Diurnal Changes in the Distribution of Ruminal Bacteria Attached to Feed Particles in Sheep Fed Hay Once Daily

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ABSTRACT : A study was made of diurnal changes in the ruminal bacteria associated with feed particles, i.e., non-associated (NAB), loosely associated (LAB), and tightly associated with particles (TAB), and the TAB concentration in different particle sizes from sheep fed orchardgrass (OG) or alfalfa (ALF) hay. Diaminopimelic acid (DAPA) was used to determine the TAB mass. Results showed that the bacterial masses in NAB and LAB were small, but comprised over 90% in TAB. The TAB mass in the ALF group sharply increased within 2 h after feeding and decreased afterward. The TAB mass showed the same trend in the OG group, increasing from 0 h to 2 h, but remained at the same level up to 14 h after feeding. The peak bacterial mass was, however, lower in the OG than the ALF group. The TAB concentration reflected the changes in total particulate tightly associated bacterial masses in both groups of hay fed sheep. Number of bacterial colonies per particle increased as the particulate size decreased in both groups. This difference, however, tended to decline as the postprandial period was prolonged. DAPA, however, tended to overestimate the TAB mass in the reticulo-rumen digesta of the hay fed sheep. (*Asian-Aus. J. Anim. Sci. 2000. Vol. 13, No. 12 : 1708-1716*)

Key Words : Diurnal Change, Particle Associated Ruminal Bacteria, Sheep

INTRODUCTION

Ruminal bacteria can be categorized into three groups according to whether they are associated with rumen wall attachment, are free in the rumen fluid and loosely, or are tightly associated with feed particles (Cheng et al., 1984; Czerkawski, 1988). These bacteria are distributed in the reticulo-rumen (RR) with different quantities, species, and enzyme activity (Cheng et al., 1984; Williams and Strachan, 1984). The microorganisms associated with feed particles constitute a large proportion of the ruminal microbial mass (Forsberg and Lam, 1977), and play an important role in the degradation of plant cell walls, because they have high polysaccharide-degrading enzyme activities (Williams et al., 1989, Martin et al., 1993). Information regarding diurnal changes in the number of microorganisms in the rumen liquid phase is available (Warner, 1966; Leedle et al., 1982). A few *in situ* (Bowman and Firkins, 1993; Olubobocun et al., 1990) and *in vivo* studies (Craig et al., 1987a) on the diurnal changes in the bacteria associated with feed particles are available. However, there is limited quantitative information regarding the dietary influence on the diurnal variations *in vivo*.

Particles in the rumen digesta have a wide range in size and differ in chemical and physical characteristics (Ueda et al., 1995). Legay-Carmier and Bauchart (1989) found that the concentrations of solid adherent bacteria differed with particle size, and were higher in the smaller effluent particles (<0.1 mm) than the larger particles (0.4-4 mm) in the rumen digesta. Since fiber fermentation is accompanied with particle size reduction in the rumen, the particulate distribution size and the associated bacteria could change after feeding. To understand the plant cell wall degradation mechanisms, it is important to understand the diurnal changes in the microorganism distribution in different sized feed particle.

Both grass and legume hay have been commonly used as a roughage source in ruminant feeding. They may differ in particle size reduction pattern and fiber fermentation in the rumen (Grenet, 1989). These differences could be attributed to differences in physical structure and chemical characteristics between the two hays, but the interaction between the two types of roughage with bacteria in the rumen may also be an important factor. Comparative information on *in vivo* bacterial attachment and colonization of feed particles of different sizes between grass and legume hay is still limited. In this study, the bacterial population was classified into three sub-populations within the RR, : (1) not associated with particles (NAB) which represents bacteria free floating in the fluid; (2) loosely associated with particles (LAB); and (3) tightly associated with particles (TAB). The diurnal changes in the mass of these sub-populations and concentration distribution of TAB according to particle size in the RR digesta were determined from sheep fed orchard grass (OG) or alfalfa (ALF) hay once daily.

MATERIALS AND METHODS

Animals, feeding, and design

Four ruminally fistulated wethers (live weight

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Received June 1, 2000; Accepted August 1, 2000

74.5-87.5 kg) were allocated into individual pens with two different dietary treatments in a switchover experimental design. Dietary treatment was orchardgrass hay (OG) vs. alfalfa hay (ALF). The wethers were fed only hay, 2 cm long, once daily and allowed to eat for 2 h. Orts were removed after a 2 h of eating. This experiment was conducted for 10 weeks. In each period, the first 3 weeks were adaptation followed by a 7 week collection period for ruminal samples. Mineralized salt and water were provided *ad libitum*.

Sample collection and behavior observations

After the adaptation period, observations for behavior continued for 48 consecutive hours. These observations included eating, ruminating and resting behavior and were recorded at 5 min intervals.

During sample collection periods, the entire RR content was evacuated each week for 7 consecutive weeks. The sampling times were before feeding (0 h), and 2, 4, 6, 10, 14 and 18 h after feeding. After mixing the entire RR contents, weight and fluid pH were measured immediately. A representative sample of approximately 2500 g was taken for microbial determination. Fluid samples were taken and stored at -20℃ for ammonia-N and total VFA analysis. After sampling the digesta was replaced into the rumen.

Determination of microbial sub-populations

Sample preparation for grouping the microorganism sub-populations was according to the modified procedure described by Craig et al. (1987a). Representative samples of the entire RR digesta were weighed (2000 g) and squeezed through 8 layers of cheesecloth. The volume of strained RR fluid was measured. The residual particulate matter was washed with 0.85% saline solution to a volume equivalent to 20% of the strained RR fluid. This washed solution was added to the strained RR fluid and defined as the non-associated microorganisms' solution (NAMS). The residual particulate matter was then washed again with saline solution (1000 ml each time) and squeezed through 8 layers of cheesecloth three times to obtain the loosely associated microorganism solution (LAMS). After removing LAMS, approximately 400-500 g of the residues after squeezing were defined as the tightly associated microorganism particles (TAMP). This residue was sampled and placed into a plastic container which contained 1000ml of the extraction solution (0.85% NaCl, 0.1% Tween-80, 1.0% methanol and 1.0% tertiary butanol) (Whitehouse et al., 1994). After refrigeration for 24 h (0℃), the extraction solution with residue was homogenized using a Waring blender for 15 min. The homogenate was squeezed through 8 layers of cheesecloth. The residue was washed and squeezed again three times with 0.85% saline (600 ml for each wash). The tightly associated microorganism solution (TAMS) was thereby obtained.

The NAB and LAB were obtained using differential centrifugation of the NAMS and LAMS. Fine particles and protozoa were removed first (500× g, 30 min, 4℃). The supernatants were then centrifuged again (30000×g, 30 min, 4℃), and the residues were freeze-dried and weighed.

The TAB mass was separated from the TAMS first, using the described procedure. Since tightly associated microorganisms could not be separated completely, the total TAB determinations were based on the concentration of DAPA in a TAB pellet and in TAMP. The TAB concentrations in the RR digesta were calculated using the following equation: (concentration of DAPA in TAMP/concentration of DAPA in separated TAB)×(content of TAMP/content of RR digesta). The DAPA in TAB pellets and TAMP were determined according to the method of Czerkawski (1974).

Particle size distribution

The particle size distribution in the rumen digesta was determined using the wet sieving procedure described by Ichinohe et al. (1994). In this procedure the particles were separated into four fractions; large particles (LP>5600 μm), medium particles (MP, 1180-5600 μm), small particles (MP, 300-1180 μm), and fine particles (FP, 47-300 μm). The LP reduction rate was calculated using a linear least square model (Mertens and Loften, 1980).

Determination of TAB contents in particle of different size

To determine the TAB concentration in different particle fractions, the DAPA concentration in each particulate fraction was determined. The TAB concentration in each particulate fraction was calculated using the following equation and assuming that the TAB contained the same level of DAPA throughout the various particulate fractions: Concentration of DAPA in particle/concentration of DAPA in separated TAB.

Statistical analyses

The data were analyzed using analysis of variance procedures (William, 1979). The diurnal changes for the mass and concentration of each bacterial population, concentration of DAPA in PAB, and rumen fluid environment indexes (pH, NH_3-N, total VFA) with time after feeding were analyzed using one-way procedures. The differences in the bacterial mass and concentrations, and NH_3-N and total VFA between OG and ALF hay, particle distribution in the rumen digesta, and the PAB concentrations in various size particles were compared using a two-factor variance analysis method. When the F test was significant

($p<0.05$), the Student-Neumann-Kuels test was used for the multiple comparatisons.

RESULTS

From the chemical composition, ALF contained higher crude protein (CP), non-fiber carbohydrates (NFC), acid detergent insoluble lignin (ADL) and lower neutral detergent fiber (NDF) than OG (CP, 19.0 vs. 12.2%; NFC, 25.6 vs. 13.9%; ADL, 6.9 vs. 3.2%; 64.0 vs. 43.2%). Sheep fed ALF ate more hay (1.3 kg/day) than those fed OG (1.1 kg/day). On the other hand, sheep fed OG took slight longer for rumination than those fed ALF (388 vs. 293 min/day). Sheep fed ALF showed a significantly larger RR digesta pool size ($p<0.05$) all of the time than those fed OG (figure 1).

Figure 2 presents the diurnal changes in the pH, NH_3-N and total VFA concentrations in the RR fluid. Sheep fed OG showed a significantly lower ruminal pH than those fed ALF ($p<0.05$). Both groups showed a significantly lower pH at 6 h post-prandial. The values were 6.3 and 6.5 for OG and ALF, respectively ($p<0.05$). Ammonia nitrogen concentration reached the highest level 2 h after feeding for all sheep, and decreased gradually up to 6 h after feeding ($p<0.05$). The concentration of total VFA in the RR fluid increased from 0 to 4 h ($p<0.05$), then decreased slightly and remained at the same level until 18 h after OG feeding. In the sheep fed ALF, the total VFA concentration increased from 0 to 2 h after feeding ($p<0.05$), remained at the same level until 6 h and then decreased gradually. The average concentrations of NH_3-N and total VFA in sheep fed ALF were significantly higher than in the OG fed sheep ($p<0.05$).

The averaged DAPA concentration (mg/g DM) in TAB pellets was 2.08 (1.44 to 2.76) and did not differ significantly between OG and ALF feeding groups. The DAPA concentrations in the TAMP were 0.53 (0.38 to 0.68) for OG, and 0.46 (0.31 to 0.62) for ALF. The DAPA concentration showed a trend towards an increase as the particle size decreased. The average DAPA concentration values (mg/g DM) in LP, MP, SP, and FP were 0.31 ± 0.04, 0.32 ± 0.04, 0.37 ± 0.06, and 0.49 ± 0.05 for OG; 0.28 ± 0.06, 0.29 ± 0.05, 0.36 ± 0.06, and 0.60 ± 0.08 for ALF.

Table 1 presents the diurnal changes in bacterial mass for different sub-populations in the RR. The NAB and LAB contained less than 10% of the total bacterial mass, while the TAB contained more than 90%. The mean total bacterial mass in the RR was 307.9 g/g DM and 339.7 g/g DM for OG and ALF fed sheep, respectively. A significant difference ($p<0.05$) between the two hay groups was apparent 2 h after feeding. Although the NAB and LAB bacterial masses showed a trend towards increasing slightly from 0 to 2 h, there was no significant difference ($p>0.05$) during the post-prandial period in both hay groups. The bacterial TAB mass in the OG group significantly increased from 0 to 2 h ($p<0.05$), but was not significantly different ($p>0.05$) from 2 to 14 h after feeding. On the other hand, the bacterial TAB mass in the ALF-fed group significantly increased to a

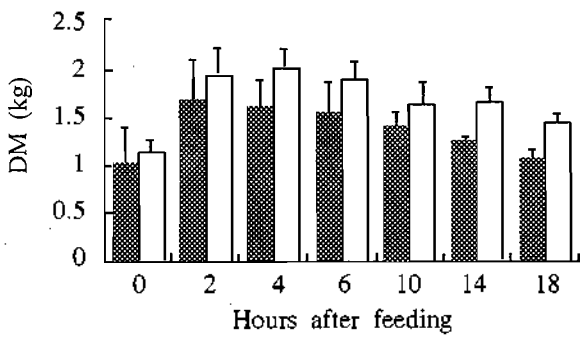

Figure 1. Diurnal changes in pool size of reticulorumen digesta in sheep fed orchardgrass (■) and alfalfa (□) hay

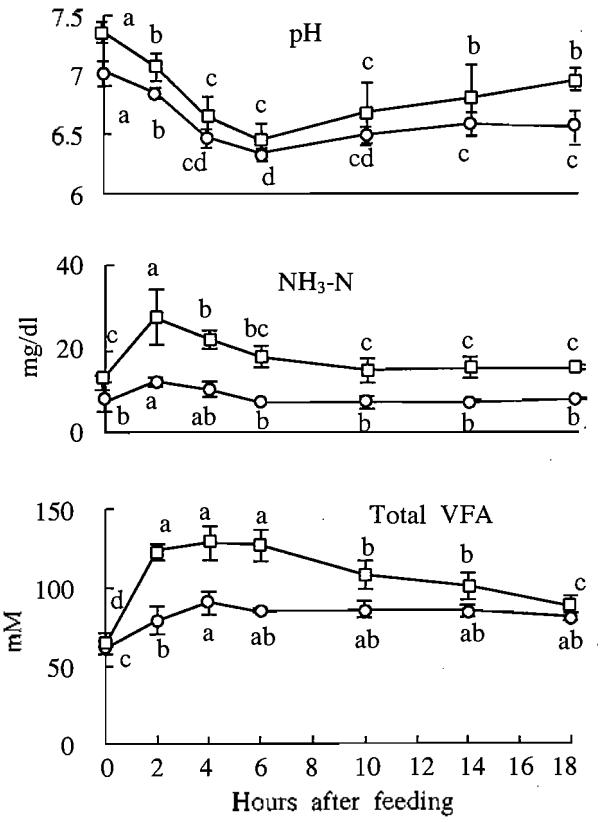

Figure 2. Diurnal changes in pH, NH_3-N, and total VFA levels in rumen fluid of sheep fed orchardgrass (○) and alfalfa (□) hay. ^{a,b,c,d} Means with non common superscripts are significantly different among each time point ($p<0.05$).

Table 1. Diurnal changes in mass of bacterial subpopulations (g dry matter) in reticulo-rumen digesta of sheep fed orchardgrass (OG) and alfalfa (ALF) hay

	Hours after feeding							Average	SE
	0	2	4	6	10	14	18		
OG									
NAB	20.6	25.1	15.7	15.1	15.8	18.4	19.5	18.2	2.56
LAB	5.1	9.8	9.5	7.5	9.0	10.0	9.1	8.7	1.28
TAB	165.8^{b}	310.9^{a}	364.6^{a}	378.4^{a}	308.0^{a}	306.4^{a}	181.8^{b}	281.0	22.25
Total B	191.5^{b}	345.7^{a}	389.8^{a}	401.0^{a}	332.9^{a}	334.8^{a}	210.4^{b}	307.9	21.15
Separated TAB	48.0	54.1	62.9	65.3	56.5	55.1	46.1	54.7	6.65
Recovery (%)	28.9	17.4	17.3	17.2	18.3	18.0	25.3	19.5	3.52
ALF									
NAB	15.6	21.8	20.4	21.2	19.9	17.9	16.0	18.6	1.65
LAB	9.5	12.1	11.9	7.7	10.4	13.2	11.0	10.8	1.15
TAB	164.8^{e}	463.4^{a}	409.2^{b}	365.5^{b}	281.3^{cd}	318.8^{c}	251.6^{d}	310.3	15.49
Total B	189.9^{f}	497.3^{a}	441.4^{b}	394.4^{c}	311.6^{de}	349.9^{cd}	278.6^{e}	339.7	15.75
Separated TAB	25.7^{c}	54.9^{a}	50.7^{ab}	51.1^{a}	44.7^{ab}	43.5^{ab}	34.2^{bc}	42.7	3.41
Recovery (%)	15.6	11.8	12.4	14.0	15.9	13.6	13.6	13.8	0.94

NAB : Bacteria non-associated with particles.
LAB : Bacteria loosely associated with particles.
TAB : Bacteria tightly associated with particles.
Total B : NAB+LAB+TAB.
Recovery : (Separated TAB)/(TAB).
^{a,b,c,d,e,f} Means with non common superscripts are significantly different ($p<0.05$).
SE : standard error.

maximum 2 h after feeding ($p<0.05$), followed by a decrease to the pre-feeding level. The diurnal change pattern in the TAB separated bacterial mass was different between sheep fed the different hays. The TAB separated bacterial mass showed a significantly increased diurnal change ($p<0.05$) during the post-prandial period in the ALF group, but no significant change was shown in the OG group. The rate of recovered TAB from feed particles (separated TAB/TAB mass) showed a trend towards an increase with the time elapse after feeding in both hay groups.

Figure 3 shows the diurnal change in the NAB, LAB and TAB concentrations in the RR digesta. The NAB concentration significantly increased up to 2 h in the ALF group ($p<0.05$), however no significant difference was apparent during the post-prandial period in the OG group. The LAB concentration significantly decreased during 0 to 6 h after feeding in the ALF fed group ($p<0.05$). The LAB concentration showed a similar trend in the OG group, but was not statistically significant. The TAB concentration showed a relatively faster increase in the ALF group, and reached the highest value 2 h after feeding. The TAB concentration in the OG group, however, only showed a trend toward a slow increase from 0 to 6 h ($p<0.1$), and then a slower change was observed until 14 h after feeding.

Figure 4 presents the diurnal particle size distribution changes in the RR digesta. The particles size proportion interaction and post-prandial time on the particle distribution were significant in both dietary groups ($p<0.05$). Both dietary groups showed a greater decrease in the ratio of large particles (LP and MP), especially in the LP, and increases in small particles (SP and FP) during the post-prandial period. However different types of hay showed different patterns from post-prandial time to large particle reduction and smaller particle formation. In the OG group, the LP and MP proportions tended to decrease from 6 h with SP and FP increasing after feeding. In the ALF group, the proportion of LP (after 4 h) showed a trend towards a decrease from 4 h with a gradual increase in SP and a slight increase in FP. However, the MP proportions in the ALF group did not show a significant change after feeding. This difference in LP breakdown was also indicated by the reduction rate, which was slightly faster for the ALF group compared to the OG group (3.19 vs. 2.95 %/h). The particle reduction pattern differed between these two hay groups as a result of the SP and FP being almost equally distributed in the OG group. The SP was more prominent than FP, LP and MP in the ALF group.

Figure 5 presents the TAB concentrations in different particulate sizes. The TAB concentration in FP showed a significant change on the post-prandial time in the ALF group and peaked at 2~6 h after feeding ($p<0.05$). The change in TAB concentration

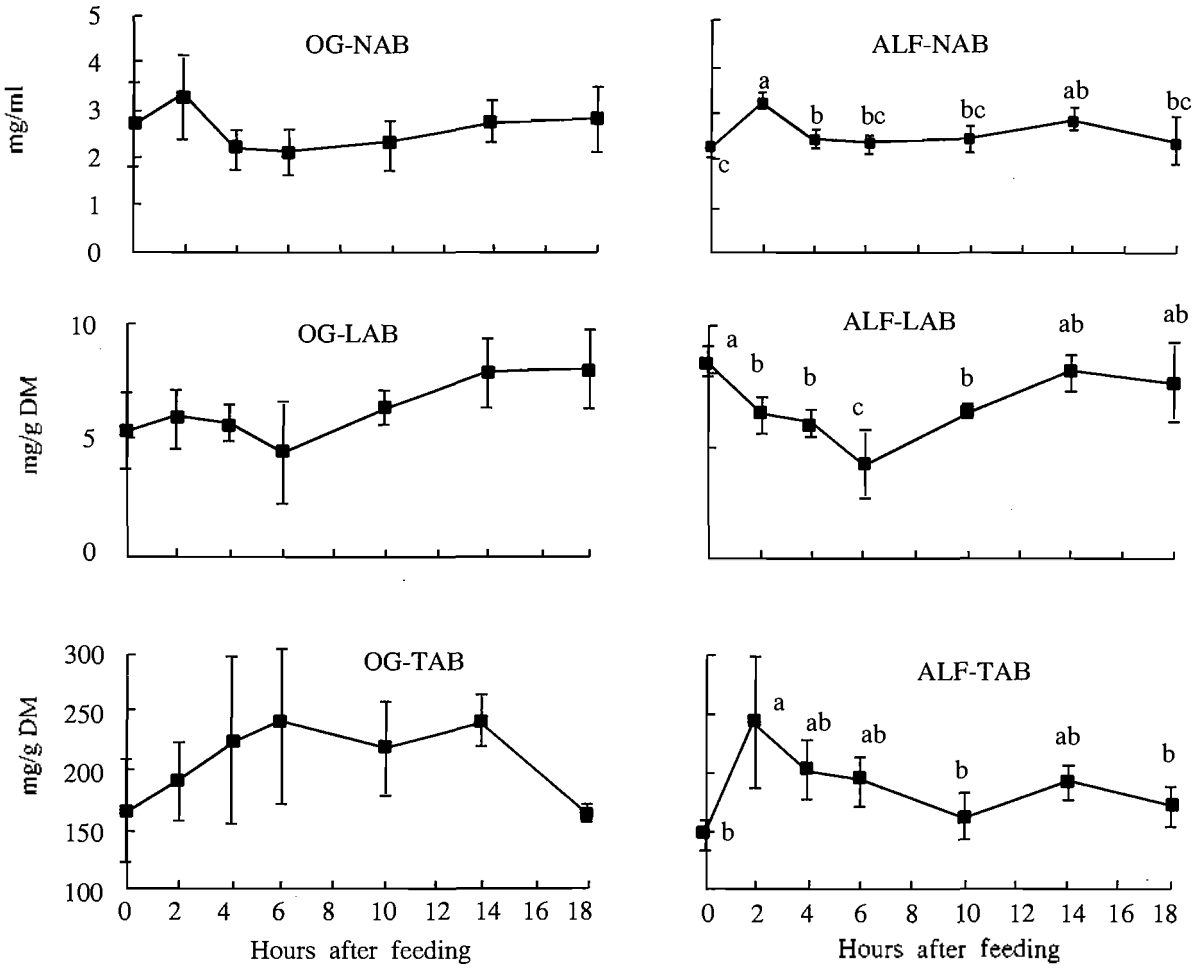

Figure 3. Diurnal changes in concentrations of non-associated (NAB), particle loosely associated (LAB), and tightly associated (TAB) bacteria in the reticulo-rumen digesta from sheep fed orchardgrass (OG) and alfalfa (ALF) hay. [a,b,c] Indicates values significantly different from 0 h ($p<0.05$).

with time after feeding for other particulate fractions, however, was not significant. The average bacterial concentrations between LP and MP did not differ significantly. Both dietary groups showed a trend toward lower bacterial mass in LP and MP than in the SP, and much lower than in the FP at all times during the post-prandial period. The TAB concentration of FP was significantly higher than LP and MP at 4, 6, 14 h, and higher than LP at 2 h after feeding of OG ($p<0.05$). In the ALF group, the TAB concentration in FP was significantly higher than other particle sizes from 2 to 18 h after feeding ($p<0.05$). The TAB concentration of FP, SP, and LP did not show significant differences 0 h after feeding, but the TAB concentration in FP at 4 h after feeding was about 2.1 times higher than the mean value of LP and MP, and 1.9 times higher than the SP at 4 h after feeding. These concentration ratios, however, decreased to 1.9 and 1.5 times at 0 h for ALF. Changes in TAB concentration of various particle sizes in the OG group also showed a similar trend towards a decrease in the late stage daily as compared to the ALF group.

From the TAB calculated summation from various size particles, the extent of TAB loss during the wet sieving procedure was similar to the averaged value of 31.3% vs. 27.4% for OG and ALF, respectively.

DISCUSSION

DAPA as a marker for TAB estimate

Since TAB could not be completely separated from feed particles, a marker was used to determine the quantity of these microbial populations. DAPA is often used as a marker for determining bacteria mass (Czerkawski, 1974; Robison et al, 1996; Faichney et al., 1997). However, some difficulties were pointed out. The first problem with using DAPA as a marker for rumen bacteria is that the DAPA concentration of isolated bacteria could vary among diets. For example, unusually high DAPA values were found in ruminal bacteria when steers were given urea (Ibrahim and Ingalls, 1972) or a high concentrate (90-100%) diet

(Whitelaw et al., 1984). An unusual diet and unstable fermentation may account for the high DAPA concentration in rumen bacteria. These results, however, do not necessarily invalidate DAPA as a marker for ruminal bacteria. In this study, no significant difference was observed in DAPA concentrations for separated TAB between the two species of hay and the value was similar to the published values (Olubobocun and Craig, 1990). In contrast to previous reports (Craig and Brown, 1987; Olubobocun and Craig, 1990), a significant difference ($p<0.05$) was observed in DAPA concentration for separated TAB at various times after feeding with the lowest 2~4 h in this study ($p<0.05$). This may be due to a DAPA proportional decline with nonstructural polysaccharides stored immediately after feeding (Craig et al., 1987b), or to different types of bacteria in TAB at different times after feeding, or different bacteria growth stages containing different DAPA concentrations (Olubobocun and Craig, 1990).

The second problem is that a large part of the DAPA resulting from bacterial lysis and degradation in the rumen digesta might be either free or bound in feed particles (Denholm and Ling, 1989). In this study, the washing and squeezing process was used to obtain TAMP. The wet sieving procedure was used to separate different particle sizes. Those processes that free DAPA could be eliminated. However, the effect of feed particle bound DAPA on the degree of accuracy in the TAB mass estimation is still not clear.

The third problem is that DAPA can be present in the feed (Czerkawski, 1974; Ling and Buttery, 1978; Rahnema and Theurer, 1986); microorganisms might be associated with the diet before it is consumed. Thus when DAPA was used as a bacteria marker, the assumption that DAPA originating in the diets would be completely degraded in the rumen (Cockburn and Williams, 1984) may not be correct. However, the dynamics of dietary DAPA degradation in the rumen, and which affects the estimated TAB mass accuracy, may require further study. We found OG and ALF hay used in this study contained 0.26 mg/g and 0.36 mg/g of DAPA, respectively. The DAPA content in the ALF hay was lower than the values determined by Rahnema and Theurer (1986) and higher than the values reported by Czerkawski (1974) and Webster et al. (1990). Since DAPA exists in the hay, it is probable that using DAPA as a marker may overestimate the bacterial mass. Using DAPA as a marker for determination of bacterial mass is still valid in a comparison but not on an absolute basis. Since the aim of this trial was a comparison of the various sizes of particulate associated bacterial mass and the diurnal changes in the rumen of sheep fed different hays, the value of the bacterial mass derived from the DAPA as a marker would be valid.

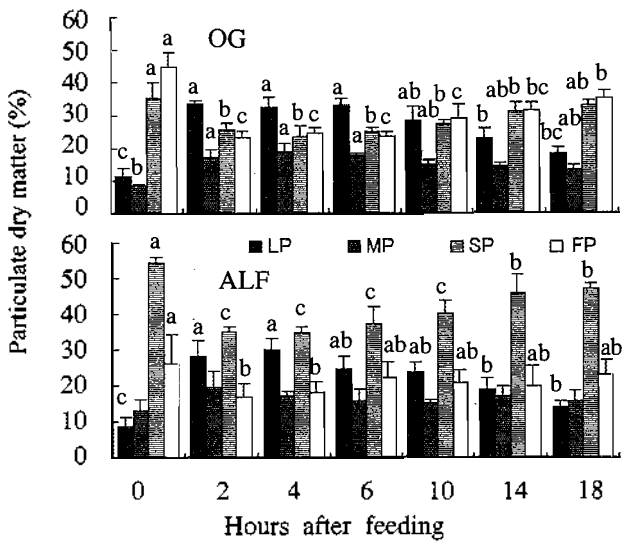

Figure 4. Diurnal changes in proportion of particulate fractions of reticulo-rumen digesta from sheep fed orchardgrass (OG) or alfalfa (ALF) hay once daily. [a,b,c] Means with non common supercripts are significantly different ($p<0.05$).

The bacteria attachment pattern to orchardgrass and alfalfa hay

Diurnal change patterns in both mass and concentration of the separated TAB and total TAB showed significant differences between sheep fed OG

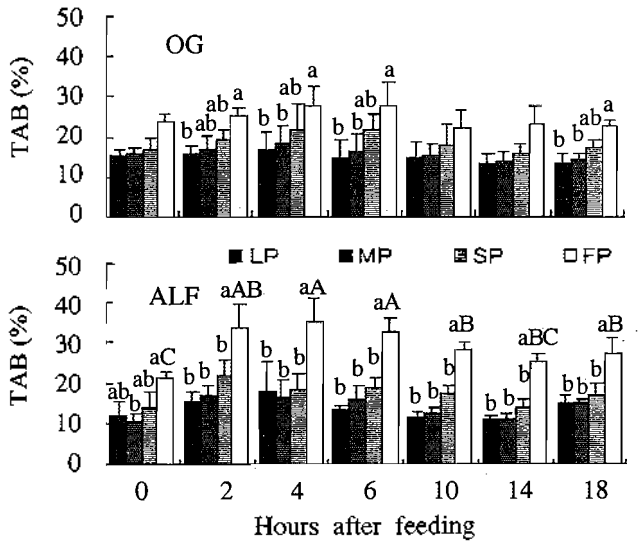

Figure 5. Concentration of TAB in various sizes of feed particle in reticulo-rumen digesta from sheep fed orchardgrass (OG) or alfalfa (ALF) hay once daily. [a,b,c] Means with non common superscripts are significantly different among particle size within a time ($p<0.05$). [A,B,C] Means with non common superscripts are significantly different between times ($p<0.05$).

and ALF (table 1, figure 3). Compared with the OG fed sheep, the TAB concentration reached a peak faster after feeding in the ALF group, suggesting that a more rapid bacterial growth and attachment to particles occurred in the ALF than the OG group.

Both microbial growth and passage rates affect the microbial mass contents in the rumen (Owens and Isaacson, 1977). Feed intake, and hence the RR content pool size, was greater in the ALF than in the OG fed sheep. This may be partly attributed to the higher mean values of TAB and total bacteria mass in the ALF over the OG group. When ALF hay is given to sheep a richer nutrient content is provided, including CP and probably minerals, as a substrate for microbial growth. Furthermore, the lignified cells centered in the xylem and tracheid cells in legumes as compared to more evenly distributed lignified cells in grasses provide a more digestible fiber for the fibrolytic bacteria in the initial stage of fermentation in legumes. These, therefore, possibly result in a favorable substrate at the initial stage after feeding for microorganism growth in the ALF fed sheep. On the other hand, the legume fed sheep showed more particle size reduction by mechanical breakdown through chewing during eating (Grenet, 1989). In this study, the sheep fed ALF (28.3%) showed slightly lower distribution in the proportions of LP at 2 h after feeding than sheep fed OG (33.5%) (figure 4). Moreover, the size reduction in feed particles through mastication both in eating and in the rumination period could increase the particle surface area for ruminal microbial attachment, as reported by Wilson and Mertens (1995). Since only 3~10% of the cell wall surface area is exposed to bacterial attachment during chewing, the surface area in the initial stage after feeding may be a limiting factor for the attachment of bacteria to the particles (Wilman and Moghaddam, 1998). The richest TAB mass in the ALF, as compared to the OG group at 2 h after feeding, therefore, might have been due to the faster bacterial growth rate and more available particle sites for bacterial attachment. As fast attachment and bacterial growth equals faster fiber digestion, rapid fermentation in the ALF-fed sheep than OG-fed group in the initial stage after feeding was also reflected in their high ruminal VFA concentration. However, the lucerne hay has a greater buffering capacity than grass hay, and exchange of more cations from feed particles with H^+ ions produced during fermentation (McBurney et al., 1983), leads to a higher ruminal pH for ALF-fed sheep than for OG-fed sheep.

Although shorter rumination occurred in the ALF fed sheep, the LP proportion in the total particle materials declined faster in the ALF compared to the OG group. Particle reduction appears to be more efficient for ALF than for OG fed sheep after feeding. In this experiment the proportion of smaller particles (SP+FP) that can pass from the rumen into the omason was higher in the ALF group as compared to the OG group ($p<0.05$). The SP passage rate was markedly faster than in the OG group (Ueda et al., 1997). These types of particles will take away large amounts of associated bacteria when passing through the rumen. Therefore, ALF could provide a higher fermentation rate with faster bacteria growth concurrent with faster passage rate from the rumen and less available fermentable fiber for bacterial growth in the latter stages of the post-prandial period. This results in a trend towards a slightly lower TAB concentration in the ALF group as compared to the OG group in latter post-prandial stage. It appears that the dynamics of bacterial attachment to feed particles might depend on the interaction between the chemical and physical characteristics of the feed, bacteria and rumen environment.

Wide varieties of bacteria exist in rumen fluid (Czrkawski and Cheng, 1988). A slightly increased NAB concentration immediately after feeding might be due to the growth of the bacteria utilizing soluble carbohydrate. However, the NAB concentration and mass showed a similar level most of the time with both types of hay fed sheep. This probably reflected the variety of bacteria species that survived in the fluid, with a relatively stable mass before feeding. Strains of the soluble carbohydrate-fermentating bacteria grew temporarily, becoming a niche in the fluid while most bacteria attach to the feed particles when feed is introduced into the rumen. Although the LAB concentration significantly changed in the ALF group, the margin was small and the bacterial mass did not show a significant change in the post-prandial period in this study. The enzyme activities from the LAB also did not show a significant change at different times after feeding (Willimans et al., 1989). Thus the LAB might obtain stable nutrients from the adjoining environment as previously hypothesized (Cheng et al., 1984; Czrkawski and Cheng, 1988; Willimans et al., 1989).

The diurnal changes in the total VFA showed different patterns in the sheep fed different hays, which agreed with the diurnal change pattern in the TAB for the respective diets, reflecting that the TAB plays an important role in fiber digestion.

Distribution of bacteria in different ruminal feed particle sizes

The feed particle associated microorganisms comprised the largest portion of the microbial mass, especially in TAB, which accounted for over 90% of the populations in the rumen. This agreed with the findings of Forsberg and Lam (1979) that particle associated microbial populations comprised up to 70%

of the rumen microbial mass. Craig et al. (1987a) also reported that the feed particle associated bacteria accounted for 70% to 80% of the microbial mass in entire rumen contents in cows receiving diets including concentrate. Different values in the proportion of TAB in the literature could be attributed to a dietary effect, animal differences, or the microbial mass determination methods. The particulate associated bacteria value would be reduced in forage fed animals supplemented with moderate level nonstructural carbohydrate concentrates (Piwonka and Firkins, 1997).

The smaller particles (SP and FP) contained a higher TAB concentration compared to the larger particles (LP and MP) (figure 5). The surface area also showed an increase as particle size decreased (Wilson and Mertens, 1995), suggesting that the surface area may be a limiting factor for bacteria attachment to feed particles. This result agreed with Legay-Carmier and Bauchart (1989), who reported that the smaller particles in the rumen digesta contained a higher TAB concentration when DAPA was used as a marker for TAB estimation. They also assumed the same level of DAPA in different particulate-size associated bacteria.

In comparison to the TAB concentration in different particle sizes among different time points post-feeding, the FP in the ALF group contained much higher TAB concentrations than the other sizes of particles as compared to the differences among particle sizes in the OG group. This is probably due to the FP in ALF being composed mainly of ALF leaves. The legume leaves contain thin walled cells that can be easily reduced to smaller particles and expose a larger surface area for the attachment of bacteria than grass leaves (Wilman and Moghaddam, 1998).

The differences in the TAB concentrations among larger size particles and smaller size particles tend to become smaller at the latter stages of the post-prandial period. This trend may be due to changes in some of the characteristics of the feed particles during rumination, mixing muscular activity by the rumen wall, and fermentation at the latter stage as compared to the initial stage of post-feeding. First, this may be due to the increase in surface area, making it easier for bacteria to attach to larger size particles. Second, the relative potential degradable fiber that exists in various sizes for bacteria development could have been changed.

The value of TAB distribution in different particle sizes obtained from this trial suggests that the surface area of the feed particles is important for bacteria attachment. This, however, did not agree with Gerson et al. (1988), who reported a slightly higher number of *R. flavefaciens* adhering to smaller particles (100-400 μm) than large particles (400-2000 μm), but the difference were not significant ($p>0.05$) although a markedly higher surface area in the smaller particles over the large particles was determined. McAllister et al. (1994) proposed a hypothesis that the bacteria attach to feed particles either randomly or through chemo-attractants. Further experiments are required to clarify the other physical and chemical characteristics of particles that may affect bacteria attachment.

In conclusion, TAB concentration reflected the changes in total bacterial mass tightly associated with particles in both groups of hay fed sheep. The number of bacterial colonies per particle increased as the particulate size decreased in both groups of hay fed sheep. This difference, however, tends to decline as the post-prandial period is prolonged. DAPA, however, tended to overestimate the TAB mass in the reticulo-rumen digesta of the hay fed sheep.

ACKNOWLEDGMENTS

We thank Prof. P. W. S. Chiou for advice in writing of this paper, and we also thank Miss M. Tanaka and Mr. M. Iida for their help in the period of ruminal digesta sampling during this study.

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