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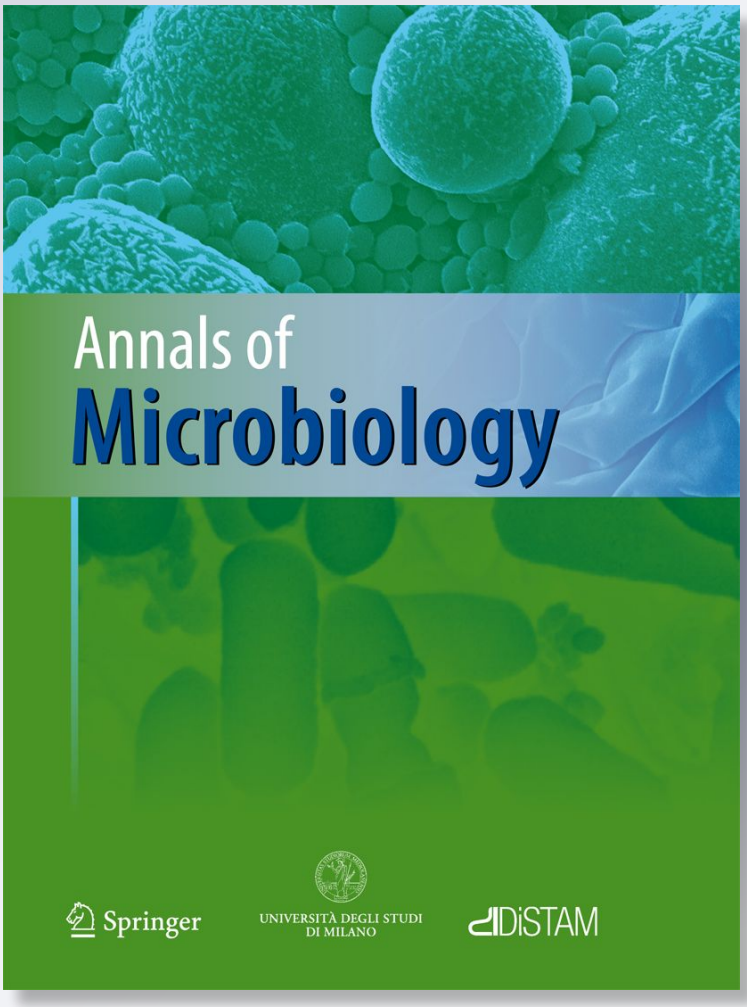

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ORIGINAL PAPER

Influence of transgenic Bt176 and non-transgenic corn silage on the structure of rumen bacterial communities

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Abstract The aim of the present study was to investigate the possible effects of a diet containing transgenic corn (*Zea mays*), cultivar *Bt*176 expressing the insecticidal protein CryIA(b), on bacterial diversity of the cow rumen in vivo. Silage prepared with *Bt*176 corn and its parental non-transgenic cultivar showed no significant differences in either composition or in vivo degradability. Four cows were fed for 35 days with the different silage in a cross-over feeding experiment. The overall structure of rumen bacterial populations was analyzed using a cultivation-independent approach—ribosomal intergenic spacer analysis (RISA)—during 3 consecutive days of each feeding trial in all cows. Planktonic bacterial populations present in the rumen fluid and biofilm grown directly on the silage were analyzed separately. Statistical analysis of RISA fingerprints did not reveal significant differences in bacterial community structure between different feedings. Small differences were nevertheless observed between individuals on the structure of bacterial communities directly colonizing the silage material. A diet constituted by transgenic *Bt*-corn silage was demonstrated not to be substantially different from non-transgenic corn silage in terms of composition, digestibility and impact on the overall rumen microbiota.

Keywords Rumen · *Bt*176 corn · RISA fingerprinting · Bacterial population structure · Silage

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Introduction

The rumen habitat is characterized by complex microbial communities with a large degree of overlap in substrate degradation capabilities and cross-feeding nets (Hungate et al. 1988; Stahl et al. 1988; Tajima et al. 2000; Ziemer et al. 2000; Sadet et al. 2007; Lanyasunya et al. 2007; Comlekcioglu et al. 2008; Shelke et al. 2009). The structure and diversity of rumen microbiota is strictly influenced by animal feeding behavior. The chemical characteristics of the forage and the type of plant cultivar used for ensiling were demonstrated to have important effects on animal performance (Folmer et al. 2002).

Insect-resistant *Bt*-corn transformed by the insertion of the *cry1A*(*b*) gene expressing the insecticidal protein CryIA(b) from *Bacillus thuringiensis* has been used widely in many countries as starting material for ensiling for livestock nutrition. Silage made with different corn cultivars has been demonstrated to host different bacterial communities and may have different nutritional values (Rossi et al. 2003; Brusetti et al. 2008). Recombinant DNA and proteins are degraded rapidly in the rumen fluid (Duggan et al. 2000, 2003; Alexander et al. 2004; Wiedemann et al. 2007; Tremblay et al. 2008) due to the pH of the rumen and to the high DNAse activity in its fluid (Flint and Thomson 1990). These findings explain why transgenic corn was demonstrated to have only limited effects on the final performances of livestock (Folmer et al. 2002; Donkin et al. 2003; Sung et al. 2006). However, little information is available on the effects of a diet based on transgenic corn silage on the microbial diversity of the rumen.

Bacteria are the most represented group among the rumen microbiota, and are responsible for various important functions such as fiber digestion, acid utilization, protein and tannin degradation (Kamra 2005). Recently, Wiedemann et al. (2007) showed that transgenic *Bt*176 corn had no effect on the density of six typical abundant cultivable bacterial species of the cow rumen. However, being limited to only a few species, this latter research cannot give a complete picture of the effects of transgenic corn on the complex bacterial population of the rumen, which remains largely unknown and composed mainly of synthrophic, non-cultivable species (Edwards et al. 2004).

This study aimed to evaluate the possible effects of a *Bt*176-based diet on the overall bacterial diversity of the cow rumen. For this purpose, a cultivation-independent DNA-fingerprinting approach was applied to describe the bacterial structure of rumen populations that, even if affected by PCR-related biases (Von Wintzingerode et al. 1997), is nevertheless not restricted to cultivable species, which are estimated to represent only 11% of the total rumen populations based on 16S rRNA gene analyses (Edwards et al. 2004).

Materials and methods

Corn ensilage

The commercial corn lines, cultivar (cv) Tundra (Novartis, class FAO 600) and its genetically modified line, cv Tundra *Bt*176 were used in the experiments. A third commercial cultivar (Novartis Proxima) was used in the pre-trial period. The different cultivars were grown in Lleida (Spain), at a density of six plants m^{-2}, fertilized with $N:P_2O_5:K_2O$ (300:100:100). Plants were harvested at two-thirds milk line stage of maturity after 174 days from seeding, chopped (average length 1 cm) and ensiled in small plastic silos (150 L) for 90 days. After the ensiling period, silage was analyzed in triplicate for chemical composition by the application of standard protocols (ASPA 1980). The presence of the transgene construct in cv Tundra *Bt*176 was verified by PCR using established protocols (Rizzi et al. 2001, 2003), both in the corn plants and in the silage.

Cow pre-feeding and feeding conditions

The feeding experiment was carried out with four Italian Friesian dry cows (average body weight 690 kg), named A–D. Access to the rumen content was assured by a rumen fistula (diameter 20 cm). Animals were fed twice daily (08:00 and 16:30 hours) with an individual dry matter intake (DMI) of 10.9 kg that consisted of corn silage (transgenic or parental; 30% of DMI), grass hay (57% of DMI) and a commercial non-transgenic feed (17 % of DMI, composed by corn grain, soybean meal and wheat bran). Before the feeding experiment (pre-trial period), rumen microbial communities were normalized by feeding the cows for 56 days with the same mixture used in the experiment, but with a silage made with a third non-transgenic corn cultivar (cv Proxima).

Experimental design and degradability analysis

The experimental plan consisted of the cross-over feeding experiment described in Fig. 1. During each feeding trial, two nylon bags (20×10 cm; 2.5 g; mesh size 53±10 μm) were inserted into the rumen, filled with 120 g corn silage, of the same type as that used for feeding. The nylon bags were fastened in a 60 cm-long cannula, completely

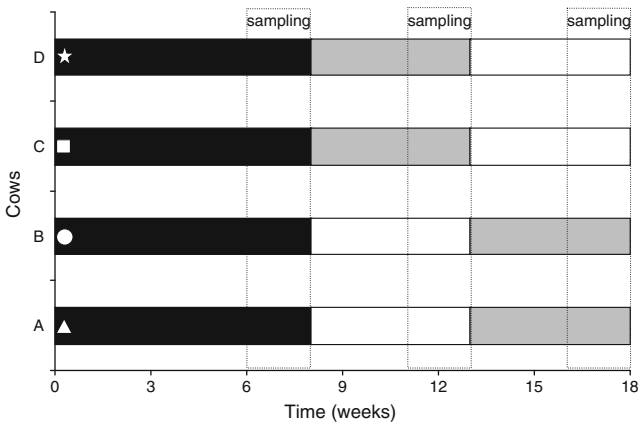

Fig. 1 Experimental plan. Type of diet administered to the four cows during pre-trial and trial periods. Sampling was performed during the last 2 weeks of each feeding period. *Black* Pre-trial feeding, *gray* parental non transgenic feeding, *white* *Bt*176 corn feeding

included in the rumen, with weights to place nylon bags in the upper and in the lower part of the rumen, on each sampling day at 08:00 hours for 4 h. During the experimental trials, the physiological status of the animals was recorded by measuring ruminal pH and ammonium concentration by the application of standard analysis protocols (AOAC 1980). Degradability of silage in the rumen was determined in a separate experiment after in situ incubation of the nylon bags in the cow rumen (up to 168 h, ASPA 1994).

Sampling and DNA extraction

For each feeding experiment, from each experimental cow, three samples of rumen fluid (10 ml) and three samples of nylon bag content (1 g) were collected on 3 consecutive days and immediately frozen at −20°C. In total, 36 rumen fluid samples and 14 nylon bags samples were analyzed for the structure of bacterial communities. Our sampling design was planned to study separately (1) the planktonic populations present in the rumen fluid, and (2) the populations attached to the nylon bag content that actively colonize the diet material and interact directly with corn silage (Hungate 1988; Stahl et al. 1988; Dehority and Orpin 1997; Tajima et al. 2000).

Total DNA extraction was performed on 100 μl rumen fluid by adding 860 μl extraction buffer (Tris-HCl pH 8 10 mM, NaCl 150 mM, EDTA 2 mM, SDS 1%), 40 μl of 20 mg/ml proteinase K, 100 μl guanidine isothiocyanate 5 M (Sigma, Milan, Italy), and incubating 3 h at 60°C. Purification of the lysate was performed with the Wizard DNA purification Prep kit (Promega, Milan, Italy) following the manufacturer's instructions. DNA extraction from nylon bag content was performed on 1 g material ground in a mortar with liquid nitrogen. EB1 buffer (4 ml; NaCl 350 mM, Tris-HCl pH 8, 50 mM, EDTA 20 mM, SDS 2%, urea 8 M) was added, and the mixture was incubated at 37°C under agitation for 10 min. The suspension was extracted with an equal volume of chloroform, and 3 ml of the resulting supernatant was mixed with an equal volume of isopropanol. After centrifugation (5,000 rpm at 4°C for 30 min), the pellet was resuspended in 2 ml of TE pH 8 (Sambrook and Russell 2001) and extracted with an equal volume of phenol:chlorophorm:isoamyl alcohol (25:24:1). Nucleic acids were then precipitated with an equal volume of isopropanol and resuspended in TE pH 8.

DNA fingerprinting analysis

Ribosomal intergenic spacer analysis (RISA) was applied to the total DNA extracted from rumen samples. Internal transcribed spacers (ITS) were amplified from 50 ng extracted DNA as previously described (Daffonchio et al. 2000) but with primers ITSF and ITSReub (Cardinale et al. 2004). Amplified fragments were separated through a mutation detection enhancement (MDE) non-denaturing acrylamide gel electrophoresis (Daffonchio et al. 2000).

The banding patterns were acquired from the silver-stained gels with the Gel Doc2000 image system (BioRad, Milan, Italy), and computer-assisted analysis of the fingerprints was performed using the Diversity Database Fingerprinting Software (BioRad, Milan, Italy). A binary matrix reporting the presence/absence of each polymorphic band was generated and analyzed using detrended correspondence analysis (DCA) (Olapade et al. 2005) using the MVSP software package (Kovach Computing Services, Anglesey, UK).

Table 1 Fermentation parameters and chemical analyses measured on transgenic *Bt*-corn silage and the parental corn silage. *dm* Dry matter, *ADF* acid detergent fiber, *NDF* neutral detergent fiber, *ADL* acid detergent lignin

Parameter	Units	Parental corn silage	*Bt*176 corn silage	Variation (%)	*P*
Ethanol	g/kg dm	8.85±0.83	7.71±0.08	−12.8	0.280
Acetic acid	g/kg dm	8.00±2.57	6.93±1.34	−13.4	0.431
Propionic acid	g/kg dm	0.52±0.35	0.34±0.18	−34.0	0.370
Isobutyric acid	g/kg dm	1.09±0.58	0.91±0.16	−16.5	0.656
N-Butyric acid	g/kg dm	0.48±0.10	0.59±0.36	+24.0	0.646
Lactic acid	g/kg dm	39.0±4.6	23.6±6.6	−39.6	0.058
Crude proteins	g/100 g dm	6.49±0.60	6.15±0.01	−5.09	0.576
Ether extract	g/100 g dm	2.59±0.01	2.51±0.30	−3.09	0.778
Crude fibre	g/100 g dm	17.7±1.9	18.0±1.9	+1.38	0.465
Ash	g/100 g dm	4.66±0.19	6.03±2.12	+29.5	0.555
NDF	g/100 g dm	40.0±2.4	39.7±3.1	−0.90	0.614
ADF	g/100 g dm	20.6±0.9	21.2±1.5	+2.6	0.455
ADL	g/100 g dm	1.24±0.05	1.02±0.16	−17.4	0.214
Starch	g/100 g dm	32.0±1.5	31.2±4.8	−2.55	0.790
Soluble sugars	g/100 g dm	0.95±0.81	2.46±1.17	+159	0.109

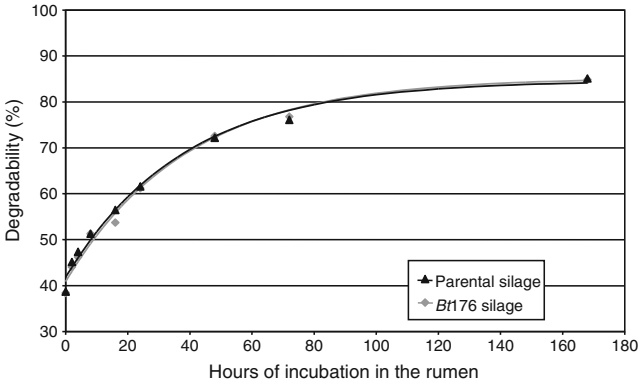

Fig. 2 In vivo degradability of the different silages used during the experimental work

Results

There were no statistically significant differences between the transgenic and the non-transgenic silages in terms of chemical analyses and fermentative parameters ($P<0.01$; Table 1). The absence of difference between silages was confirmed by the parameters describing the physiological status of the animals, i.e., ruminal pH and ammonium, ($P<0.01$, data not shown). Degradability was very similar for transgenic and non-transgenic silage, reaching a value of 85% after 168 h of incubation in the rumen (Fig. 2), with no significant difference between the two corn cultivars.

The structure of bacterial communities was analysed by RISA fingerprinting (Daffonchio et al. 2000). Internal transcribed spacers are commonly exploited as molecular markers for the analysis of the genetic relationships between bacterial species (Gürtler and Stanisich 1996; Daffonchio et al. 1998a, b). The ITSF and ITSReub primers used for ITS amplification are theoretically able to amplify more than 19,000 bacterial strains from 18 different bacterial divisions (Cardinale et al. 2004). Moreover, ITS separation on an MDE acrylamide matrix increases the informativity of the fingerprints obtained, allowing the detection of additional bands produced by heteroduplex molecules due to the annealing of long and short ITS single strands during PCR amplification (Daffonchio et al. 2000). As a prerequisite for a robust comparative analysis of the bacterial diversity in rumen samples, the reproducibility of the RISA method had to be checked. Three samples of ruminal fluid for each cow were subjected to DNA extraction in two separated parallel replica experiments. RISA fingerprinting was applied to each DNA aliquot and the fingerprints obtained were analysed. The percentage of the average similarity between replicated samples was 81.0±9.2. Based on these results, we considered 71.8% as the minimum threshold to define significantly different RISA profiles.

RISA fingerprinting was then applied to the total DNA extracted from samples of ruminal fluid and from the nylon bag content. The fingerprints contained 57.9±7.6 and 56.3±6.7 bands for the liquid and solid fraction, respectively, for a total of respectively 263 and 219 polymorphic bands, confirming the high informativity of the method and indicating that the bacterial populations, planktonic or directly colonising the silages, have similar size. Samples of ruminal fluid showed 32.7% of the bands common between all the animals tested, while samples of nylon bag content had only 15.5%, indicating that individual variability has a stronger influence in the selection of silage colonising populations, rather than the planktonic species.

DCA of the similarity matrix generated from the fingerprints was performed to describe the variability existing between the animals and the diets. The results indicated that samples of both liquid and solid ruminal content are not

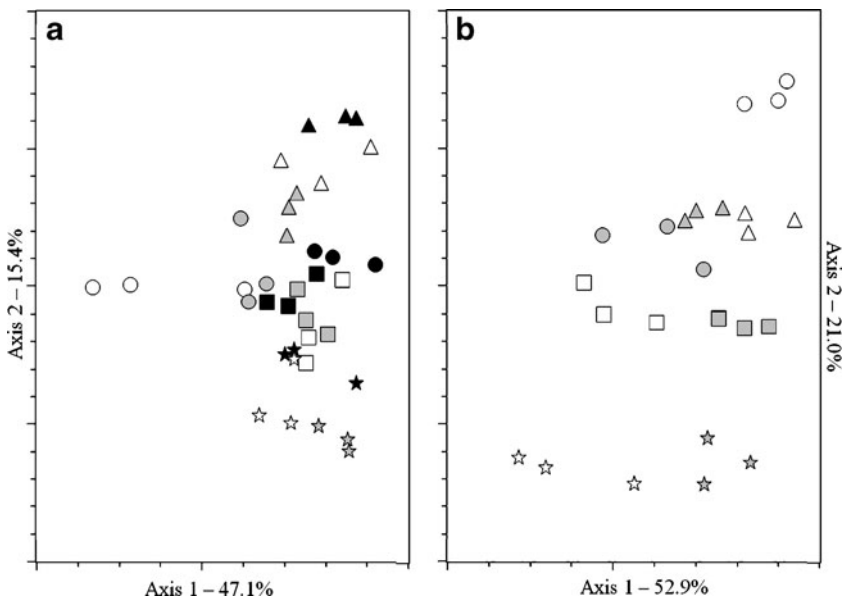

Fig. 3 Detrended correspondence analysis (DCA) of ribosomal intergenic spacer analysis (RISA) fingerprinting of rumen fluid (**a**) and of silage contained in nylon bags placed directly in the rumen (**b**). The two-dimensional plots explain 62.5% (**a**) and 73.9% (**b**) of the total inertia. Different individuals are indicated by different shape symbols, while colors distinguish different diets as reported in Fig. 1. *Triangles* Cow A, *circles* cow B, *squares* cow C, *stars* cow D

significantly differentiated depending on the diet, i.e., that the different diets supplied to the cows did not induce substantial changes in the bacterial communities (Fig. 3). In particular, the *P* values between the different diets were 0.156 and 0.357, respectively, for axis 1 and 2 in the analysis of the rumen fluid samples, while they were 0.296 and 0.468, respectively, for axis 1 and 2 in the analysis of the nylon bags samples. In the rumen fluid samples, no significant differences were seen between the pre-trial and the trial period, indicating that the conventional corn cv Proxima also had no distinct influence on the structure of the planktonic rumen microbiota. No significant differences were observed between individuals along axis 1 (P=0.135) and axis 2 (P=0.558) for the analysis of the rumen fluids. Although no differences were visible along axis 1 (P= 0.165), weak significant differences were seen along axis 2 of the nylon bags analysis (P=0.076), indicating that diverse individuals could induce a weak selection only on the populations directly interacting with the plant material.

Discussion

Variation in bacterial species composition between individuals fed with different diets has been demonstrated for various ruminant species (Ferme et al. 2004; Lanyasunya et al. 2007; Boguhn et al. 2008; Goel et al. 2008), and even small changes in chemical composition of forages were strong enough to alter the bacterial population structure in the rumen, both in artificial systems and in vivo (Ferme et al. 2004; Grabber et al. 2009). Several authors have demonstrated that transgenic *Bt*-corn plants have an unexpectedly different chemical composition with respect to the parental non-genetically modified cultivars, having a higher lignin content (Masoero et al. 1999; Saxena and Stotzky 2001; Poerschmann et al. 2005), higher starch content, and lower soluble nitrogen and protein contents (Masoero et al. 1999). Our result nevertheless demonstrated that these differences are not maintained after corn fermentation by means of silage composition and degradability, physiological status of the animal fed by the silage, and structure of the rumen bacterial communities evaluated in vivo. Our data were in agreement with those of other authors. Donkin et al. (2003) found no differences in dry matter intake, milk production and milk composition in lactating dairy cows fed with *Bt*MON810 and Roundup Ready grains and silages. They similarly observed no differences in rumen degradability, as shown also by Sung et al. (2006), Hendrix et al. (2000), Folmer et al. (2002), Barrière et al. (2001). Rossi et al. (2003) found differences in rumen degradability of genetically modified 7821*Bt* corn when compared with the non-transgenic cv. However, these changes were transient and had disappeared within 24 h of ingestion.

Using a cultivation-independent technique like RISA fingerprinting, we observed no significant diet-effect on the structure of rumen bacterial populations, confirming the findings of Wiedemann et al. (2007) on the population dynamics of only six bacterial species. The populations directly colonising the diet material, when compared with the planktonic bacterial communities, were nevertheless, albeit with weak significance, influenced more by the individual, as demonstrated by a smaller number of common bands between RISA fingerprints and significant differences on axis 2 of the DCA analysis. These observations could be due to the strict contact between plant material and rumen microbiota, which can have an influence in the selection of specific microbial functional groups in diverse individuals. In the absence of any differences in the physiological status of animals fed by different silages, as demonstrated by digestibility, ruminal pH and ammonium content, the rumen constitutes therefore a more stable environment for planktonic bacteria, the diversity of which is not influenced by the individual.

These findings underline the need to investigate in detail the differences in composition of the planktonic and silage-colonizing microbial functional groups. Other open questions also remain to be answered with in vivo experiments, such as the effects of transgenic silages on archaeal and fungal populations, the effects of new generation transgenic cultivars, such as transplastomic varieties, or the interaction between transgenic cultivars and host species under different livestock management.

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