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DO NAÏVE RUMINANTS DEGRADE ALKALOIDS IN THE RUMEN?

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Abstract—Three different methods for the culture of rumen microorganisms (Hungate's technique, the Hohenheim *in vitro* gas production method, and the semicontinuous rumen simulation technique) were employed to study the influence of various alkaloids (sparteine, lupanine, cytisine, atropine, quinidine, lobeline, harmaline, arecoline, nicotine, caffeine, pilocarpine, gramine, senecionine, and monocrotaline) on rumen microorganisms. Rumen microorganisms from naïve ruminants (sheep, cattle) that had not been exposed to the alkaloids before were generally not able to degrade most of the alkaloids. Only the alkaloids pilocarpine, gramine, and monocrotaline appeared to be degradable. Rumen microorganisms from a sheep preconditioned to lupin alkaloids tolerated lupanine much better than nonadapted microorganisms, but no degradation occurred. The findings indicate that the main site of detoxification in naïve ruminants is not the rumen but more likely the liver and kidneys as in nonruminants.

Key Words—Rumen, ruminants, detoxification, alkaloids, alkaloid degradation, adaptation.

INTRODUCTION

Herbivores are often confronted with toxins in their diet and have evolved various mechanisms during evolution to overcome plant defenses (Harborne, 1993; Wink, 1993). Whereas some poisons are detoxified or degraded in the digestive tract, many others are metabolized by enzymes primarily after absorption. In vertebrates, the liver is the first postabsorptive organ and thus, responsible for the detoxification of xenobiotics. In most animals, the inducible

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microsomal mixed function oxidase (MFO) system, including the cytochrome P-450, is the major metabolizing enzyme system (Freeland and Janzen, 1974; Smith, 1992). These enzymes are substrate nonspecific and are predominantly concentrated in the liver and kidney of vertebrates. Some mammalian herbivores avoid toxicity of plant secondary metabolites by inactivating them before they reach the digestive tract. Deer are known to produce a salivary protein that binds tannins, thus allowing the animals to feed on tannin-rich plants (Robbins et al., 1987). The digestive tract itself is another potential site of detoxification. Ruminants and other foregut fermenters, for example, have evolved complex stomachs with a fermentation chamber, e.g., the rumen, where food particles are degraded by a diverse bacterial and protozoal community. The main benefits of a foregut fermentation system are the microbial degradation of cellulose and the microbial *de novo* biosynthesis of proteins. Nevertheless, many authors suggest that microbial detoxification of xenobiotics has played an important role in the evolution of the foregut fermentation system (Freeland and Janzen, 1974; Van Soest, 1994).

Plants containing alkaloids are widespread in the plant kingdom, and due to their toxicity (review: Wink 1993, 2000), many of them represent an important farming problem and are responsible for extensive poisoning of both humans and livestock. In the western USA, severe livestock losses result from the poisoning by tansy ragwort, *Senecio jacobaea*, which contains pyrrolizidine alkaloids (Huxtable, 1979; Wachenheim et al., 1992a,b). Larkspur (*Delphinium* sp.) is responsible for more cattle death than any other poisonous plant in the USA (Cheeke, 1998), and the plants known as locoweed, *Astragalus* sp. and *Oxytropis* sp., which contain swainsonine and other indolizidine alkaloids, are responsible for the neurological syndrome known as "locoism" in mammals (Molyneux and James, 1982). The lupin alkaloids anagyrine and ammodendrine are present in numerous lupin species in the USA and are of particular importance because of their teratogenic effect in cattle, causing skeletal deformities referred to as "crooked calf disease" (Cheeke and Kelly, 1989). In Afghanistan, over 1600 people were poisoned in 1974 by consuming wheat contaminated with seeds of *Heliotropium*, which contain high amounts of pyrrolizidine alkaloids (Mohabbat et al., 1976). *Heliotropium europaeum* and *Echium plantagineum*, both containing pyrrolizidine alkaloids, are responsible for poisoning of poultry (Pass et al., 1979), cattle (Harper et al., 1985), sheep (Seaman, 1985), horses (Giesecke, 1986), and pigs (Jones et al., 1981) in Australia. Often ruminants are the target of poisoning. This is somewhat surprising since ruminants have several potential detoxification systems (rumen, liver). We were interested in studying the role and efficiency of the rumen in detoxifying dietary toxins, especially alkaloids.

According to the position of the enlarged chamber in the digestive tract, herbivores are divided into two groups. In hindgut fermenters, the microbial

action occurs after the stomach and the small intestine and takes place in the cecum or large intestine (e.g., rabbits and horses), which is anatomically similar to the organs of carnivores and omnivores but much enlarged. In foregut fermenters, the chamber precedes the stomach with its pepsin digestion and the small intestine and is either a separate organ, e.g., the crop in the hoatzin (*Opisthocomus hoazin*), the only avian with foregut digestion, or part of a complex stomach not found in carnivores or omnivores, e.g., the rumen in ruminants (Hungate, 1988; Van Soest, 1994). Other ruminant-like digestive systems without a rumen have been found in a wide variety of other herbivores: the hoatzin, *Colobus* and *Macaca* monkeys, and camelids, and many of them are regarded as "functional ruminants" (Van Soest, 1994).

Cellulose, hemicellulose, pectins, fructans, starches, and other polysaccharides are hydrolyzed by rumen microbes to monomeric and dimeric sugars, which are further fermented to acetic, butyric, and propionic acids, methane, and carbon dioxide (Bryant, 1977; Van Soest, 1994). The gases are released by the animal and are essentially waste products, but the acids are absorbed through the rumen wall into the bloodstream and finally converted into sugars and lipids required for energy and tissue building. Proteins are hydrolyzed to amino acids and peptides, which are then deaminated to ammonia and fatty acids. The latter are further metabolized, whereas most of the bulk of ammonia is absorbed through the rumen wall to be converted into urea (Stewart et al., 1997).

The rumen ecosystem is composed of a large number of different species of bacteria, protozoa, and fungi. Rumen bacteria are predominantly strict anaerobes, although a few facultative anaerobes exist (Van Soest, 1994). Strict anaerobic bacteria are present in the rumen in numbers of about 10^{10} ml^{-1} and, together with the rumen Archaea (methanogens), are considered as the true rumen bacteria (Stewart et al., 1997). Methanogens form methane from H_2, CO_2, or formate (Hungate et al., 1970). The bacteria account for about half of the total biomass in a normal rumen but are responsible for most of the metabolic work (Van Soest, 1994; Stewart et al., 1997). There is little information concerning geographical and interspecific differences in the composition of rumen bacterial communities. Most studies in wild ruminants have been limited to microscopic observations on morphology and the Gram stain. According to Yokohama and Johnson (1988), the major species of bacteria are ubiquitous in ruminants, and there is no evidence of host specificity. *Synergistes jonesii*, a bacterium capable of detoxifying the hydrolysis products of nonprotein amino acids, such as mimosine, 3-hydroxy-4(1*H*)-pyridone (3,4-DHP), and 2,3-dihydroxypyridine (2,3-DHP), is the only well documented case of geographical differences in rumen bacterial community composition (Jones and Megarrity, 1983, 1986; Allison et al., 1990; Hammond, 1995). *S. jonesii* was isolated from the rumen of Hawaiian goats and was absent in Australian ruminants.

Protozoa are the most conspicuous organisms in the rumen. Unlike bacteria, they are not essential for the survival of the host ruminant. The most obvious and most important protozoa in the rumen are the ciliates, of which two different groups are present, both in the subclass *Trichostomatia*, the holotrich protozoa that belong to the order *Vestibuliferida*, and the entodiniomorphs that belong to the order *Entodiniomorphida* (Williams and Coleman, 1992). The latter are well adapted to the rumen environment and utilize particulate rather than soluble food materials. In contrast, the holotrichs can use mostly soluble food materials and are more aerotolerant. Most protozoa, if not all, have bacteria in vesicles in their cytoplasm, and there is evidence that the species present reflect those in the surrounding medium and include methanogens (Finlay et al., 1994; Lloyd et al., 1994). Population densities of protozoa in the rumen under normal conditions are on the order of 10^4–10^6 ml^{-1} (Williams and Coleman, 1992) and form a large proportion of the rumen microbial biomass (20–40% of net microbial nitrogen); yet their output may be minimal because of high retention and slow turnovers (Williams and Coleman, 1992; Van Soest, 1994).

The presence of anaerobic fungi in the rumen has been known since the 1970s (Orpin and Joblin, 1997). Before that, swimming zoospores were mistaken for flagellated protozoan. The contribution of fungi to the microbial biomass may be small, and their major function in the rumen may be in causing or facilitating cell wall disappearance (Van Soest, 1994).

The benefits derived from symbiosis with rumen microbes have long been recognized and include predominantly the transformation of dietary constituents into nutrients needed for growth, milk production, and energy. Because of the observation that ruminants are often more resistant to plant toxins and other xenobiotics than nonruminants, several authors have proposed that the degradation of toxic compounds present in the diet of the ruminant is conducted by the rumen microorganisms (Carlson and Breeze, 1984; Dawson et al., 1997; Cheeke, 1998; Weimer, 1998). Similarly, other authors proposed that these detoxification activities might have been a driving force during the evolution of the foregut fermentation digestive system (Freeland and Janzen, 1974; Van Soest, 1994). Sometimes, degradation activities are related to adaptive changes in the rumen microorganism populations, which result in an acquired resistance to specific toxins (Duncan et al., 2000; Duncan and Milne, 1992; Blythe and Craig, 1994; Dawson et al., 1997; Newbold et al., 1997; Odenyo et al., 1997).

In this investigation, we examined the hypothesis that rumen microbes are involved in detoxification processes in ruminants (Freeland and Janzen, 1974; Van Soest, 1994). For this purpose, the degradation of a set of 14 alkaloids (sparteine, lupanine, cytisine, atropine, quinidine, lobeline, harmaline, arecoline, nicotine, caffeine, pilocarpine, gramine, senecionine, and monocrotaline), as one of the most prominent groups of toxins, by rumen microorganisms was analyzed *in vitro*. Three more widely used methods in the study of rumen processes were

employed: batch cultures using Hungate's technique, the Hohenheim *in vitro* gas production method (HFT, "Hohenheimer Futterwert Test"), and the semi-continuous rumen simulation technique (RUSITEC).

METHODS AND MATERIALS

Alkaloids. Except for lupanine and senecionine, all alkaloids were purchased from Sigma (Munich, Germany) or Roth (Karlsruhe, Germany). Senecionine was a gift from R. Molyneux (USDA). Lupanine was isolated and purified from seeds of bitter lupins (*Lupinus angustifolius*). Alkaloids were dissolved in PBS buffer. In order to improve the solubility of some alkaloids, the pH of the stock solution was acidified with HCl. The effect of the pH of the alkaloid solutions on the respective assays was tested using the same volume of test solution without alkaloid but at the same pH.

Isolation and Quantification of Alkaloids. Samples were dissolved in 20 ml, 0.5 M HCl and incubated overnight under agitation and in darkness. In order to detect the potential presence of N-oxides, 100 mg of zinc powder were added to the corresponding samples, and these were stirred for another 3 hr under continuous agitation. The solution was made alkaline with 6 M NaOH (pH 12). Samples were extracted using a solid–liquid phase system with Isolute (ICT) as solid and CH_2Cl_2 as liquid phase. The dichloromethane extracts were collected in round-bottom flasks and evaporated under vacuum (890 mbar) at 40°C. Alkaloids were recovered from the flask with 2–3 ml CH_2Cl_2 and transferred to a vial. The solvent was evaporated under N_2 flow or overnight in a hood and protected from light. Dried alkaloid samples were stored at −20°C until GLC analysis.

Two Carlo Erba gas chromatographs were used for the GLC analysis. The first gas chromatograph (series 5100) was equipped with a DB-1 capillary column (J&W, 15 m long and 0.25 mm inner diam) and the second GLC (series 6000) with an OV-1 column (Ohio Valley, 15 m long and 0.25 mm inner diam). Both GLCs were equipped with flame ionization detectors (FID). The split was 1:5 and 1:10, respectively. Helium was used as carrier gas. The temperature of the injector and the detector were 250 and 300°C, respectively. A 1-µl aliquot of each sample was injected into the gas chromatograph for analysis. The oven temperature program used for each alkaloid was 150°C for 3 min, 150–250°C at 15°C/min, and 250–300°C at 25°C/min for lupanine, sparteine, atropine, quinidine, harmaline, senecionine, monocrotaline, and pilocarpine, and 80°C for 2 min, 80–150°C at 10°C/min, and 150–300°C at 20°C/min for arecoline, caffeine, nicotine, gramine, and lobeline. Alkaloids were quantified by using external standards consisting of a solution in methanol.

Alkaloids were identified by GLC-MS using authentic alkaloids as reference. For GLC-MS, an OV-1 fused silica capillary column (30 m × 0.25 mm) was used coupled to a quadrupole Finnigan Mat 4515 mass spectrometer. EI-MS were recorded at 40 eV and evaluated with the INCOS data system. The conditions are as follows: carrier gas He; splitless injection; temperature 250°C; oven temperature program—initial temperature 120°C, 3 min isothermal, 120–300°C, 8°C/min.

Rumen Microbial Culture Techniques. Rumen microbes are sensitive to their environment, and successful cultures must satisfy both environmental and nutritional requirements. In *in vitro* cultures, all products accumulate in the system; thus, it is necessary that the maximum levels of acidic products remain below the limiting levels of osmotic pressure and buffer capacity, especially in closed systems. For this reason, *in vitro* cultures must be diluted by about an order of magnitude with respect to both feed and organisms to avoid hyperacidity and high osmotic pressures (Van Soest, 1994).

Diluting the substrate in the culture medium increases the susceptibility of fermentation to traces of oxygen. Therefore special precautions must be taken to protect dilute concentrations of rumen microorganisms from oxygen contamination. The methanogens and pure cultures are especially sensitive to traces of oxygen (Van Soest, 1994), but batch cultures in small volumes, such as the Hungate tubes and the syringes in the "Hohenheim *in vitro* gas production method" (HFT), also require an oxygen-free atmosphere (Hungate, 1969; Van Soest, 1994). Some anaerobes and the methanogens cannot initiate growth at redox potentials greater than -0.33 V, which corresponds to 10^{-75} of the concentration of oxygen in the atmosphere or 2.5×10^{-80} mmol O_2/l (Hungate, 1969). Normally, most of the oxygen is removed from the medium by boiling followed by gassing out with CO_2, and any residual oxygen is removed by adding cysteine. Commercial sources of CO_2 usually contain traces of oxygen and need to be purified by passing the gas through heated copper turnings (Hungate, 1969).

Hohenheim In Vitro Gas Production Method or HFT. The assay consisted of rumen batch cultures kept in glass syringes and incubated for up to 2 d in a water bath. The method was based on Menke et al. (1979). To determine the fate of lupanine and sparteine incubated *in vitro* with rumen liquor and their effect on fermentation parameters, 500-mg dry matter of hay has incubated in triplicate in 100-ml graduate glass syringes (Fortuna, Germany) containing 40 ml of the *in vitro* medium with bovine or ovine rumen liquor. Syringe pistons were lubricated with Vaseline to facilitate their movement allowing space for the increasing gas volume in the syringe. Two control sets were prepared: control 1 containing no hay and control 2 containing no rumen liquor. Alkaloids were added to the treatment and control syringes at a concentration of 1 mM. Three syringes were used as blank and were incubated without alkaloids. All

syringes were incubated under anaerobic conditions at 37°C for 36 hr and shaken manually every hour for the first 6 hr and every 3 hr for the rest of the incubation period in order to avoid the accumulation of hay on the superficies of the medium. Three syringes were collected every 6 hr for the treatment group and every 12 hr for each control group. Blank syringes were incubated for 36 hr. The content of each syringe was immediately acidified to pH 3 with 0.5 ml, 7 M HCl in order to stop any microbial reaction and then stored at −20°C for later analyses. These assays were performed at the Institute for Animal Production in the Tropics and Subtropics, University of Hohenheim.

Preparation of Culture Medium for HFT. Rumen inoculum was collected from a cow and two sheep fitted with permanent rumen fistulas, kept at the University of Hohenheim (Germany), and fed on a roughage-based diet. Rumen contents were obtained through the fistulas and immediately strained through sterile cheesecloth into insulated prewarmed thermoses, avoiding formation of air bubbles. Thermoses were transported to the lab within 15 min, where the experiments were started immediately.

Culture medium was prepared under anaerobic conditions. Constantly bubbling the medium with CO_2 (Messer Griesheim GmbH 4.5), previously passed through a column of heated cooper (350°C) in order to remove any oxygen traces (Hungate, 1969), provided acceptable anaerobic condition in the medium. Rumen liquor was added to the warm culture medium (37°C) under constant agitation and gas bubbling. The composition of the culture medium was as outlined by Menke et al. (1979).

Adaptation of Rumen Microorganisms to Alkaloids Present in the Diet. To investigate the possible induction of lupanine and sparteine degradation metabolism in rumen bacteria, two merino sheep were fed during 4 wk on a diet containing increasing amounts of seeds of alkaloid-rich *L. angustifolius* (lupanine was the major, 13-hydroxylupanine and angustifoline were additional alkaloids). During the first week, the sheep (40-kg weight) obtained 2.5 mg/kg alkaloids; the dose was increased via 5, 10, and 20 mg/kg during weeks 2, 3, and 4, respectively.

Rumen Batch Cultures using Hungate's Technique. Alkaloids were studied *in vitro* following the method described in Hungate (1969). Hungate tubes with butyl rubber stoppers (Bellco Glass) were filled with 5-ml culture medium 2 (Hobson, 1969), medium RA-1, or medium RA-2 under anaerobic conditions, and inoculated with 100-µl diluted fresh rumen liquor. An aliquot of concentrated alkaloid solution was injected into each tube in order to reach a final concentration of 1 and 10 mM. Rumen liquor was obtained as explained before and diluted 1:9 in medium 2. Controls without bacteria (blanks) or alkaloids were performed. All cultures were incubated at 37°C. Three tubes from each treatment and from the control group without rumen inoculum were collected after 0, 2, and 7 d in order to measure the alkaloid contents. An aliquot (100 µl)

of each 7 d-old culture was inoculated in fresh medium under the same conditions. The alkaloid content of the refreshed cultures was measured 0, 2, and 7 d later. An aliquot of 200 μl, 6 M HCl was added to each collected tube to stop any bacterial activity. Samples were then stored at −20°C.

Culture solution was boiled and bubbled with CO_2 (Messer Griesheim GmbH 4.5) under constant agitation with a magnet until the pink color of the resazurine disappeared (10–15 min). Resazurine is used to indicate oxidation–reduction potentials above −0.042 that are known to inhibit the growth of most ruminal strict anaerobic bacteria (Hungate, 1969). The culture medium was allowed to cool to about 60°C under CO_2 flow using a cold-water bath before cysteine was added as a reducing agent. Culture medium was distributed with a glass pipette into Hungate tubes, which were gassed for 30 sec with CO_2. Gassing was maintained for 30 additional sec after the medium was inoculated. The tubes were hermetically closed with the butyl rubber stoppers and autoclaved for 15 min at 121°C. Afterwards, culture tubes were stored at room temperature and darkness until used. Tubes in which the medium had turned to a reddish color were discarded. Purified rumen fluid was obtained by autoclaving (121°C, 15 min) and centrifuging rumen liquor at 4000 rpm for 30 min.

Rumen Simulation Technique. Since the rumen is a form of open and continuous culture, many researchers have attempted to establish *in vitro* continuous cultures of mixed rumen microorganisms with a volume of more or less whole rumen contents as the starting "inoculum." The most important of these methods is the RUSITEC (Czerkawski and Breckenridge, 1977, 1979a,b; Cheng and McAllister, 1997), allowing the culture of bacteria, protozoa, and fungi that inhabit the different physiological compartments of rumen for an almost indefinite period of time (Van Soest, 1994).

The RUSITEC technique is a semicontinuous rumen fermentation system consisting of four to six reaction vessels (fermenters). Fermentation patterns and processes observed in a RUSITEC closely resemble the fermentation patterns and processes observed in the rumen *in vivo*. The control fermenters of an already going 30-d-long assay were used in this study during the last 4 d. Fermenters were kept in a steady-state fermentation phase during at least 20 d before the assays. Control fermenters were then inoculated with alkaloids and treated as explained below. Values from the five previous days (before alkaloid addition) were used as control values.

A RUSITEC assay is started by placing in each fermenter 80 g of solid rumen digesta in one nylon bag (1-mm mesh), and the experimental diet (12-g dry matter of hay, and 3.4-g calf food concentrate pellets) in a second nylon bag. Both bags are inserted (the one with rumen content above) in a perforated cage and this cage into the reaction vessel with 500-ml fresh collected rumen liquor, 200-ml RUSITEC buffer (Czerkawski and Breckenridge, 1977), and

100-ml deionized water. The fermenters are placed in a water bath at 39°C, and the perforated cages are fixed to a motor that slides them with 6- to 7-cm amplitude at about 8–9 cycles/min. The fermenters are connected to a buffer reservoir allowing a buffer flow rate of 280–290 μl/min and then filled with CO_2 (Messer Griesheim GmbH 4.5). Buffer overflow is collected in a 2 l Erlenmeyer (filled with 40 ml, 7 M HCl), which is also connected to a collection gas bag (Linde, Art. No. 037660006).

After 24 hr incubation, the buffer flow is stopped, the gas bag changed, the overflow collected, and the solid inoculum bag removed and replaced with a new bag of experimental diet. The fermenter is filled again with CO_2 and connected again to a new Erlenmeyer and a new gas bag. The buffer flow is restarted. Subsequently, the older food bag is replaced with a new one each day, and the needed samples are collected to measure the fermentation parameters of interest. The content of the food bag is washed with RUSITEC buffer and pressed to squeeze out excess liquid, which is returned to the fermenter.

The fermentation parameters decrease during the first days but reach a steady state after 5–7 d, which can be maintained indefinitely with a balanced diet and correct handling of the fermenter. The steady state must be reached before an experiment can be started. Normally, two fermenters are used as control in an experiment, feeding them with the same diet and maintaining them in the steady state. In these experiments, the rumen liquor and the solid inoculum were collected from a fistulated cow 3 hr after the morning feeding.

Fate of Alkaloids in Rumen Simulation Technique. A concentrated alkaloid buffer solution was inoculated into each fermenter to a final alkaloid concentration of 2 mM during the daily change of the food bag. Alkaloid stock solutions were as concentrated as possible in order to use the smallest possible aliquot, but in such a way that every fermenter received the same volume of stock solution. Because the RUSITEC is a semiopen system, the alkaloid concentration in each fermenter constantly decreased even without alkaloids being degraded. The concentration decrease can be predicted with the equation $C_2 = (1-(\mathrm{fr}/\mathrm{Vt})^t)C_1$, where C_1 and C_2 are the initial and final concentrations, respectively, fr is the buffer flow rate, Vt is the total volume in the fermenter, and t is time. To measure the actual alkaloid concentration in each fermenter, 40 ml of fermenter content were taken at 0, 6, 12, 24, 48, and 72 hr. Every sample was divided into two 20-ml aliquots and stored at −20°C until they were processed for GLC analyses. Any microbial activity was stopped in each sample by adding 0.5 ml, 6 M HCl.

Determination of Fermentation Parameters

pH Determination. The pH value of a freshly taken aliquot (10 ml) of the fermenter content was measured using a calibrated pH meter (WTW, Type pH-91).

NH_3 Determination. The same aliquots, used for pH measurements, were immediately used to determine ammonia concentration. A calibrated ion-sensitive electrode (Orion 9515) and an ion analyzer (Orion 920-EA) were used. Samples were first treated with 100 µl, 2 M NaOH in order to change all ammonium ions in solution to free ammonia because the electrode can measure only the latter.

Volumetric Determination of Total Gas Production. The volume of gas produced during 24 hr in each fermenter and collected in hermetic gas bags (Linde, Art. No. 037660006) was determined by using the principle of water displacement. Three 1-l calibrated cylinders were filled with water and placed upside down in a bowl with water. The content of each gas bag was transferred with a plastic tube into the cylinders. The amount of water displaced corresponds to the amount of gas produced in the respective fermenter. During this procedure, a sample of gas was collected for the gas composition analysis.

Chromatographic Analysis of the Gas Composition. A sample was taken with a 1-ml syringe (Hamilton, TLL 1001) from the gas bag to determine the composition of the gas mixture. Each sample was collected immediately before GLC analysis. Samples (1 ml each) were injected manually into a gas chromatograph (Shimadzu, GC-8A). The impulses produced by the thermal conductivity detector (TCD) were processed on an integrator (Shimadzu, CR-3A). The GC was calibrated every day with a special gas mixture (Messer Griesheim). A correction factor for each gas was calculated by the integrator from the mean value of two consecutively injected calibration samples (1 ml each).

RESULTS AND DISCUSSION

Influence of Alkaloids on Gas Production in Bacterial Cultures from Rumen. The "Hohenheimer Futterwert Test" (HFT) and the Hungate anaerobic technique were used in a first approach to follow the fate of alkaloids in the rumen ecosystem that mainly targets the bacterial members. The total gas production in the rumen correlates linearly to the production of short-chain fatty acids (Van Soest, 1994; Blümmel et al., 1997b). Its reduction is indicative of a negative effect on ruminal fermentation processes. Since rumen microbes, especially bacteria, hydrolyze plant polysaccharides to monomeric or dimeric sugars, which are further fermented to acetic, propionic, and butyric acids, methane, and carbon dioxide, any effect on the total gas production in the rumen can be interpreted as an effect on the rumen microbial community.

The quinolizidine alkaloids lupanine and sparteine, which exhibit antimicrobial activities at higher concentrations (Wink, 1984), were selected for the first pilot experiments. At a physiological concentration of 1 mM, neither lupa-

nine nor sparteine had a significant effect on *in vitro* gas production (HFT) of microbial cultures derived from rumen liquor of cattle (Table 1, column c). Both alkaloids slightly decreased gas production when rumen liquor from a sheep fed on a roughage-based diet was used (Table 1, column a). The known antibacterial properties of lupin alkaloids (Wink, 1984) could be responsible for the negative effect of both alkaloids on the *in vitro* microbial fermentation of sheep.

Using rumen liquor from a sheep fed for 1 mo on a diet containing up to 3.2% of lupin seeds (containing lupanine as main alkaloid and no sparteine), we observed that lupanine increased *in vitro* gas production whereas sparteine had a negative effect (Table 1, column b). The effect of sparteine on *in vitro* gas production is four times stronger than the effect of lupanine. An even more pronounced adaptation of the bacterial community to lupanine was observed after feeding the sheep increasing amounts of lupin seeds (diet II) for 1 mo: Rumen bacteria from a sheep fed on a diet without alkaloids (diet I) were not able to grow at an alkaloid concentration of 10 mM in contrast to bacteria from a sheep fed on diet II, which showed an even better growth at 10 mM than at 1 mM (Table 2). After 7 d of incubation in media containing alkaloids as a sole carbon source (RA-1 and RA-2), only the microbes from diet II were able to grow after reinoculation in fresh medium. This finding suggests an acquired resistance to the added lupanine (Table 2).

Feeding a ruminant on a diet containing plant toxins can modify the microbial composition of its rumen, favoring those microorganisms that can tolerate or even metabolize such toxins (Duncan and Milne, 1992; Blythe and Craig, 1994; Newbold et al., 1997; Odenyo et al., 1997; Duncan et al., 2000). This could also be the case in our experiment, thus favoring the growth of those microbes able to tolerate the presence of lupanine in the rumen. If the favored microbes play an important role in the fermentation processes, this is observed as an enhancement of total gas production (Table 1). The higher negative effect

TABLE 1. EFFECT OF LUPANINE AND SPARTEINE ON THE TOTAL GAS PRODUCTION *In vitro* IN HFT

	Gas production (ml/200 mg hay)		
	a	b	c
Control	42.8 ± 0.28n	42.9 ± 0.99n	49.9 ± 1.04n
Lupanine 1 mM	41.6 ± 0.53m	44.5 ± 0.23m	47.9 ± 1.14n
Sparteine 1 mM	41.7 ± 1.45m	36.9 ± 0.14o	48.7 ± 0.92n

Rumen liquor was obtained from a sheep fed on a roughage-based diet (a) from a sheep fed on an adaptation diet, including increasing amounts of lupin seeds (b), or from a cow fed on a roughage-based diet (c).
Values are given in means and standard error. Assays were done in triplicate. Means with different letters in a column differ at $P < 0.05$ (ANOVA).

TABLE 2. GROWTH OF RUMINAL BACTERIA IN BATCH CULTURES WITH DIFFERENT CONCENTRATIONS OF ALKALOIDS

		Lupanine		Sparteine	
Culture medium	Treatment	1 mM	10 mM	1 mM	10 mM
M2	a	+++	−	+++	−
	b	++	+++	++	+++
RA-1	a	n.d.	−	n.d.	−
	b	n.d.	**	n.d.	**
RA-2	a	n.d.	−	n.d.	−
	b	n.d.	**	n.d.	**

Bacterial inocula were obtained from a sheep fed on a diet without (a) or with (b) lupin seeds. −: Neither tolerance nor growth; ++: good growth; +++: very good growth; **: tolerance without growth; n.d.: not determined.

of sparteine after the adaptation feeding trial can be interpreted as a differential activity of both alkaloids on the rumen microbial community. Although both lupanine and sparteine have a similar molecular structure (differing only by a ketone group in position 2 of lupanine), both have different biological activities. Sparteine is more active on Na^+ channels and muscarinic acetylcholine receptors than lupanine, whereas lupanine is more active on nicotinic acetylcholine receptors (Wink, 1993, 2000; Schmeller et al., 1994). Sparteine is also more toxic to mice but less toxic to insects (Wink, 1992).

Fate of Lupanine and Sparteine in HFT. Lupanine and sparteine were incubated *in vitro* in glass syringes. Incubations were conducted in triplicate and by using rumen fluid from only one animal in each assay. Both lupanine and sparteine were stable in control incubations for 36 hr. No degradation of alkaloids was found during an incubation period of up to 36 hr in the assays with rumen liquor, neither from a cow nor from a sheep fed on a roughage-based diet. In addition, in experiments with the Hungate technique, no degradation of either alkaloid could be detected.

The recovery data were about 94% in the assays using rumen liquor from a sheep preconditioned for 4 wk on a roughage-based diet with increasing amounts of lupin (*L. angustifolius*) seeds (Table 3). Although these values were lower than those of the corresponding experiments and could indicate some degradation, the differences from controls were not statistically significant.

Fate of Various Alkaloids in Anaerobic Batch Cultures using Hungate's Technique. Rumen inocula from a "naïve" cow that had not been exposed to alkaloids showed good growth in Hungate tubes together with alkaloids of different structures and biological activities (1 mM each; sparteine, lupanine, atropine, quinidine, harmaline, cytisine, and senecionine). GLC analyses showed no degradation of alkaloids after 7 d of incubation. All tested alkaloids were also

TABLE 3. FATE OF LUPANINE AND SPARTEINE IN HFT

	Recovery of alkaloids		
Alkaloid	a	b	c
Lupanine	108.3 ± 2.5	94.5 ± 3.75	124.5 ± 17.6
Sparteine	124.5 ± 17.6	93.8 ± 7.93	97.8 ± 2.49

Rumen liquor was obtained from a sheep fed on a roughage-based diet (a), from a sheep fed on an adaptation diet including increasing amounts of lupin seeds (b), or from a cow fed on a roughage-based diet (c).
Alkaloids were determined by GLC. Values are given in means and standard error of the percent of alkaloids recovered after 36-hr incubation. Assays were done in triplicate. No statistical differences were found at $P < 0.05$ (*t*-student), compared to the initial concentrations.

stable for 7 d in control incubations without active rumen inoculum (Figure 1). It is remarkable that the pyrrolizidine alkaloid (PA) senecionine is not degraded; several authors have reported that PAs can be degraded by rumen microorganisms. We suggest that they had used rumen microorganisms from preconditioned ruminants that had been exposed to PAs before (Swick et al., 1983; Craig et al., 1986, 1992; Cheeke, 1988, 1998; Wachenheim et al., 1992a,b).

Rumen Simulation Technique. It could be argued that the negative results from our HFT and Hungate experiments are due to the fact that rumen protozoa

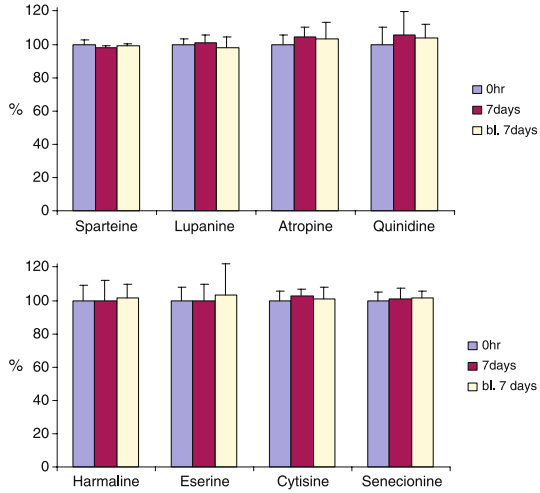

FIG. 1. Recovery of alkaloids after 7-d incubation in Hungate tubes inoculated with culture medium M2 and bovine rumen liquor. Values are given in mean and standard deviation. No significant differences were found between 0 hr and 7 d either for treatment or for blank (bl.) incubations at $P < 0.05$ (*t*-student).

and their associated bacterial consortia cannot be cultured by using Hungate's technique and that their culture in HFT is unstable. In addition, bacteria that live attached to food particles cannot be cultured using Hungate's technique or in HFT but in RUSITEC fermenters. In order to overcome the technical problems, we designed a second set of experiments with RUSITEC to study the fate of alkaloids in the rumen.

The following alkaloids of various structures and bioactivities (2 mM each of sparteine, lupanine, atropine, quinidine, lobeline, harmaline, arecoline, nicotine, caffeine, pilocarpine, gramine, and monocrotaline) were added on day 5 to the RUSITEC culture vessels. Several physiological parameters were determined during the run of the fermentation experiments, such as hydrogen ion and ammonia concentrations and gas and protein production.

Changes in hydrogen ion concentrations were analyzed continuously from the start of the cultures and 3 d after the alkaloids had been added to monitor any adverse effects. Hydrogen ion concentrations (pH values) varied between 6.56 and 6.99, being mostly between 6.7 and 6.9, both during the alkaloid-free control days (d 1–5) and the posttreatment days (d 6–8). These variations are within the normal pH variation found in previous RUSITEC experiments done in the Rumen Laboratory at the Veterinary School of Hannover (TiHo), indicating that alkaloids did not cause any drastic effect, thus confirming the findings from our Hungate experiments.

The concentration of ammonia in the fermenters showed a high variation already during the alkaloid-free days varying from 15 to 23 mmol/l but were within the range found in previous RUSITEC experiments. After addition of the alkaloids on d 5, a reduction of ammonia concentrations was observed in most fermenters (Figure 2). This effect was more pronounced for lobeline, harmaline, and atropine. Ammonia production in the rumen fluid is a consequence of amino acid and urea breakdown by both rumen bacteria and protozoa. Amino acids are deaminated to ammonia and fatty acids by most rumen protozoa (Allison, 1970) and a broad group of rumen bacteria, the latter being responsible for most of the ammonia production. Urea is broken down in the rumen only by bacteria (Sakurada et al., 1994). Considering this and the fact that weak antimicrobial activities seem to be exhibited by the applied alkaloids (Wink, 1993; Verpoorte, 1998), it is not surprising that the addition of alkaloids to the RUSITEC fermenters has a negative effect on fermentation parameters. The alkaloid concentration used was not high enough to have a more pronounced antimicrobial effect. The reduction of ammonia in the fermenters agrees with the results reported by Wiedmeier et al. (1987), who found that the ammonia concentration in cattle rumen decreased by 37% when the animals were fed on a diet containing the alkaloid pilocarpine at a dose of 4 mg/kg body weight.

Effect on Ruminal Gas Production. All fermenters showed a high variation in the daily total gas production. The amount of variation extends from 250 ml

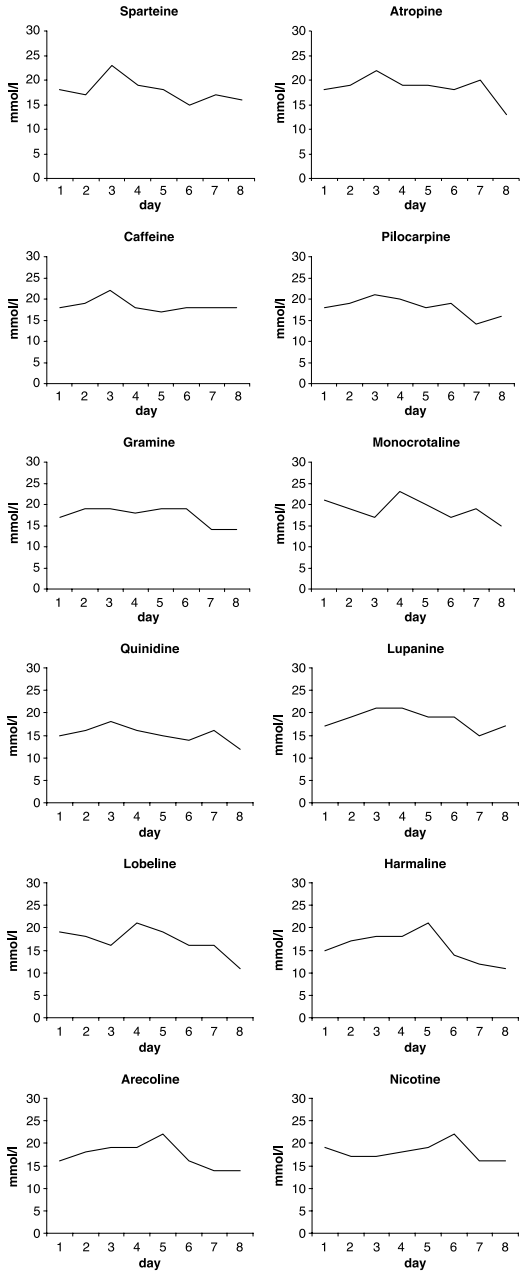

FIG. 2. Effect of alkaloids on ruminal NH_3 concentration measured in RUSITEC fermenters for 72 hr. Alkaloids were added on day 5 at a final concentration of 2 mM.

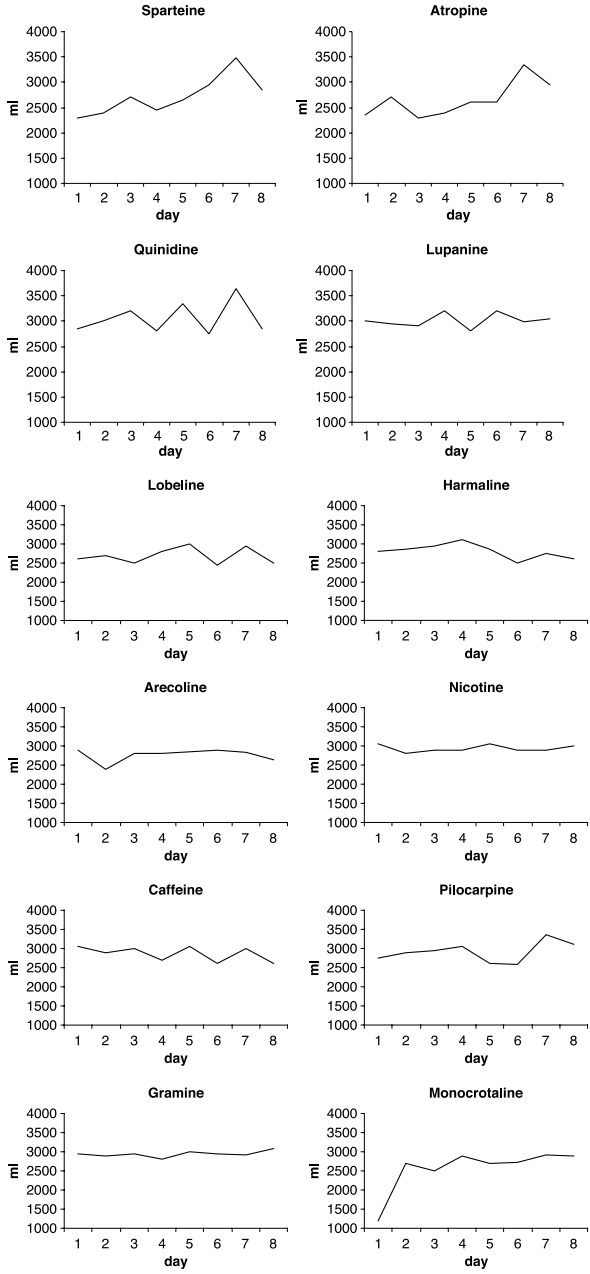

FIG. 3. Effect of alkaloids on ruminal total gas production measured in RUSITEC fermenters for 72 hr. Alkaloids were added on day 5 at a final concentration of 2 mM.

for arecoline and nicotine to 900 ml for quinidine (Figure 3). The extreme low value on the first day of the fermenter treated with monocrotaline is probably an artifact due to incorrect handling of the gas bag. However, a significant increase in total gas production is observed after the addition of sparteine, atropine, and pilocarpine (Figure 3). Harmaline, in contrast, has a negative effect on fermentation (it has pronounced antimicrobial properties; Wink, 2000), thus reducing total gas production.

A reduction of ammonia production in the fermenters was interpreted as a result of the antimicrobial activities of the employed alkaloids. This interpretation seems contradictory to the observation that some alkaloids also increased total gas production in the fermenters, which corresponds to an improvement of the microbial fermentation processes in the reaction vessels. Although total gas production correlates linearly to the production of short-chain fatty acids and consequently resembles the efficiency of the microbial fermentation, the latter is not only dependent on the microbial biomass, but also on the microbial fermentative capacities. It has been demonstrated that *in vitro* gas production can have an inverse relationship to microbial biomass production (Makkar et al., 1995a,b; Blümmel et al., 1997a,b). The addition of sparteine, atropine, and pilocarpine could favor those bacteria with the highest fermentative capacities. Similar results have been reported for caffeine (Campbell et al., 1976) and pilocarpine (Wiedmeier et al., 1987).

In contrast to total gas production, gas composition was almost constant for all fermenters during experiments (data not shown). Only in the fermenters treated with atropine, quinidine, and caffeine a slight increase in the proportion of CO_2 after addition of alkaloids was observed (data not shown), which suggests a slight improvement in the carbohydrate fermentation. In addition, quinidine appears to be the only tested alkaloid with a negative effect on the methanogen community with its concomitant reduction of methane production. Since total gas production was not affected by this alkaloid and since carbon dioxide, together with hydrogen, is the major precursor of methane in the rumen (Wolin et al., 1997), the observed increase of CO_2 can be interpreted as a direct effect of methane reduction.

Effect on Ruminal Protein Concentration. The addition of quinidine and caffeine (Figure 4) decreased the protein concentration in the fermenters by 43 and 47%, respectively. No significant effect was observed after addition of the other alkaloids. Since only bacterial protein was measured, a reduction of protein concentration resembles a decrease in the bacterial biomass and can correspond to antimicrobial activities of the alkaloids. Most alkaloids had a negative effect in at least one fermentation parameter, which can be interpreted as a result of antimicrobial activities (Verpoorte, 1998). Yet the bacterial biomass and most fermentation parameters were not disturbed; we can, therefore, assume that the microbes were alive and should have been able to degrade alkaloids.

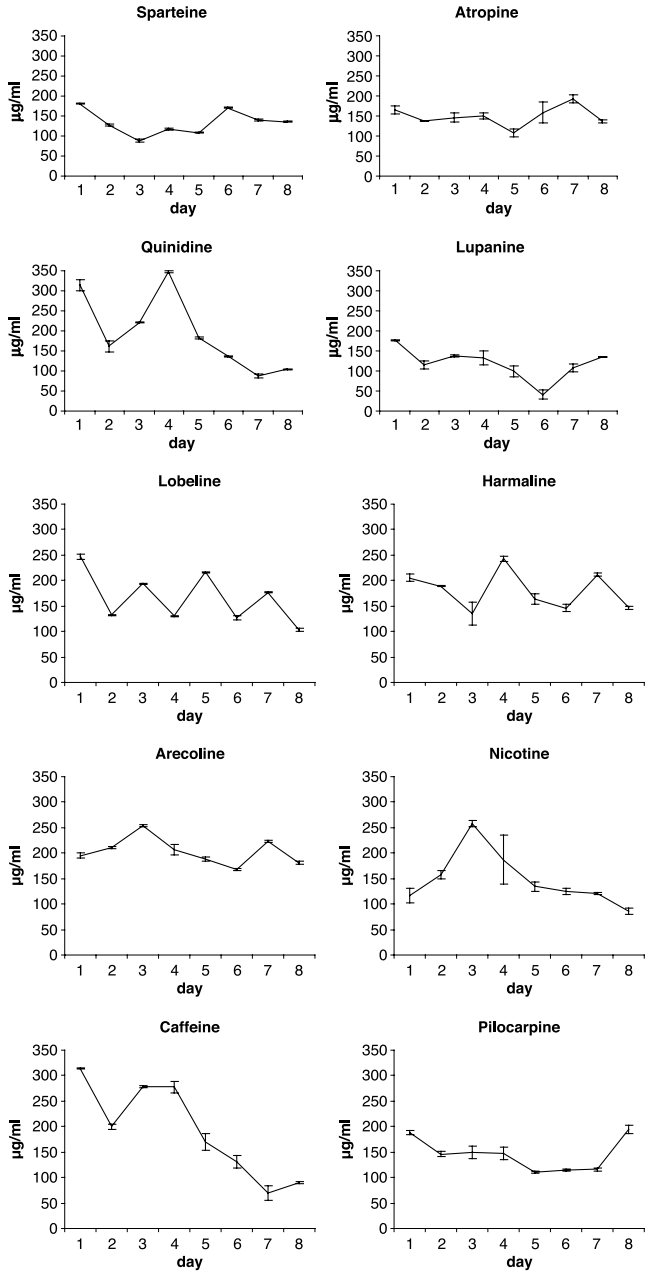

FIG. 4. Effect of alkaloids on ruminal protein concentration measured in RUSITEC fermenters for 72 hr. Alkaloids were added on day 5 at a final concentration of 2 mM.

Fate of Alkaloids in Rumen Simulation Technique Fermenter. All tested alkaloids were stable in control incubations that used sterilized rumen liquor to avoid possible degrading metabolism from rumen microorganisms. Most alkaloids were stable during the incubations and did not change (Figure 5). A decrease in the concentration was observed only for pilocarpine, monocrotaline, and gramine (Figures 6–8).

Pilocarpine showed the highest degradation rate being totally degraded after 12 hr incubation (Figure 6). The concentration of monocrotaline decreased to 38.9% within the first 12 hr and to 4.7% within the next 12 hr (Figure 7), which represents a degradation of 49.4 and 92.1%, respectively (in relation to the expected concentration). In Figure 7B, the gas chromatograms of the monocrotaline samples collected at time 0, 24, and 48 hr incubation are illustrated. It is possible that an ester hydrolysis of monocrotaline is involved in the degradation of this alkaloid by rumen microbes. Similar reaction mechanisms have been postulated for jacobine (Wachenheim et al., 1992a,b) and for the monoester heliotrine, along with modification of the necine base (Russell and Smith, 1968; Lanigan, 1971, 1976). Enzymatic hydrolysis of monocrotaline has been reported from hepatic microsomal incubation of guinea pigs, leading to the nontoxic necic acid and necine base (Dueker et al., 1992). There are substantial evidence and counterevidence for detoxification pathways in liver vs. rumen in the literature concerning pyrrolizidine alkaloids (Swick et al., 1983; Craig et al., 1986, 1992; Cheeke, 1988, 1998; Wachenheim et al., 1992a). Wachenheim et al. (1992b) found that pyrrolizidine alkaloids (PAs) were degraded more rapidly when incubated *in vitro* with ovine or caprine than

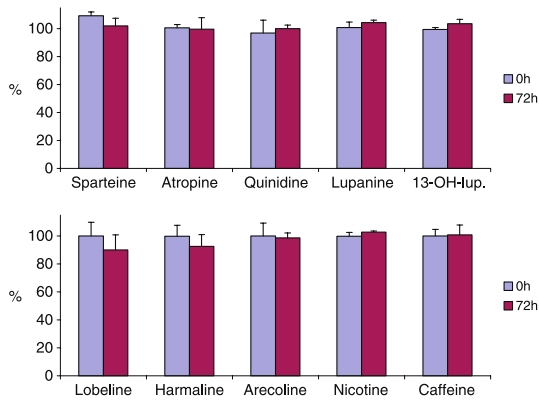

FIG. 5. Recovery of alkaloids after 72 hr incubation in RUSITEC fermenters. Values are given in mean and standard deviation. No significant differences were found between 0 and 72 hr at $P < 0.05$ (*t*-student).

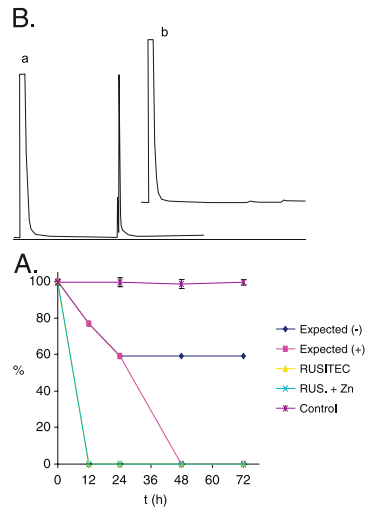

FIG. 6. Fate of pilocarpine in RUSITEC fermenter. (A) Kinetics of pilocarpine contents during fermentation; (B) GLC profiles of the cultures after 0 hr (a) and 12 hr (b); 1 = pilocarpine.

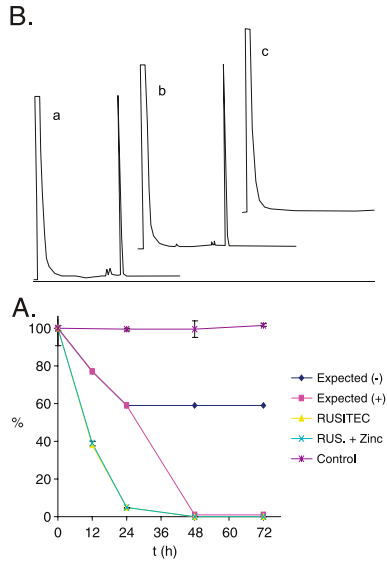

FIG. 7. Fate of monocrotaline in RUSITEC fermenter. (A) Kinetics of monocrotaline contents during fermentation; (B) GLC profiles of the cultures after 0 hr (a), 24 hr (b), and 48 hr (c); 1 = monocrotaline.

with bovine rumen fluid, and by using the "most probable number procedure" for estimating PA-degrading bacteria in rumen, they demonstrated that cattle have these bacteria, but in much reduced numbers than sheep and goats. In contrast, Shull et al. (1976) and Swick et al. (1983) found that *S. jacobaea* is still toxic to rats after incubation with sheep rumen fluid. Nevertheless, the presence of alkaloid-degrading organisms in the rumen is far from ubiquitous in all ruminants. The apparently high frequency of reports on PA-degrading microbes found in the literature, compared with other toxins, seems to be rather a result of the intense investigation of this group of alkaloids and their economical importance in the range management (Swick et al., 1983; Craig et al., 1986, 1992; Cheeke, 1988, 1998; Wachenheim et al., 1992a).

Gramine suffered a lower degradation rate, 35.7 and 38.3%, respectively, of the expected concentration after 12 and 24 hr (Figure 8). The gas chromatograms of the gramine samples collected 0, 24, and 48 hr after incubation in RUSITEC are shown in Figure 8B. Since gramine occurs in young leaves of cereals and these plants are the main component of a roughage-based diet for

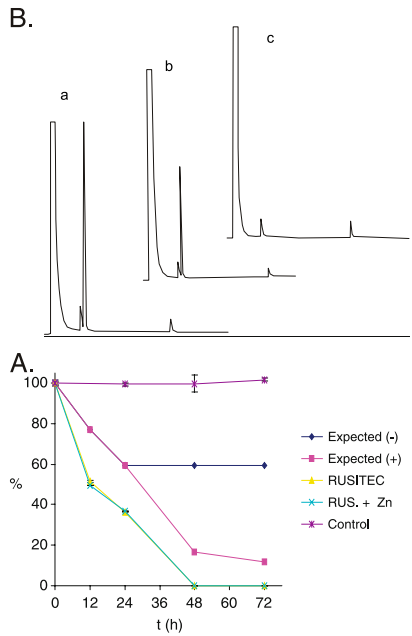

FIG. 8. Fate of gramine in RUSITEC fermenter. (A) Kinetics of gramine contents during fermentation; (B) GLC profiles of the cultures after 0 hr (a), 24 hr (b), and 48 hr (c); 1 = gramine.

ruminants, we would expect to find that rumen microbes from cattle kept in stables and fed on this kind of diet are able to metabolize this alkaloid.

We found no evidence for alkaloid N-oxide formation in any fermenter, which agrees with the hypothesis of Dawson et al. (1997) that oxidative reactions are not to be expected in the rumen because of the relatively low oxygen concentrations present.

In summary, our experiments using the Hungate and HFT approach provide no evidence for alkaloid degradation when rumen microorganisms from naive and unexposed sheep and cattle were assayed. Using the RUSITEC method, which takes a more complex rumen microbial community into account, most alkaloids appear to be stable and were not degraded. Evidence for degradation was obtained for gramine, pilocarpine, and monocrotaline, however, indicating a broader metabolic activity than would have been assumed from the bacterial cultures using Hungate and HFT alone.

Feeding sheep for a period of 4 wk on a diet containing increasing amounts of bitter lupin seeds was not sufficient to induce alkaloid-degrading metabolism in ovine rumen microbes. Nevertheless, rumen microbes could adapt to the presence of lupanine, and the fermentation processes, determined by total gas production, were, therefore, enhanced. Feeding ruminants a diet containing plant toxins can thus modify the composition of the microbial rumen community either by selecting those microorganisms able to tolerate higher concentration of the toxins consumed by the herbivore or by allowing the microorganisms to become adapted to the new conditions.

Most experiments are conducted *in vitro* using rumen fluid from animals (mainly cattle and sheep) kept in stables and fed on controlled grass-based diets. Many authors have reported that previous exposure to a diet containing the plant toxin is, in most cases, a requisite for the rumen microbes to be able to metabolize it or for the ruminant not to be affected by the toxin, suggesting an adaptation of the rumen microorganisms for metabolizing it (Smith, 1992). Allyl cyanide is degraded in the rumen of sheep fed on cabbage, but not in the rumen of grass-fed sheep (Duncan and Milne, 1992). Ethiopian sheep gradually adapted to a diet containing *Acacia angustissima* are protected from its toxins through rumen detoxification, whereas non-adapted animals die within a few days (Odenyo et al., 1997). The rumen flora of nonadapted deer is severely inhibited by the essential oils of Douglas fir needles, whereas no inhibition is found on rumen flora from deer previously adapted to a diet containing up to 50% Douglas fir needles (Oh et al., 1967). The antiprotozoal factor (saponins) of *Sesbania sesban*, an African leguminous tree, affects negatively the rumen protozoa of Scottish sheep but not of feral Ethiopian sheep, and *in vitro* experiments suggest that bacteria are adapted to detoxify this antiprotozoal agent (Newbold et al., 1997; Teferedegne et al., 1999). Culvenor et al. (1984) found that the *in vitro* degradation of *Echium* pyrrolizidine alkaloids incubated

with ovine rumen fluid can be improved up to 22 times when the donor sheep are previously fed with *Echium* for 12 wk. Sheep that have been reared on pyrrolizidine alkaloids can degrade them, but inexperienced sheep cannot. Similarly, the rumen flora of deer that have not previously been fed with Douglas fir needles are severely inhibited by it, but experienced deer can consume a diet of up to 50% needles without showing negative effects (Oh et al., 1967).

The results obtained in this study clearly emphasize that degradation of alkaloids does not occur to a substantial degree in the rumen of naïve ruminants, thus suggesting a prominent role of liver detoxification instead. More work is needed to understand the specific role of the rumen and the liver in the different susceptibility of ruminants to plant toxins and other xenobiotics. More attention should be paid to detoxification activity in hepatic tissues of ruminants and on their previous feeding conditions. In addition, the nature of the ruminant species should be taken into account when comparing interspecific differences. Grazing ruminants, e.g., cattle, have evolved on food plants (e.g., grasses) that are, in general, poorly chemically defended (Cheeke, 1998), depending more on growth habit and physical defenses. In contrast, browsers, e.g., sheep, goat, and deer, have evolved on food plants that are generally well equipped with chemical defenses. Thus, different detoxification mechanisms may have evolved in both groups, and their rumen floras have been subject to different evolutionary pressures.

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