)
TC-R445	cry1F/anti-sense			
NK-F163	Hsp-70/sense	NK603	231 bp	Lee et al. (2004)
NKR-393	epsps/anti-sense			
CRTA7-F	Cry3Bb1/sense	MON863	111 bp	Lee et al. (2006)
CRTF7-R	Tahsp17/anti-sense			

TC1507, NK603 and MON863) with each primer pair (Table 1). The amplification of target DNA was monitored with TaqMan Probe (Eurogentec, s.a., Seraing, Belgium) using ABI PRISM 7700 SDS (Applied Biosystems, Foster City, CA), as described by Kuribara et al. (2002) with some modification. A reaction solution (25 μl) contained 12.5 μl of TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA), 2.5 μl of template DNA and 10.0 μl of primer probe mix containing 1.25 μM primer and 0.5 μM TaqMan Probe. The reaction conditions were set with the following PCR step-cycle program: 2 min at 50°C, and 95°C for 10 min and 40 cycles, 30 s at 95°C, and 1 min at 59°C. The copy number of each sample was calculated as the mean value of triplicate amplification results, which were compared with the calibration curves established with 0, 20, 125, 1,500, 20,000, 250,000 copies of reference molecules per reaction. Salmon testis DNA (Sigma, MO, USA) was used as negative control for analysis. GM corn amounts (%) were calculated from the copy number as previously reported (Kuribara et al., 2002).

For qualitative analysis of transgenic corns, the PCR products were separated using 0.8% (wt/vol) and 2.0% agarose gels containing 1 μg/ml of ethidium bromide. Electrophoresis was carried out at approximately 100 V for 1.5 to 2.0 h, and the DNA was then visualized under UV light.

Analysis of fermentative parameters and digestibility

The collected samples were used to analyze digestibility, pH change, NH_3-N, gas accumulation and VFA production. DM digestibility was calculated from the reduced amounts of dry matter after incubation. NDF and ADF were analyzed by Van Soest et al. (1991) for the determination their digestibility. The pH value was measured in culture medium by Mettler Delta 340 (Mettler Electronics, UK). Accumulated gas in the head space of the serum bottle was detected by detachable pressure transducer and digital readout voltmeter (Laurel Electroincs, USA) and then calculated from a standard curve by plotting digital value obtained from serially different amounts of gas. CH_4 and H_2 were measured by gas chromatography (Varian 38000, USA) with a Carbosieve S 8100 mesh column (Supelco Inc., Bellafonte, PA, USA). VFAs were determined by gas chromatography (HP6890 GC System, Hewlett Packard, USA) with a flame ionization detector and a 30 m×0.32 mm ×0.25 μm high performance capillary column (HP-FFAP, Hewlett Packard, USA) after removed particle in liquid sample according by Kumar and Dass (2005).

Quantification of *F. succinogenes* and *S. bovis* by RT-PCR

RT-PCR reactions for quantifying rumen bacteria were completed with the iCycler iQ real-time PCR detection system (Bio-Rad Laboratories Inc., CA). The PCR amplification was performed by the iQ Syber Green Supermix (Bio-Rad Laboratories Inc., CA) with species-specific primer sets: 5′-GGTATGGGATGAGCTTGC-3′ and 5′-GCCTGCCCCTGAACTATC-3′ targeting *F. succinogenes* and 5′-CTAATACCGCATAACAGCAT-3′ and 5′-AGAAACTTCCTATCTCT AGG-3′ targeting *S. bovis* (Tajima et al., 2001). RT-PCR conditions involved one cycle at 95°C for 3 min for initial denaturation, and 40 cycles of denaturation at 95°C for 30 s followed by annealing at 62°C (*F. succinogenes*) and 57°C (*S. bovis*) for 30 s and then an extension at 72°C for 30 s. Thereafter, the melting point of PCR product was analyzed to detect specificity of application. The melting curve was obtained by a 0.1°C/s increase of heating temperature from 65 to 95°C with fluorescence detection at 0.1°C intervals.

Bacterial population was defined as log copy number of 16S rDNA which was calculated from the standard curve of 16S rDNA plasmid. Standard curves were made by plotting

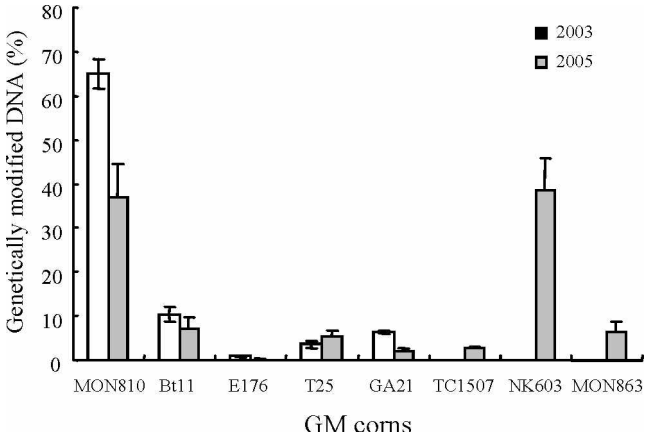

Figure 1. Proportion of transgenic corn in feed corn.

C_t value resulting from the serial dilution of 16S rDNA plasmid. The 16S rDNA plasmid inserted space-specific fragment was constructed using pGEM-T and pGEM-T Easy Vector System (Promega, USA) according to the manual description.

Statistical analysis

The differences in bacterial populations, digestibilities, pH values, amounts of gas accumulation and VFA production were determined using PROC ANOVA of SAS (1989). When the F-test yielded significant (i.e. $p<0.05$) results, multiple comparisons were made using the least significant difference (LSD) test (Steel and Torrie, 1986).

RESULTS AND DISCUSSION

Detection of transgenic DNA from corns

At present, it is possible to qualitatively and quantitatively analyze eight lines of transgenic maize: MON810, Event176, Bt11, T25, GA21, NK603, TC1507 and MON863 (Kuribara et al., 2002; Lee et al., 2004; 2006). Feed corns imported from the USA were used for transgenic DNA monitoring, and the results are summarized in Figure 1. It was found that six of the above lines of transgenic traits excluding TC1507 and MON863 were introduced to feed corns on 8 sampling times in the year 2003, and all traits to feed corns on 6 sampling times in the year 2005. The transgenic corns comprised more than 80% of the imported feed corns in the year 2003 with the highest mixed rate being MON810 (65%). However, the imported feed corns in the year 2005 contained over 90% of transgenic corns including TC1507 and MON863 transgenic traits. Major transgenic traits were MON810 (37%) and NK603 (38%), and other traits were less than 10%, respectively. Transgenic corns might have increased around 25% in feed corn imports of year 2005 compared with that of 2003.

These results suggested that various transgenic corns for herbicide tolerance and/or insect resistance were imported

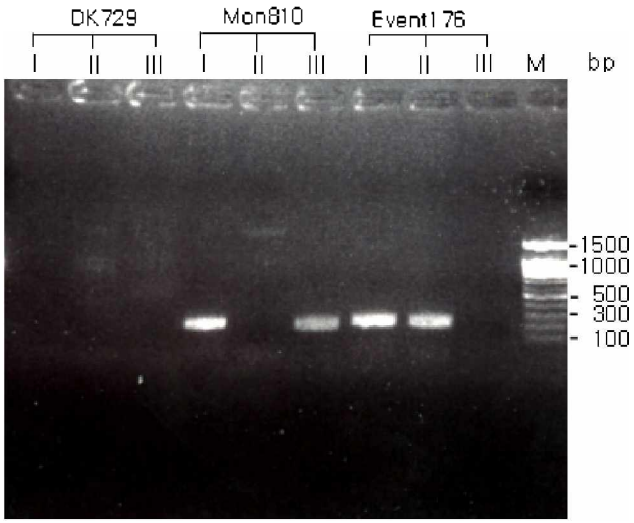

Figure 2. Detection of transgenic DNA for identification of isogenic and transgenic corn. Lane I (220 bp) was amplified by general primers for GM corn (CM01 and CM02). Lane II (211 bp) and Lane III (196 bp) were amplified by specific primers for Event176 (CDPK-cry03 and CDPK-cry04) and Mon810 (HS01 and cry-CR01), respectively. Lane M was standard ladder.

to Korea and incorporated into animal feeds. Recently, a new GM maize line, insect-resistant MON863, was added to the approved transgenic maize line for commercialization and importation in Korea (Lee et al., 2006). Also, another GM line is waiting for approval for importation and more new GM lines will be developed by many agricultural biotechnology companies. Accordingly, constant monitoring of imported maize including other feed sources is needed to provide the consumers with the option to select their preferred products. Many countries have established labeling systems based on their own criteria, with thresholds for unintentional mixing of GM crops restricted to 0.9% in the EU (The European Parliament and the Council of the European Union, 2003), 3% in Korea (Ministry of Agriculture and Forestry of Korea, 2000), and 5% in Japan (Ministry of Agriculture, Forestry and Fisheries of Japan, 2000).

During the PCR analysis of transgenic maize, the grains showing positive reaction on specific primer pairs of MON810 and Event176 were separately collected for *in vitro* studies. Collected transgenic corns (Mon 810 and Event176) and isogenic corn (DK729) was confirmed to be contaminated by other traits and their qualitative analysis are presented in Figure 2. At this time, the general primer was CM01 and CM02 for transgenic corns, and specific primers were CDPK -cry3 and CDPK-cry 04, and HS01 and cry-CR01 for Event 176 and Mon810, respectively (Chiueh et al., 2001). Transgenic corns (Mon 810 and Event176) positively responded on general primer and separately acted only for each specific primer. However, in

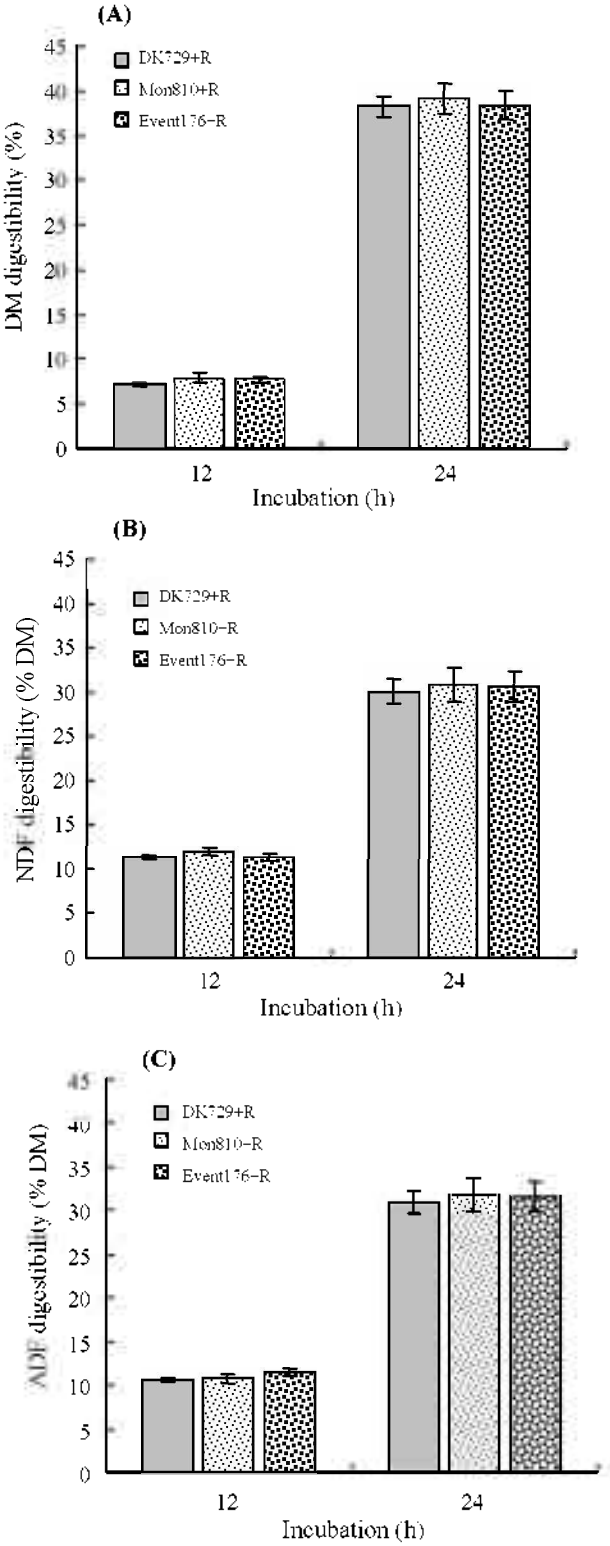

Figure 3. Digestibility of dry matter (A), NDF (B) and ADF (C) of experimental diets with non- and GM corn in rumen fermentation *in vitro*.

isogenic corn (DK729), no band was found in all primer pairs. Therefore, these collected corns in their pure forms were used in the next *in vitro* experiments.

Digestibility of fiber as influenced by transgenic corns

This study was undertaken to compare the *in vitro* digestibility of basal diets that involved rice straw as fiber source and either isogenic or transgenic feed corns. The digestive values for the *in vitro* diets containing isogenic corn and rice straw (DK729+R), and two traits of transgenic corns and rice straws (Mon 810+R and Event176+R) are presented in Figure 3. There were no significant ($p>0.05$) differences in digestibility of dry matter, NDF and ADF at 12 h and 24 h of incubations, respectively. These results indicate that transgenic corn did not influence rumen microbial digestion of corn or rice straw.

Similar digestibility was observed between non-*Bt* and *Bt* hybrid pairs for *Bt* Mon810 transgenic event (Faust, 1999). *In vitro* digestibility of DM and cell wall from Bt-corn (73.6 and 45.6%, respectively) and non-Bt corn silage (72.6 and 45.6%, respectively) harvested at 1/4 to 1/3 milk line were not significantly ($p>0.05$) different. Also, there was no significant difference at black layer stages of development. In another study, Folmer et al. (2002) reported no effect of *Bt* trait on *in situ* digestion kinetics of NDF. The fractional rate of NDF digestion averaged 0.03 h^{-1}, and the potential extent of NDF digestion averaged 59.2%. Donkin et al. (2003) reported no difference in rumen degradability, determined separately for corn grain and corn silage, for RR-GA21 or Bt-MON810 hybrids compared with their respective controls. We also observed a similar trend in our experiment which is further supported by data on nutritional composition (Brake and Vlachos, 1998; Sidu et al., 2000; Phipps et al., 2005).

In contrast, Masoero et al. (1999) observed increased stover degradation in the CR-Bt$^-$ variety (cry1A(b) addition by Monsanto Italy), probably as a consequence of higher content of lower structural carbohydrates. A major structural component of plant cells, lignin was significantly higher (39-97%) in all hybrids of *Bt* corn than in their non-*Bt* isolines (Saxena and Stotzky, 2001).

Effects of transgenic corns on rumen fermentation parameters

Rumen fluid and fermented gas were examined to see if transgenic corns had an influence on fermentative characteristics during *in vitro* rumen fermentation. The pH and NH_3-N concentrations during the incubation are presented in Figure 4 and 5, respectively. VFA concentration and gas production are summarized in Table 2.

As shown in Figure 4 and Table 2, there were no significant differences ($p>0.05$) between isogenic and transgenic corns on pH or VFA throughout the *in vitro* rumen fluid sampling. The pH was unaffected by the trait of corn and decreased with VFA accumulation for isogenic and transgenic corns with incubation time. The VFA concentrations were increased during the incubation in all

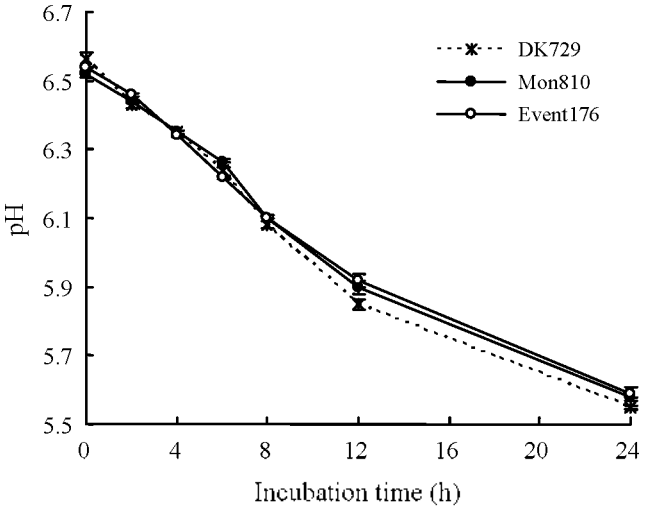

Figure 4. The pH change during rumen fermentation *in vitro* with isogenic and transgenic GM corn.

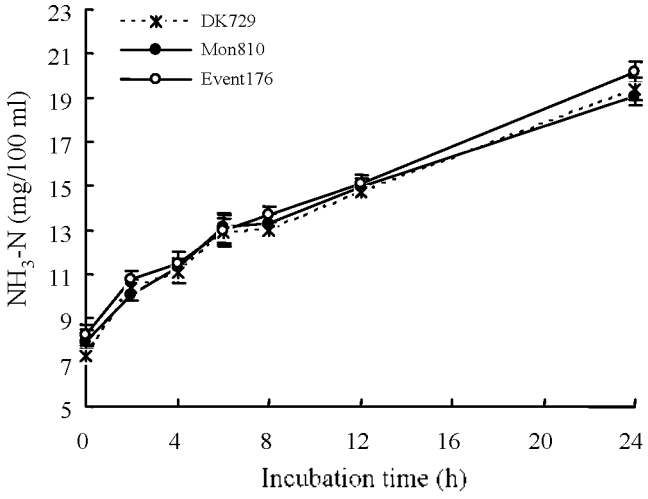

Figure 5. NH_3-N concentration during rumen fermentation *in vitro* with isogenic and transgenic GM corn.

treatments and the trend was also similar. There was no influence of the corn trait on concentrations of total VFA, acetic, propionic, butyric and valeric acids; therefore only means after 24 h of incubation are presented.

These findings were confirmed when rations containing isogenic or transgenic event CBH351 (Starlink)-derived hybrid corn (SL) were fed to dairy cows by Yonemochi et al. (2002), with no adverse effects on rumen fermentation. The rumen pH ranged from 7.1 to 7.3 and total VFA from 200.6 to 209.9 mmol/L during the experimental periods. Fomer et al. (2002) also examined the rumen fermentation characteristics of *Bt* and non-Bt maize silage from either early or late maturing hybrids and did not detect any adverse effects on rumen pH, rumen VFA concentrations and *in situ* NDF degradability.

The effects of transgenic corns on fermented gas accumulation and NH_3-N production are presented in Table 2 and Figure 5. Gas production increased with time course of incubation in all the treatments (DK729+R, Mon 810+R and Event176+R). There was no significant difference ($p>0.05$) in the total gas production as well as CH_4 and H_2 concentrations at all sampling times; therefore, only means after 24 h of incubation are presented. No significant difference ($p>0.05$) was found in the NH_3-N production between isogenic and transgenic corn. These results reflect that transgenic corn had no influence on corn as well as on the *in vitro* rumen fermentation.

Folmer et al. (2002) also reported nitrogen and protein fractions (A, B_1, B_2, B_3 and C) of hybrid maize silage in beef and dairy experiments. They found similar protein fractions between N7333Bt and non-N7333Bt corn silage, 3.13 vs. 3.49 and 2.83 vs. 2.85 for non-protein nitrogen and insoluble protein, respectively. No significant difference was observed in the amino acids between isogenic and transgenic corn by Brake and Vlachos (1998) and Clark and Ipharraguerre (2001). These reports confirm our results on the NH_3-N production by microbial fermentation.

The population of *F. succinogenes* and *S. bovis*

F. succinogenes and *S. bovis*, the major cellulolytic and amylolytic bacterial species, respectively, were quantified to compare the influence of transgenic corn on rumen microbial populations (Figures 6 and 7). Their populations were not affected by transgenic corn or its trait. *F. succinogenes* did not show any significant difference between isogenic (DK729+R) and transgenic corn (Mon810+R and Event174+R) after 12 and 24 h of incubation ($p>0.05$) with minor increase in the population. Similarly, there was no significant ($p>0.05$) difference in *S. bovis* population between isogenic and transgenic corn.

Table 2. *In vitro* gas and VFA production after 24 h of incubation with isogenic and transgenic GM corn

Item	DK729+R	Mon810+R	Event176+R	SEM	Significance
Total VFA (mM)	111.92	104.98	107.19	11.135	NS
Acetic acid (mM)	59.42	56.87	57.04	6.788	NS
Propionic acid (mM)	23.21	21.37	22.51	2.170	NS
Butyric acid (mM)	23.81	22.34	22.98	2.140	NS
Valeric acid (mM)	1.55	1.40	1.55	0.152	NS
Total gas (ml)	53.08	57.90	58.23	3.839	NS
CH_4 (mM)	10.00	11.35	10.43	1.466	NS
H_2 (mM)	0.07	0.07	0.08	0.013	NS

NS: Non-significant ($p>0.05$).

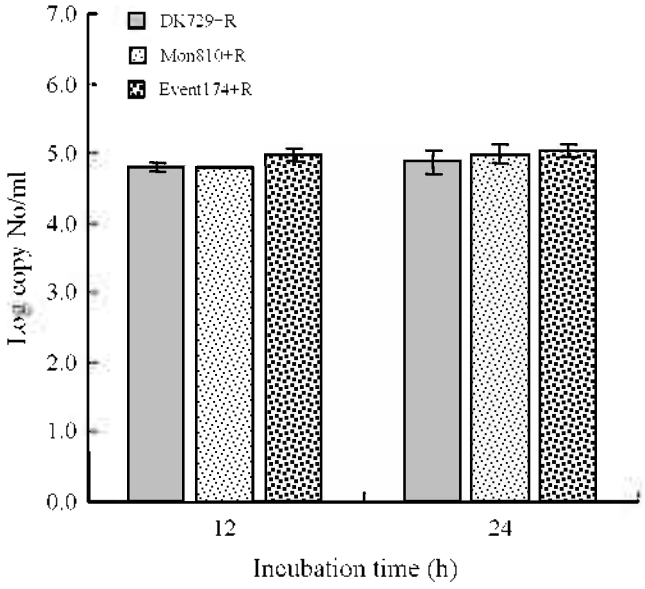

Figure 6. Comparison of *F. succinogenes* population between isogenic and transgenic GM corn.

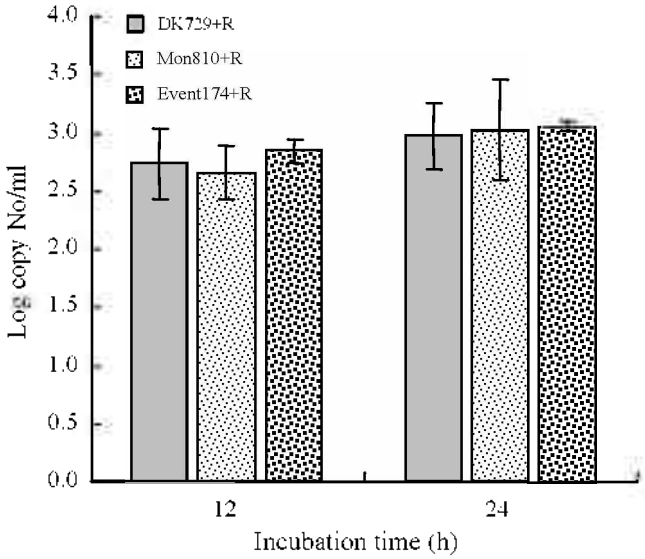

Figure 7. Comparison of *S. bovis* population between isogenic and transgenic corn.

Yonemochi et al. (2003) observed no effect of transgenic corn on cell density of protozoa when dairy cows were fed diets contained non-SL or SL, respectively, for 5 weeks. When the influence of Cry1Ab transgenic maize on rumen bacterial microflora was investigated to compare isogenic material through analysis of 497 individual bacterial 16S rDNA sequences, no significant influence of Bt176 maize feed was found on the composition of the microbial population (Einspanier et al., 2004).

CONCLUSION

The current study showed that the various transgenic corns having herbicide tolerance and/or insect resistance are being utilized as animal feeds. In the present experiment transgenic corns (Mon810 and Event176) did not influence rumen bacterial fermentation as compared to isogenic corn (DK729). However, there are other transgenic lines waiting to be approved for importation and new diverse traits are being developed by many agricultural biotechnological companies. There are increased public and scientific concerns about the safety of transgenic feeds for animals and consequently for humans consuming animal products. Accordingly, GMO monitoring of animal feed is constantly needed with rigorous evaluation of their long-term influence on animal production and health to provide consumers with options to select their preferred products.

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