

The effect of ciliate fauna composition on murein content and mureinolytic activity in the rumen of sheep*

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ABSTRACT

The effect of the ciliates, *Eudiplodinium maggii*, *Diploplastron affine* and *Entodinium caudatum*, and natural protozoal fauna on the ruminal murein concentration and mureinolytic activity was examined on three rams, repeatedly defaunated and refaunated with *Eudiplodinium maggii*, *Diploplastron affine*, *Entodinium caudatum* and natural protozoal fauna. The number of ciliates varied from 18 (*E. maggii*) to 334×10^3 /g rumen content (natural fauna). The murein concentration fluctuated between 180 and 277 mg/g dry matter (DM). The establishment of ciliates in the rumen of defaunated sheep decreased the murein content by 28-35% ($P < 0.05$). Mureinolytic activity varied from 2.2 and 5.7 $\mu\text{g/g DM}$ of rumen fluid/min and was the lowest in defaunated sheep and the highest in animals faunated with *E. caudatum*. The protozoa increased this activity from 32 (*E. maggii*) to 159% (*E. caudatum*). All examined parameters showed diurnal variations. The ciliate number was the greatest just before feeding and the smallest 4 h thereafter. The fluctuation pattern in murein content was inverse to that of protozoa concentration and mureinolytic activity.

KEY WORDS: rumen ciliates, murein content, mureinolytic activity, sheep

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INTRODUCTION

The rumen is a unique ecosystem inhabited by entirely unicellular organisms. The most numerous are representatives of three different taxonomic groups, i.e. bacteria, fungi and protozoa. The establishment and maintenance of a stable microbial population is dependent on the diet, level and frequency of feeding, and microbe-microbe interactions (Wolin et al., 1997). According to biomass, the most abundant microbial groups in the rumen are bacteria followed by protozoa. The latter can, however, sometimes contribute to almost 50% of the total microbial biomass of rumen microbiota (Jouany, 1991). The relationships between these two groups of ruminal microorganisms have been the subject of numerous studies; the most common interaction seems to be predation (Coleman, 1989). Predation is a two-step process. In the course of the first step bacteria become engulfed by ciliates. During the second, the protozoa digest engulfed bacteria starting from degradation of murein, an important component of the bacterial cell wall (Hoogenraad and Hird, 1970; Morgavi et al., 1996).

Murein (a peptidoglycan) is a heteropolymer consisting of linear polysaccharide strands cross-linked by oligopeptides. Each carbohydrate strand is composed of N-acetylmuramic acid and N-acetylglucosamine. On the other hand, the cross-linking oligopeptides are usually tetrapeptides consisting of L-alanine, D-glutamate, meso-diaminopimelate and D-alanine (Ling, 1990). Murein contributes to about 3-10% and 20-60% of the dry matter of Gram-negative and Gram-positive bacteria, respectively (Litzinger and Mayer, 2010). On the other hand, Hoogenraad and Hird (1970) showed that the amino acid residue per 100 mg of freeze-dried cell wall was 29.2 mg. Thus, murein can be considered a potentially important source of energy and nitrogen in the rumen ecosystem.

Numerous studies published to date have shown that the drop in bacteria concentration in the rumen results from their engulfment by protozoa (Eadie and Hobson, 1962; Kurihara et al., 1968). Thus, the changes in bacteria number reflect the changes in murein content in the rumen. No publications concerning either murein concentration or its turnover in the rumen, as well as the contribution of protozoa to its metabolism, have been found in the available literature, however.

The objectives of our studies were thus: 1. to characterize the influence of common species of ciliates, such as *Entodinium caudatum*, *Eudiplodinium maggii* and *Diploplastron affine*, and of natural protozoal fauna on the murein content and mureinolytic activity in the rumen fluid of sheep, and 2. to examine the effect of the time of sampling on the examined parameters.

MATERIAL AND METHODS

Animal, feeding and experiment design

Three adult rams of Żelazieńska breed with an average 63 ± 2.5 kg body weight were fitted with large rumen fistulae and kept in separate pens. They were fed meadow hay (750 g) and ground barley (130 g) every 12 h. Water was available *ad libitum*.

The study consisted of 5 experimental periods in the course of which the sheep were either ciliate free (control period) or were inoculated with a population consisting of either only a single species of protozoa or natural rumen fauna (experimental periods). The study began with defaunation and collection of samples of rumen fluid from ciliate-free sheep (period I). The control period was followed by the refaunation of rams with *Eudiplodinium maggii* and an appropriate sampling (period II). The sheep were then defaunated again and refaunated with *Diploplastron affine* ciliates (period III). After the collection was finished, the sheep were again defaunated and refaunated with *Entodinium caudatum* (period IV). Finally, an inoculum consisting of mixed fauna was introduced into the rumen of animals already having *Entodinium caudatum* in the rumen (period V). Each period was composed of two sub-periods. The first lasted 3 weeks starting from the day when the defaunation procedure was finished or the day of inoculation of ciliates into the rumen. The development of the ciliate population was monitored every 2 days during this sub-period. The second sub-period was when rumen fluid was collected. It was sampled just before the morning feeding and 4, 8 and 12 h thereafter. The sampling was performed with a metal probe. A 5-6 cm filter was tightly screwed onto the end of the probe that was inserted into the rumen. The filter wall possessed numerous pores about 2 mm in diameter. To obtain representative material, the fluid was taken from 5 different places in the rumen and pooled. It was thoroughly mixed and sampled to determine the ciliate number (2 x 5 ml) and to perform chemical analysis (about 100 ml). The first of the samples was preserved with an equal volume of 4% aqueous formaldehyde while the second was lyophilized and stored at -80°C . The collection was repeated three times on three different days. The same animal was subjected to sampling no more than 2 times a week, however.

Defaunation and refaunation of sheep

The sheep were defaunated by evacuation of reticulo-rumen contents followed by the washing of both chambers according to Jouany and Senaud (1979). Additionally, the omasum was also washed as described by Michałowski

et al. (1999). The evacuated digesta was frozen and kept for 3 days at -20°C . The washing procedure was repeated 2 times a day and was followed by the filling of the reticulo-rumen with a buffer solution according to Michałowski et al. (1999). The frozen digesta was thawed on the 4th day and then heated to 70°C for 15 min to kill the protozoa that could have survived the freezing. Finally the content was returned to the rumen after adjustment of the temperature to 40°C . To refaunate the sheep with the single-species population, the ciliates *Entodinium caudatum*, *Eudiplodinium maggii* and *Diploplastron affine*, were isolated from the rumen of other sheep and cultured *in vitro* (Michalowski et al., 1991). Sheep possessing a natural mixed ciliate fauna were the source of the rumen fluid introduced as an *inoculum* into the rumen of rams at the beginning of the last experimental period. The ciliates isolated to begin the *in vitro* cultures were identified according to Dogiel (1927).

Ciliate counts

The protozoa were counted according to Michalowski and Muszynski (1978). The preserved sample, 0.1 ml in volume, was placed on a microscopic slide and all individuals were counted. The analysed material was diluted with a 1% aqueous formaldehyde solution if necessary.

Determination of murein content

The murein content in the rumen fluid of sheep was calculated from the concentration of N-acetylmuramic acid that was determined according to King and White (1977). Briefly, 200 mg samples of lyophilized rumen fluid were hydrolysed in 6 ml 6M HCl for 12 h and evaporated at 60°C to dryness. The residues were suspended in 6 ml distilled water and evaporated again. This procedure was repeated twice. Finally, the dry residue was suspended in 1M NH_4OH and incubated at 38°C for 30 min to liberate lactic acid. Its quantitative determination was performed by an enzymatic method using a Megazyme D-Lactic Acid Assay Kit (Cat. No. K-DATE). The obtained results were compared with a standard curve to calculate the content of murein in the analysed samples. Samples of purified murein of known weight were used to prepare the standard curve (Figure 1). These samples were hydrolysed and the liberated lactate quantified as above. The murein used to construct the standard curve was isolated from lyophilized cells of bacteria *Micrococcus lysodeikticus* ATCC (Sigma M 377) according to Glauner (1988).

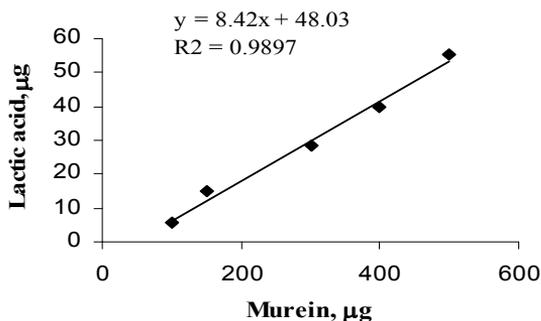


Figure 1. The relationship between the hydrolysed murein and the released lactic acid

The calculated recovery of murein was 67%, thus, a multiplication factor of 1.492 was applied to calculate the true quantity of murein in the analysed samples (Figure 2).

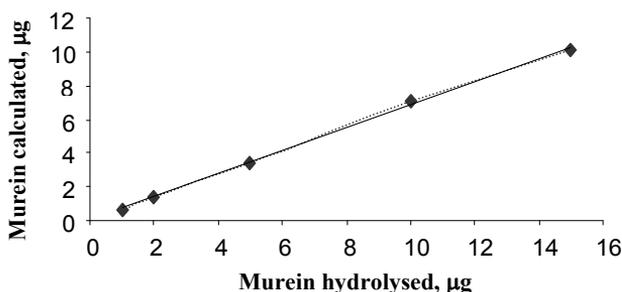


Figure 2. The relationship between the murein hydrolysed and calculated on the basis of liberated lactic acid

Determination of mureinolytic activity

Mureinolytic activity was determined according to Mörsky (1983) after extraction of enzymes from rumen fluid samples with the use of carbon tetrachloride (Huhtanen and Khalili, 1992). The extracted enzymes were incubated with a suspension of lyophilized *Micrococcus lysodeikticus* ATCC (Sigma M377) in a 0.02 M sodium phosphate buffer for 1 h at 40°C and this was followed by measurement of the turbidity of the reaction mixture. The activity of mureinolytic enzymes was expressed as the disappearance of the digested substrate µg/g DM of rumen fluid. The samples of enzyme extract and substrate were used as controls. They were incubated separately in the same buffer and their turbidity was measured as described above.

Statistical analysis

The analysis results of rumen content samples taken at the particular time points on each collection day were used to calculate the mean values for each sampling day and each animal. These data were then used to calculate the overall means characterizing ciliate number (periods II-V) as well as murein content and mureinolytic activity (periods I-V). Similar values characterizing the ciliate count as well as murein content and mureinolytic activity related to each animal and each time of sampling were also calculated. These data were used to calculate the mean values for analysing the influence of time of sampling on the examined parameters. To characterize the effect of protozoa on murein content and mureinolytic activity, the significance of differences between the ciliate-free and differently faunated sheep was analysed. The influence of particular groups of protozoa on the same parameters was characterized by the significance of differences between the differently faunated animals. The effect of time of sampling on protozoa population density was analysed by calculating the differences between their numbers in successive sampling points. Similar calculations were made in relation to murein content and mureinolytic activity. Student's t-test was used to calculate the statistical significance of differences between the compared means; all calculations were performed according to Parker (1979).

RESULTS AND DISCUSSION

Effect of protozoa on murein content and mureinolytic activity

The sheep were ciliate-free during the first (control) period of the experiment and were successively inoculated with three single species of these microorganisms during the next three periods, followed by faunation with natural rumen fauna. The protozoa counts in subsequent periods of the study accompanied by the murein content and mureinolytic activity of rumen fluid are presented in Table 1. The concentration of protozoa varied from about 18 to 334×10^3 cells/ml and the number of *Eudiplodinium maggii* ciliates was the smallest of all populations developed in the rumen of the sheep. The number of these protozoa was almost 3 times lower than of *Diploplastron affine* and over 12 times lower in comparison with the number of *Entodinium caudatum*. The most numerous protozoa were found during the last period of the experiment, i.e. when the sheep were inoculated with natural rumen fauna. Representatives of the genus *Entodinium* contributed there to 86-96% of the total protozoa count, whereas the ciliates *Eudiplodinium maggii* and *Diploplastron affine*, to only 1-5% and 1-8%, respectively. Ciliates from the genus *Epidinium* were also present

Table 1. The ciliate number ($\times 10^3/\text{ml}$), murein content (mg/g DM) and mureinolytic activity (mg disappeared murein/g DM/min) in the rumen fluid of ciliate free sheep (I) and in the same animals faunated with *E. maggii* (II), *D. affine* (III), *E. caudatum* (IV) and a natural rumen fauna (V). Overall means for each period (n=9)

Item	Experimental period				
	I	II	III	IV	V
Ciliate concentration	0	17.8 \pm 0.97 ^a	51.3 \pm 2.90 ^b	223 \pm 5.54 ^c	334 \pm 8.82 ^d
Murein content	277 \pm 13.90 ^a	180 \pm 11.49 ^b	185 \pm 11.81 ^b	184 \pm 13.78 ^b	200 \pm 14.86 ^c
Mureinolytic activity	2.2 \pm 0.08 ^a	2.9 \pm 0.29 ^b	3.1 \pm 0.28 ^b	5.7 \pm 0.97 ^c	5.7 \pm 0.56 ^c

values with different superscripts differ significantly at $P < 0.05$

there and contributed up to 5% of the total number of mixed fauna. According to Michalowski (1990), *Entodinia*, with the exception of *Entodinium bursa* (synonym *Entodinium vorax*), belong to the group of 'small ciliates'. The biomass of a single cell of a protozoan from this group is many times less than of *Diploplastron affine* and, in particular, of *Eudiplodinium maggii* which belongs to the 'large ophryoscolecids'. Thus, in spite of the relatively high count, the protozoal biomass was probably the lowest when only *Entodinium caudatum* ciliates were present in the rumen of rams.

The concentration of murein varied from 180 to 277 mg/g rumen fluid in the relation to the absence or presence of protozoa and their populations established in the rumen. The development of a ciliate population resulted in a decrease in the concentration of murein in the rumen fluid of rams by about 28-35%. This decrease could be explained as a result of a diminishing of bacteria number due to their engulfment and digestion by protozoa (Coleman, 1989). Such an explanation is in agreement with the findings obtained by Eadie and Hobson (1962) and Kurihara et al. (1968) who observed a negative effect of ciliates on bacteria number in the rumen. It disagrees, however, with the results of the experiment performed by Jouany et al. (1981) who found no such effect of protozoa on bacteria count. Some other factors should thus also be taken into consideration; one of them seems to be competition between bacteria and ciliates for nutrients. Interestingly, the negative effect of natural rumen fauna was significantly less in comparison with the influence of single-species populations. This suggests that rumen fauna composition can be considered one of the factors influencing murein content in the rumen. The data presented in Table 1 also show that murein contributed to about 18-28% DM of rumen fluid. Litzinger and Mayer (2010) reported that the content of murein in Gram-negative and Gram-positive bacteria varied in the range of 2-10% and 20-60% of their DM, respectively. Ling (1990) claimed, however, that Gram-negative species are most numerous in the rumen, whereas Gram-positive species account for 5-30% of the total number of bacteria. On the other hand, the cell wall of very abundant *Butirivibrio fibrisolvens*, and predominant cellulolytic species, i.e. *Ruminococcus flavefaciens* and *Ruminococcus albus*, exhibits the

cell wall structure of Gram-positive species despite being Gram-negative strains (Ling, 1990; Stewart et al., 1997). Thus, the relevant data presented in Table 1 seem not to be overestimated.

Mureinolytic activity was estimated as the disappearance rate of the lyophilized bacteria *Micrococcus lysodeikticus* following their incubation with the enzymes extracted from the rumen fluid (Huhtanen and Khalili, 1992). The disappearance rate of the substrate was influenced by the presence and composition of the ciliate population and was the lowest in ciliate-free sheep. The differences between defaunated and faunated sheep were statistically significant independent of the protozoal population established in the rumen. The examined degradation rate was the highest when the sheep were faunated with *Entodinium caudatum*. No difference was found, however, between the activities measured in periods IV and V. The obtained results thus confirm the earlier findings of Morgavi et al. (1996) concerning the mureinolytic activity of *Entodinium caudatum* and of natural ciliate fauna. No difference was also found between the mureinolytic activity in the rumen of animals inoculated with *Eudiplodinium maggii* and *Diploplastron affine*. Both species are considered to be involved in fibre digestion (Dehority, 1993; Michalowski, 1997; Wereszka et al., 2006). On the other hand, their ability to degrade murein was lower by 46-50% in comparison with the fauna composed of *Entodinium caudatum* and with mixed rumen fauna consisting mainly of small ciliates. This suggests that small protozoa may be decisive for the conversion of murein in the rumen. Such a suggestion needs experimental confirmation, however.

Effect of sampling time on ciliate number, murein content and mureinolytic activity

The population density of ciliates at different times after feeding is presented in Table 2. The number of ciliates varied from 14 to 360 x 10³/ml rumen fluid depending on the species and time after feeding. Statistically significant diurnal fluctuations in population density were observed in the case of all of the groups

Table 2. The concentration of ciliates (x10³/ml) in the rumen fluid of selectively faunated sheep at different time after morning feeding (h). Mean values (n=3)

Ciliate population	Periods	Time after feeding, h			
		0	4	8	12
<i>E. maggii</i>	II	20.3 ^a	14.1 ^b	17.3 ^c	19.3 ^a
<i>D. affine</i>	III	57.7 ^a	42.9 ^b	45.6 ^c	58.9 ^a
<i>E. caudatum</i>	IV	234 ^a	217 ^b	200 ^b	238 ^a
Natural fauna	V	350 ^a	307 ^b	318 ^b	360 ^a

values with different superscript differ significantly at P<0.05

of protozoa inoculated into the rumen of sheep. The most numerous ciliates were found just before feeding and the least, at 4 or 8 h afterwards ($P < 0.05$). Our findings are thus in accordance with the results of Michalowski and Muszynski (1978).

The changes in the concentration of murein in the rumen fluid of sheep are presented in Table 3. The smallest content of this bacterial cell wall component in the rumen fluid of sheep was found just before feeding and the largest, at 4 h after ($P < 0.05$). The diurnal variations in the murein concentration were thus of a reverse pattern in comparison with that of protozoa numbers and reflect the changes in the number of bacteria. They followed perhaps the changes in the quantity of available nutrients and the changes in multiplication velocity of these microorganisms.

Table 3. The concentration of murein (mg/g DM) in the rumen fluid of ciliate free and selectively faunated sheep in relation to the time after morning feeding (h). Mean values (n=3)

Established population	Periods	Time after feeding, h			
		0	4	8	12
None	I	278 ^a	359 ^b	298 ^c	273 ^a
<i>E. maggii</i>	II	157 ^a	226 ^b	180 ^c	158 ^a
<i>D. affine</i>	III	158 ^a	230 ^b	186 ^c	161 ^a
<i>E. caudatum</i>	IV	161 ^a	234 ^b	200 ^b	148 ^a
Natural fauna	V	171 ^a	246 ^b	246 ^b	175 ^a

values with different superscript differ significantly at $P < 0.05$

As pointed out in the Introduction, murein is a regular component of the bacterial cell wall and one of the constituents of this peptidoglycan is muramic acid. This acid was used by King and White (1977) to assess the biomass of bacteria in estuarine and marine samples. The results presented in our report support the possibility of using it as a natural marker of bacteria in the rumen. For many years, diaminopimelic acid (DAPA) has played this role. In fact, this amino acid is also a component of bacterial cell walls. The quantitative determination of muramic acid is, however, much simpler in comparison with DAPA quantification methods (King and White, 1977; Michalowski, 1990; Czuderna and Kowalczyk, 1999). Nowadays, molecular methods, e.g., qPCR, have often been used to assess bacterial mass or/and number in the rumen (Vasta et al., 2010). Nonetheless, this method requires very expensive equipment and chemicals. Conversely, no such equipment is required to quantify muramic acid or murein by the method used in our study.

The changes in mureinolytic activity with the time after feeding, as shown in Table 4, indicate that the digestion rate of murein was influenced by the presence and composition of the ciliate population.

Table 4. The mureinolytic activity (mg disappeared substrate/g DM rumen fluid/min) in ciliate free and differently faunated sheep in the relation to the time after feeding (h). Mean values (n=3)

Established population	Experiment periods	Time after feeding, h			
		0	4	8	12
None	I	2.34 ^a	1.87 ^b	2.06 ^c	2.37 ^a
<i>E. maggii</i>	II	3.80 ^a	1.89 ^b	2.04 ^b	3.90 ^a
<i>D. affine</i>	III	3.81 ^a	2.06 ^b	2.81 ^b	3.63 ^a
<i>E. caudatum</i>	IV	8.15 ^a	3.17 ^b	3.56 ^b	8.05 ^a
Natural fauna	V	7.51 ^a	4.37 ^b	4.29 ^b	6.45 ^a

values with different superscript differ significantly at $P < 0.05$

The highest activity was found just before feeding, the lowest, 4 h after. The decline in activity was observed independently of the presence or absence of protozoa. The magnitude of the decline differed, however. Mureinolytic activity in the rumen fluid of ciliate-free and faunated animals decreased during the first 4 h after feeding by about 20% and 42-61%, respectively. The changes in mureinolytic activity were opposite to the changes in bacteria number, as suggested by the changes in murein content. On the other hand, this activity was positively correlated with the changes in the number of protozoa. This suggests that protozoa are responsible for mureinolytic activity in the rumen.

CONCLUSIONS

The quantitative determination of murein (a peptidoglycan) enabled us to consider this component of the bacterial cell wall as a potentially important source of nutrients in the rumen. The enzymatic study showed that ciliates are the microorganisms involved in murein digestion and perhaps play the main role in conversion of this bacterial cell wall component in the rumen. The mureinolytic activity of rumen protozoa does, however, seem to be species dependent. The reported findings suggest a dominant role of small protozoa like *Entodinium caudatum* in the degradation of murein in the rumen.

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