EFFECT ON RUMEN MICROBIAL POPULATIONS OF AMMONIA TREATMENT OF RICE STRAW FORAGE FOR STEERS

HAJIME MINATO,* SHIGENOBU ISHIZAKI,¹ YOSHIKAZU ADACHI,
AND MAKOTO MITSUMORI

National Institute of Animal Health, Tsukuba, Ibaraki 305, Japan

¹*Chiba Prefectural Livestock Experiment Station, Yachimata, Chiba 289–11, Japan*

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The microbial populations and fermentation characteristics were compared in the rumina of two rumen-cannulated Holstein steers fed untreated rice straw (RS) and concentrate or ammonia-treated rice straw (ARS) and concentrate. The total volatile fatty acid concentrations in the rumina of both steers were significantly higher when they were fed ARS than when fed RS. The molar proportion of butyric acid when ARS was fed increased significantly, compared with the periods when was fed RS. The population density of protozoa varied only insignificantly with RS or ARS diets. But *Dasytricha* spp. increased with RS and *Entodinium* spp. increased with ARS. The viable counts of cellulolytic bacteria were higher with ARS than with RS. The viable counts of sulfate reducing bacteria were lower with ARS, compared with the RS diet. Feeding ARS increased the viable bacterial populations such as *Eubacterium* spp., *Fibrobacter succinogenes*, *Ruminococcus albus*, and *Succinivibrio* spp. But *Bacteroides* spp. and *Butyrivibrio* spp. were dominant when RS was fed.

Various physical and chemical treatments of low-quality roughages to improve their nutritive values for ruminants have been tried (11, 21). Such physical treatments as grinding, milling and chopping have increased the intake of roughages, but has not improved their nutritive value. Moreover, these treatments had the disadvantage of high costs. On the other hand, chemical treatment with calcium hydroxide, sodium hydroxide or ammonia has been tried to improve their nutritive values and to avoid spoilage during storage. Of the treatments with alkali, the ammonia treatment has the advantages of being relatively inexpensive, simply applied on the farm, and it increases the digestibility and the nitrogen content of the herbage. The

* Address reprint requests to: Dr. Hajime Minato, National Institute of Animal Health, 1–1, Kannondai 3-chome, Tsukuba, Ibaraki 305, Japan.

treatment of herbage with ammonia has been extensively studied in Europe and Canada (27).

The improvement in digestion of alkali-treated straws has been attributed to the hydrolysis of linkage between lignin and other cell wall carbohydrates, saponification of bound acetic acid and phenolic acids, swelling of cell walls, and the partial solubilization of hemicelluloses, lignin, protein and silica (11). It was reported that ammonia treatment increased the digestibility of barley straw by up to about 20% in cattle feeding experiments (28). Similar results in increased digestibility of herbages with ammonia treatment have been found by comparing the digestibility of untreated and ammonia-treated barley straw incubated with rumen contents in vitro or enclosed in nylon mesh bags placed in vivo rumen (9). Moreover, it was shown that ammonia treatment of barley straw increased its susceptibility to solubilization by the predominant cellulolytic bacteria from the rumen, *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens* (5, 16). However, a comparison of the constitution of ruminal micro-organisms between animals fed untreated and ammonia-treated straw has not yet been investigated. The aim of the present investigation was to clarify the influence of ammonia treatment of rice straw given to steers as a forage on the constitution of micro-organisms established in the rumen.

MATERIALS AND METHODS

Treatment of rice straw with anhydrous ammonia. Four hundred and fifty kg of rice straw were reconstituted to 30–40% moisture in tap water and piled on a large wooden draining board and wrapped with a 0.2-mm thick vinyl sheet. About 15.5 kg of anhydrous NH_3 was applied to the rice straw as NH_3 gas. After being stored for 3 weeks, the ammonia-treated straw was opened, and dried outside in the sun. Treating the straw with 3.4% anhydrous ammonia increased the content of its crude protein 2.8 times, and its hemicellulose was reduced by 35.2% compared with untreated rice straw.

Animals, feeding and sampling regimen. Two Holstein steers, 14 months of age and weighing approximately 280 kg, were used in this experiment. Each steer was fitted with a permanent cannula in the rumen before the start of the experiment. The composition of the control diet and two experimental diets are shown in Table 1. Rice straw and ammonia-treated rice straw, added as roughage in the experimental diets, were chopped into about 5 cm lengths with an electric cutter. At 9 a.m. and 4 p.m. each steer was fed a daily ration of concentrate and roughage divided into two equal parts. Mineral lick and water were available ad libitum. The experiment took place in the National Institute of Animal Health, beginning on May 22, 1986, and ending on November 20, 1986. The rations were offered to two steers in the following sequence: for steer No. 1, 28 days of control (C)-diet, 28 days of ammonia-treated rice straw (ARS)-diet, 42 days of C-diet, 30 days of untreated rice straw (RS)-diet; for steer No. 2, 28 days of C-diet, 28 days of RS-diet, 42 days

Table 1. Composition of rations fed to steers.

Ingredient	Control (C)-diet	Untreated rice straw (RS)-diet	Ammonia-treated rice straw (ARS)-diet
Concentrate	3 kg	3 kg	3 kg
Untreated rice straw	—	4 kg	—
Ammonia-treated rice straw	—	—	4 kg
Timothy hay	2 kg	—	—
Alfalfa hay cubes	1 kg	—	—

of C-diet, 30 days of ARS-diet. The adaptation period from a preceding diet to a next diet was a week and roughage only in the diet was changed gradually during this period. Samples of rumen content, which contained solids and liquid, were collected through the rumen cannula before morning feeding on the 21st day of each feeding period.

Sample preparation. Immediately after each sample collection, the rumen contents were transported to a laboratory. Two hundred grams of the rumen content, containing equal weights of solids and liquid portions, were processed under a stream of oxygen-free CO₂ for 1 min with a homogenizer (Nihon Seiki Manufacturing Ltd., Tokyo; Model Ace), and the 1 min homogenizing treatment was repeated once more after halting the machine for 1 min. Then the homogenized rumen content was strained through one layer of gauze.

A portion of the strained rumen fluid was used to enumerate viable rumen bacteria. Another portion was blended with an equal volume of saline solution containing 20% formalin (v/v), for total bacterial enumeration by microscopic examination.

The remaining portion of the strained rumen fluid was stored in a refrigerator following acidification with 24% (w/v) *meta*-phosphoric acid in 5N H₂SO₄, for volatile fatty acid (VFA) analysis, or for non-VFA analysis such as lactic acid or succinic acid. After centrifuging, the VFA in samples was analyzed by gas chromatography by the method of Packett and McCune (22), and non-VFA was analyzed by the method of Holdeman et al. (12). The concentration of VFA and non-VFA in the samples was determined using respectively 2-methyl-valeric acid and fumaric acid as internal standards.

Enumeration of bacteria. For anaerobic bacterial enumeration, ten fold serial dilutions of the strained rumen fluid were made in the anaerobic dilution solution of Bryant and Burkey (2), to which agar was added to 0.1% (w/v). Three sets of tubes containing agar medium or liquid medium were inoculated with 0.2 ml from each of the appropriate dilutions.

Total viable counts of bacteria in the strained rumen fluid were made by the techniques of Hungate (14) with the rumen fluid-glucose-cellobiose-maltose-starch-agar medium (RGCMSA) identical to the complete medium of Scott and Dehority (25) except for the carbohydrates. Colonies in the roll tubes were counted

after a 5-day incubation period at 38°C, and the viable count was taken as the average of the three roll-tube counts at the appropriate dilution.

Viable counts of the following functional groups of bacteria were made: cellulolytic bacteria, amylolytic bacteria, xylan-fermenters and pectin-fermenters. The basal medium to prepare the liquid media used for enumerating those bacteria contained ingredients in the following percentages: mineral solution A (0.6% K_2HPO_4), 7.5; mineral solution B (0.6% KH_2PO_4 , 1.2% $(NH_4)_2SO_4$, 1.2% NaCl, 0.12% $MgSO_4 \cdot 7H_2O$, 0.12% $CaCl_2 \cdot 2H_2O$), 7.5; sodium resazurin, 0.0001; trypticase (BBL Microbiology Systems, Cockeysville, MD), 0.5 (or 0.2 for cellulolytic bacteria); clarified rumen liquor, 30; sodium carbonate, 0.4 (or 0.06 for the xylan- or pectin-decomposing bacteria); L-cysteine-HCl $\cdot H_2O$, 0.05. Cellulolytic bacteria were enumerated with the basal medium supplemented with 0.12% yeast extract, a strip of Toyo No. 2 filter paper and 0.02% cellobiose (18). Amylolytic bacteria were enumerated with the basal medium supplemented with 0.1% soluble starch (Difco Laboratories, Detroit, MI). Starch hydrolysis was detected by the addition of iodine solution to the cultures after a 5-day incubation period at 38°C. Xylan-fermenters were enumerated in the basal medium supplemented with 0.5% xylan (Aldrich Chemical Company, Inc., Milwaukee, Wis.) from oat spelts. Pectin-fermenters were enumerated with the basal medium supplemented with 0.5% pectin (Wako Pure Chemical Industries, Ltd., Tokyo) from citrus. The gas phase in the liquid media for enumerating xylan- or pectin-fermenters was 10% CO_2 -90% N_2 . The presence of xylan- or pectin-fermenters was determined by measuring the pH of the cultures with a pH meter after a one-week incubation period at 38°C. Sulfate-reducing bacteria were enumerated with the liquid medium of Butlin et al. (4), to which 10% clarified rumen liquor, 0.1% yeast extract (Difco), 0.0001% sodium resazurin, 0.01% sodium thioglycolate and 0.01% L-ascorbic acid were supplemented. The gas phase of the medium was 100% CO_2 . The presence of sulfate-reducing bacteria was determined by the appearance of black color in the cultures after a one-week incubation period at 38°C. In the liquid culture, the count of each functional group of bacteria was taken as the most probable number (MPN). Methanogens were enumerated with the liquid medium of Paynter and Hungate (23), supplemented with 0.2% sodium formate. The methanogens and preparation of the medium were cultured using a high pressure- O_2 free gas injection device (Sanshin Industrial Co., Ltd., Yokohama) according to the method described by Balch et al. (1). The presence of methanogens was determined by the detection of methane in the gas phase after a three-week incubation period at 38°C. Methane was determined by using a GC-8A gas chromatograph (Shimadzu Corp., Kyoto), equipped with a thermal conductivity detector and fitted with a column of molecular sieve (5A, 60 to 80 mesh; Guild Corp., Bethel Park, Pa.) operated at 50°C. Total counts of bacteria in the strained rumen fluid were carried out according to the method described by Minato and Suto (19, 20).

Primary identification of rumen bacteria isolated. Approximately 35 strains

were isolated by picking colonies at random from roll tubes used in the total viable count containing 20–50 colonies per tube. After confirming the purity of all strains isolated, they were examined for their morphological and biochemical characteristics. Tests for their gram reaction, motility, oxygen relation, H₂S production, carbohydrates fermentation (glucose, maltose, cellobiose, xylose, xylan and pectin), and milk coagulation or peptonization, were performed as described by Bryant et al. (3). Tests for their starch hydrolysis and cellulose digestion were made as described above. Organic acids such as VFA and non-VFA produced from carbohydrates were analyzed using a gas chromatograph as described above. Primary identification of bacterial isolates was carried out according to the VPI Manual (12) and Bergey's Manual of Systematic Bacteriology (17, 26).

Enumeration of protozoa. A liquid portion of rumen content was blended with an equal volume of saline solution containing 20% formalin (v/v). A portion of the sample fixed with formalin was stained with staining solution containing 0.05% methylgreen in 10% formal saline. The protozoa in the samples were counted and identified using a plankton counter deck glass as described by Imai and Katsuno (15).

Statistical analyses. Data were subjected to analysis of variance and compared with F tests.

RESULTS

Relationship between VFA concentrations and proportions in the rumen contents and diets

VFA concentrations and proportions in the rumen of steers at the periods of sampling for the estimation of the microflora are presented in Table 2. The total VFA concentrations in the rumina of both steers fed C-diet were affected not only by the periods of sampling but also by individual steers. On the other hand, the molar proportion of VFA in the rumina of the two steers fed C-diet were relatively similar. Total VFA concentrations in the rumina of both steers fed ARS-diet were significantly ($p < 0.2$) higher than when fed RS-diet. The molar proportion of butyric acid in the rumen of both steers fed ARS-diet increased significantly ($p < 0.1$), compared with the periods of feeding the RS-diet.

Influence of diets on protozoal population

The two steers had the same protozoa genera, except *Diplodinium* spp. which was absent in steer No. 1. The total numbers and constitution of protozoa in the rumen of steers are shown in Table 3. The total numbers of protozoa were not affected by individual steers. But the protozoal constitution was significantly affected by feeds. The proportion of *Dasytricha* spp. was higher ($p < 0.1$) in feeding RS-diet than in feeding ARS-diet. And, the proportion of *Entodinium* spp. was higher ($p < 0.1$) in feeding C-diet than in feeding ARS- or RS-diet.

Table 2. Concentration and composition of VFA in the rumina of steers fed different diets.

	C-diet	ARS-diet	RS-diet	F test		
				Diet		Animal
				C:(ARS+RS)	ARS:RS	
Total VFA concentration (mm) ^a	75.3±6.2	95.5±1.1	81.0±3.5	6.4°	4.0	8.5*
VFA composition (mol%) ^a						
Acetic acid	68.6±1.1	66.5±0.7	67.5±1.1	1.2	0.2	5.1°
Propionic acid	10.8±0.5	13.8±1.2	13.4±1.0	5.1°	0.2	2.0
Isobutyric acid	1.7±0	1.0±0	1.4±0	88.2**	19.6**	0.2
Butyric acid	15.4±1.1	15.8±1.1	13.4±0.9	2.1	9.3*	0
Isovaleric acid	2.2±0.1	1.5±0.2	1.9±0.1	8.8*	2.0	1.6
Valeric acid	1.0±0.1	1.0±0.3	0.6±0.2	0.2	0.4	1.1
Caproic acid	0.4±0.1	0.3±0.1	0.2±0.1	0.5	0.3	1.2

VFA: volatile fatty acids. ° $p < 0.1$, * $p < 0.05$, ** $p < 0.01$. For C-diet, RS-diet and ARS-diet, see Table 1.

^a Mean and standard error of the mean. Each mean in the C-diet column is based on four determinations, and on two determinations in the ARS or RS-diet column.

Table 3. Total count and generic constitution of protozoa in the rumina of steers fed different diets.

	C-diet	ARS-diet	RS-diet	F test		
				Diet		Animal
				C:(ARS+RS)	ARS:RS	
Total count (Log No./g rumen content) ^a	5.9±0.1	5.6±0.3	5.6±0.3	1.6	0	0
Constitution of protozoa (%) ^a						
<i>Dasytricha</i> spp.	6.4±2.9	1.2±0.1	14.1±1.4	0.1	4.7°	0.4
<i>Isostricha</i> spp.	2.0±1.0	1.6±0.4	4.3±1.0	0.4	1.4	0.1
<i>Entodinium</i> spp.	63.9±7.0	58.6±1.8	36.1±3.8	5.4°	2.3	0.2
<i>Epidinium</i> spp.	11.7±1.8	15.1±4.0	17.6±1.7	1.4	0.2	0.2
<i>Eremoplastron</i> spp.	11.8±5.6	19.0±2.9	24.8±2.2	1.5	0.5	0
<i>Eudiplodinium</i> spp.	0.7±0.3	4.1±0.7	1.1±0.7	4.5°	1.0	0
<i>Eodinium</i> spp.	0	0.6±0.4	2.2±1.5	1.4	1.0	0.5
<i>Diplodinium</i> spp.	3.2±2.7	0	0	0.7	0	0.7

° $p < 0.1$, * $p < 0.05$, ** $p < 0.01$. For C-diet, RS-diet and ARS-diet, see Table 1.

^a Mean and standard error of the mean. Each mean in the C-diet column is based on four determinations, and on two determinations in the ARS or RS-diet column.

Table 4. Total count of bacteria and viable count of some bacteria in the rumina of steers fed different diets.

	C-diet	ARS-diet	RS-diet	<i>F</i> test		Animal
				Diet		
				C : (ARS + RS)	ARS : RS	
Total count (Log No./g rumen content) ^a	10.5 ± 0.1	10.9 ± 0.1	10.3 ± 0.2	2.1	0.1	0
Viable count of some bacteria (Log No./g rumen content) ^a						
Total viable count	9.3 ± 0.1	9.3 ± 0.2	9.1 ± 0.2	0.1	0.4	0
Cellulolytic bacteria	8.0 ± 0.1	8.2 ± 0.2	7.3 ± 0.1	1.3	7.4°	0.1
Methanogens	7.3 ± 0.1	7.7 ± 0.3	7.5 ± 0	1.5	0.2	2.8
Sulfate reducing bacteria	6.7 ± 0.1	5.9 ± 0	6.6 ± 0.1	5.1°	6.7°	0.3
Amylolytic bacteria	9.6 ± 0.1	9.4	9.4	0.1	0.3	0.1
Xylan fermenters	NC	9.7	9.4			
Pectin fermenters	NC	9.1	9.1			

° $p < 0.1$, * $p < 0.05$, ** $p < 0.01$. NC = not counted. For C-diet, RS-diet and ARS-diet, see Table 1.

^a Mean and standard error of the mean. Each mean in the C-diet column is based on four counts, and on two counts in the ARS or RS-diet column.

Influence of diets on viable count of some bacteria

Total counts of bacteria and viable counts of some bacteria in the rumen of steers at the periods of feeding different diets are shown in Table 4.

Total counts of bacteria and viable counts of some bacteria were higher in September than in June in feeding the same C-diet. For all samples examined, the total counts of bacteria by direct microscopic observation were clearly higher than the total viable counts. This suggests that a number of rumen bacteria did not make a colony on the RGCMSA medium.

On the other hand, viable counts of a given functional group of bacteria were affected by diets. The viable counts of cellulolytic bacteria were higher ($p < 0.1$) in feeding ARS-diet than in feeding RS-diet. Contrary to this, the viable counts of sulfate reducing bacteria decreased in feeding ARS-diet, compared with feeding RS-diet ($p < 0.1$). The viable counts of methanogens were not changed by feeding different diets. The viable counts of amylolytic bacteria, pectin-fermenters and xylan-fermenters was at the level of 10^9 .

Influence of diets on the constitution of rumen microflora

The constitutions of predominant bacteria isolated from the rumen contents of both steers at the periods of feeding different diets are presented in Table 5. The constitutions of rumen microflora of both steers fed the same C-diet were different to a certain degree between two seasons. In June, the viable bacterial population

Table 5. The constitution of bacteria isolated at high dilutions from the rumen content of steers fed different diets.

	C-diet (%)	ARS-diet (%)	RS-diet (%)	F test		
				Diet		Animal
				C: (ARS+RS)	ARS:RS	
<i>Bacteroides</i> spp.	22.7±7.8 ^a	16.5±5.6	53.5±0	0.1	0.3	0.1
<i>Ruminobacter amylophilus</i>	1.0±0.8	3.7±2.6	1.8±1.3	0.6	0.3	0
<i>Fibrobacter succinogenes</i>	0.8±0.7	2.5±1.7	0	0.1	1.3	0.1
<i>Selenomonas</i> spp.	13.0±5.1	1.2±0.8	0	3.0	0.1	0.1
<i>Succinivibrio</i> spp.	0	3.9±0.7	0	1.9	3.7	0.1
<i>Fusobacterium</i> spp.	5.7±1.3	2.6±0.1	5.4±1.2	0.8	1.0	2.4
<i>Butyrivibrio</i> spp.	35.2±5.1	23.9±1.3	32.2±2.5	0.9	0.6	2.1
<i>Ruminococcus</i> spp.	4.4±2.1	1.2±0.9	0	1.6	0.1	0.5
<i>Ruminococcus albus</i>	0.8±0.7	2.9±2.0	0	0.1	1.4	0.1
<i>Eubacterium</i> spp.	3.2±1.1	30.2±11.0	5.4±1.2	3.3	4.8 ^o	0.8
<i>Clostridium</i> spp.	1.6±0.8	2.5±1.7	0	0.1	1.1	0.6
<i>Streptococcus</i> spp.	3.2±1.3	2.6±0.1	3.6±2.5	0.1	0.1	0.2
<i>Lactobacillus</i> spp.	1.6±1.3	0	0	0.7	0	0.7
Unidentified bacteria	6.9±2.4	6.5±0.6	7.2±2.5	0.1	0.1	0.1

^o $p < 0.1$, * $p < 0.05$, ** $p < 0.01$. For C-diet, RS-diet and ARS-diet, see Table 1.

^a Figures show the mean and standard error of the mean. Each mean in the C-diet column is based on four determinations, and on two determinations in the ARS or RS-diet column.

in feeding C-diet was dominated by *Butyrivibrio* spp. and *Selenomonas* spp. but the population was predominantly *Butyrivibrio* spp. and *Bacteroides* spp. in September. In spite of this, the constitutions of rumen microflora of both steers fed the same diet such as ARS or RS-diet at the different periods were similar. Clearly, the constitutions were affected by diets. The viable bacterial population in the rumen of both steers fed ARS-diet was dominated by *Eubacterium* spp., *Butyrivibrio* spp. and *Bacteroides* spp. On the other hand, the bacterial population in feeding RS-diet was dominated by *Bacteroides* spp. and *Butyrivibrio* spp. Feeding ARS-diet significantly increased ($p < 0.1$) the viable count of *Eubacterium* spp., compared with RS-diet. *Eubacterium* spp. was present at 3.1–7.1% of the bacterial population with RS-diet or C-diet. The cellulolytic bacteria such as *F. succinogenes* and *R. albus* were predominant with ARS-diet, but was not detected at high dilutions of the rumen contents of either steer with RS-diet. *Fusobacterium* spp. remained a small but constant proportion of the microflora with three different diets. *Ruminobacter amylophilus* was detected in the rumen of both steers at the sampling period of November 8, independently of the diets. *Streptococcus* spp. was present at 2.4–7.1% of the bacterial population in the rumen of one steer on three different diets.

Utilization of polysaccharides by bacteria isolated from the rumen

The starch-, xylan- and pectin-digesting ability of the predominant bacteria

Table 6. Utilization of polysaccharides by bacteria isolated at high dilutions from the rumen content of steers.

Bacteria	Starch fermenters	Xylan fermenters	Pectin fermenters	Total number of strains examined
<i>Bacteroides</i> spp.	74.2 ^a	74.2	86.4	66
<i>Ruminobacter amylophilus</i>	100	0	0	5
<i>Fibrobacter succinogenes</i>	0	0	66.6	3
<i>Selenomonas</i> spp.	56.3	31.2	87.5	16
<i>Succinivibrio</i> spp.	33.3	0	66.6	3
<i>Fusobacterium</i> spp.	58.3	41.6	100	12
<i>Butyrivibrio</i> spp.	50.6	63.3	100	79
<i>Ruminococcus</i> spp.	0	16.6	66.6	6
<i>Ruminococcus albus</i>	0	66.6	33.3	3
<i>Eubacterium</i> spp.	13.8	93.1	24.1	29
<i>Clostridium</i> spp.	0	50	50	4
<i>Streptococcus</i> spp.	100	0	100	8
<i>Lactobacillus</i> spp.	0	50	50	2

^a Figures show the percentage of strains of each bacterial group which digested the carbohydrates indicated.

isolated from the rumina in this investigation is presented in Table 6. Many strains of *Butyrivibrio* spp., *Bacteroides* spp., *Selenomonas* spp. and *Fusobacterium* spp. were able to digest starch, xylan and pectin. Almost all strains of *Eubacterium* spp., a butyric acid producer, digested xylan, but strains capable of digesting starch and pectin were very limited. The cellulolytic *F. succinogenes* strains did not digest starch and xylan. Other cellulolytic *R. albus* strains did not digest starch. The amylolytic *R. amylophilus* strains did not digest xylan and pectin. Some strains of *Succinivibrio* spp. and *Streptococcus* spp. digested pectin and starch.

DISCUSSION

The total bacterial cell count, total viable count and constitution of bacteria in the rumen of the same steer fed C-diet containing timothy hay, alfalfa hay cubes and concentrate, varied at the two sampling months. The variations in the bacterial population density and the protozoal population density in the rumen contents of both steers agreed well with the principle that in the absence of protozoa there is frequently a compensatory rise in the number of bacteria (8). Furthermore, the predominant bacteria in the rumen contents in the two steers fed C-diet were somewhat different at the two sampling months. This suggests that there are some monthly or seasonal variations in the constitution of rumen bacteria in steers fed on the same ration.

Although the constitution of rumen microflora apparently changed with time, the kinds of the predominant bacteria established in the rumina of both steers in the periods of feeding the same diet were quite similar regardless of sampling months,

whether untreated or ammonia-treated rice straw was fed. And more kinds of bacteria were isolated at high dilutions from the rumen contents of both steers fed ARS-diet than in feeding RS-diet. The viable counts of cellulolytic bacteria were higher in feeding ARS-diet than in feeding RS-diet. The cellulolytic bacteria such as *F. succinogenes* and *R. albus* were isolated at high dilutions from the rumen contents of both steers fed ARS-diet, but were not isolated in feeding RS-diet (Table 5). These results were supported by the findings that treatment of barley straw with anhydrous ammonia increased its digestion by *F. succinogenes* and *R. albus* in vitro (16) and ammonia-treatment of barley straw increased microbial colonization on straw (24). However, the kind of cellulolytic bacteria isolated at high dilutions varied with steers. *R. albus* and *F. succinogenes* were isolated at high dilutions from the rumen contents of steer No. 1 and No. 2, respectively. It would appear, therefore, that *R. albus* and *F. succinogenes* must be responsible for most of the cellulolytic activity in the rumina of these steers. The proportions of *Butyrivibrio* spp. among bacteria isolated were relatively similar in the four diet periods for each steer. None of the butyrivibrio strain isolated in this investigation were cellulolytic. *Eubacterium* spp. constituted a large proportion of the ruminal bacterial population of both steers in the period of feeding ARS-diet (Table 5). The increase of *Eubacterium* spp. in feeding ARS-diet can be explained by the fact that ammonia treatment broke down the partial linkages between lignin and other cell wall carbohydrates of the straw, so these bacteria gained easy access to xylan and other carbohydrates of the cell wall carbohydrates.

The constitution of protozoa in the rumen contents of both steers varied in the different diet periods. The proportion of *Dasytricha* spp. in the protozoal population increased with RS-diet compared with the ARS-diet. This agrees with the report that *Isotrichids* became abundant in ruminants fed hay rich in soluble sugars (13). While, *Dasytricha* spp. decreased with the ARS-diet (Table 3). The decrease in the numbers of *Dasytricha* spp. may have been due to partial losses of some soluble sugars from the straw during the treatment with ammonia. It has been reported that ruminal protozoal populations can be divided into three groups: type A, characterized by the presence of *Polyplastron* spp., *Diploplastron* spp. and *Ophryoscolex* spp.; type B, characterized by *Epidinium* spp., *Eudiplodinium* spp. and *Eremoplastron* spp.; common type, characterized by *Isotricha* spp., *Dasytricha* spp. and *Entodinium* spp., which coexist satisfactorily in either type A or type B populations (6, 7). Both steers used in this investigation had a type B populations. And feeding ARS-diet did not affect the establishment of type A or type B population.

The concentrations of VFA in the rumen contents of both steers were significantly higher with ARS-diet than RS-diet (Table 2). This may be related to the findings that the treatment of straw with ammonia increased its susceptibility to solubilization, the production of microbial protein, and fermentation products by the rumen cellulolytic bacteria (16) and by mixed rumen micro-organisms (10). The composition of VFA in the rumen contents of both steers fed ARS-diet was

characterized by an increased proportion of butyric acid, compared with the period of feeding RS-diet. Butyric acid is produced in vitro by a relatively few kinds of rumen bacteria, the common one being *Butyrivibrio* spp. However, these bacteria did not increase particularly with the ARS-diet, compared with the RS-diet while the other butyric acid-producing bacteria, *Eubacterium* spp., increased significantly with the ARS-diet (Table 5). Therefore, the metabolism of *Eubacterium* spp. must contribute significantly to the butyric acid production in the rumen contents of both steers. The proportion of acetic acid in the VFA of the rumen contents of both steers was very high in each diet period. Acetic acid is produced in vitro by many kinds of rumen bacteria and protozoa. Therefore, it is probable that most of the flora and fauna in the rumen contents of both steers had a considerable capacity to produce acetic acid.

In conclusion, the treatment of rice straw with ammonia improved its digestibility by rumen micro-organisms, increased the intake by cattle, and improved cattle productivity.

The numbers of sulfate reducing bacteria in the rumen contents of both steers were significantly higher with the RS-diet than with the ARS-diet (Table 4). Dissection of both animal bodies after this experiment was finished revealed that the color of the rumen epithelium of the steer on the ARS-diet was faintly brown, while that of the steer on the RS-diet was black. A problem for the future is whether there is a relationship between the numbers of ruminal sulfate-reducing bacteria and the color of rumen epithelium.

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