Journal of Animal and Feed Sciences, 6, 1997, 485–497

The effect of diet on the microbial mass, and xylanase and CMC-ase activities in whole rumen digesta and in different fractions of the rumen contents of cows*

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(Received 5 March 1997; accepted 24 October 1997)

ABSTRACT

Three cows fitted with large rumen fistulas were used in investigations of relationships between growth of microbial populations and activity of fibrolytic enzymes in relation to diet. The diet consisted of barley grain and hay and was supplemented with rapeseed meal or fish meal. The total number of ciliates varied from $121.3x10^3$ and $223.2x10^3$/g of rumen contents and was significantly higher when the feed was supplemented with fish meal. Bacterial mass was 11.0-14.9 mg/g rumen contents irrespective of diet. The determinations were based on the DAPA concentration in bacteria and in rumen digesta. The biomass of protozoa in the whole rumen varied from 208.2 to 358.6 g, that of bacteria ranged from 986.3 to 1391.6 g. Fish meal supplement increased the biomass of protozoa before the morning meal and 4 h thereafter and the biomass of bacteria before feeding. Xylanase activity in whole rumen digesta varied from 68.4 to 96 μmol reducing sugars released from xyian/g DM/min and was higher when the feed was supplemented with rapeseed meal. The activity of CMC-ase was 6.2-6.9 μmol reducing sugars released from CMC/g DM/min. No relationship was found between either enzyme activity and total bacterial mass in the rumen contents The xylanase and CMC-ase activities associated with particulate fractions varied from 34.0 to 63.3 and from 1.9 to

* Supported by the State Committee for Scientific Research, Grant No 5 S305 021 05

4.6 μmol of reducing sugars released from xylan or CMC respectively. The activity was higher when cows were was fed diet supplemented with fish meal, and this was accompanied by higher counts of protozoa. A negative correlation was found between the activity of CMC-ase associated with particulate fractions and ADL content in this material.

KEY WORDS: xylanase, CMC-ase, bacteria, protozoa, rumen digesta, particle fractions

INTRODUCTION

Cell wall carbohydrates are the main source of energy for ruminants fed forage diets. The breakdown rate of the two basical components of structural polysaccharides, i.e. cellulose and hemicellulose in the rumen depends on the activity of enzymes degrading these substances and/or the number of cellulolytic and hemicellulolytic microorganisms (Silva et al., 1987; Huthanen and Khalili, 1992). It is generally accepted that microbial attachment to the feed particles is an important factor influencing degradation of the structural polysaccharides in the rumen (Cheng et al., 1984; Weimer, 1996) and there is a suggestion that the surface area of feed particles influences their colonization by cellulolytic and hemicellulolytic bacteria (Huthanen et al., 1992). According to Silva et al. (1987) the rate of colonization of the feed particles by cellulolytic microorganisms can be determined by measurement of the CMC-ase activity associated with the particulate fraction of the diet. It has been already found that supplementation of forage with a high proportion of sucrose or starch (barley grain) decreased activity of cellulolytic and xylanolytic enzymes (Huthanen and Khalili, 1992, Noziere et al., 1996). The influence of some other components of the diet is not well established.

The two most numerous populations of rumen microorganisms are bacteria and protozoa. Interrelationships exist between these two groups (Coleman, 1975) and it is well known that the presence of ciliates in the rumen decreases the number of bacteria in the liquid phase of rumen contents which pass down from the reticulo-rumen (Jouany, 1996). The influence, however, of ciliates on the rate of colonization of feed particles by fibrolytic bacteria is not known.

The aim of this experiment was to investigate the activity of fibrolytic enzymes like xylanase and CMC-ase in the rumen of cows fed a hay-barley diet supplemented with two different sources of protein. Measurement of activity of both enzymes in the whole rumen digesta and in particulate fractions of different size, as well as the analysis of chemical composition of the particulate fractions and the measurement of density of the bacterial and protozoal population were performed in order to gain insight into the mechanism of the fibre degradation in the rumen.

MATERIAL AND METHODS

Animals and feeding

Three cows fitted with permanent 110 mm rumen cannulas were used. The animals were fed with isonitrogenous and isoenergetic diets based on hay and barley grain and supplemented with rapeseed meal (diet R) or fish meal (diet F). A detailed description of the diets is given in an earlier paper in this issue (Pająk et al., 1997). The feed was given in two equal meals at 8.00 and 16.00. Water was freely available. An adaptation period of 3-4 weeks to each kind of feed was allowed before the sampling period was started.

Sampling

Samples of the rumen contents, approximately 9 kg, were taken after emptying the rumen. The digesta was weighed, thoroughly mixed and returned to the rumen immediately after sampling. The sampled contents were then subsampled for protozoa counts (2x5 g), for enzyme activity and DAPA measurement (2x20 g) and for fractionation of digesta to the particles of different size (2x100 g). The remaining part of each sample was frozen and used for fractionation as described above and for chemical analysis. The relevant results are presented in the preceding paper (Pająk et al., 1997). The sampling was performed just before the morning feeding and 4 and 8 h thereafter. The time interval between subsequent samplings from the same animal was about one week. No repetition was made.

Samples of rumen fluid, about 2 liters, were taken before the morning feeding of cows and used for preparation of the suspension of protozoa and bacteria.

Incubation in the rumen

Particles of different size obtained following grinding of hay were incubated in the rumen of cows with the use of nylon bags with a pore size of 50 μm. Hay was ground with the use of a hammer grinder. Particle fractions of three different size (5 mm, 1-5 mm and 0.25-1 mm) were obtained by a sieving technique. Samples of each fraction (5 g) were incubated in triplicate in the rumen of cows for 12 h starting from the morning feeding. The bags were taken from the rumen at 2, 4, 8 and 12 h of incubation and washed in cold water in a household washing maschine (Wiatka Automat 12). The washing program lasted 15 min and consisted of 5 rinsing cycles. After washing, the particles were collected, stored at -20^{0}C and used for determination of xylanase and CMC-ase activities.

Preparation of the particulate fractions of rumen digesta, purified protozoa and bacteria

Fractionation of digesta into particles of different size (see above) was carried out using a wet sieving technique with an use of Analysette 3 (Fritsch) apparatus (Pająk et al., 1997).

Preparation of the suspension of ciliates free of feed debris and external bacteria, as well as separation of protozoa of different cell dimensions was done according to Michałowski (1990).

To obtain a purified fraction of bacteria, the rumen fluid was strained through 4 layers of cheese cloth and centrifuged at 200 g for 5 min. The supernatant was collected and centrifuged at 25.000 g for 20 min at 4°C. The obtained pellet composed of bacteria was then washed two times with cold distilled water and dried at 105°C.

Analytical methods

Extraction of enzymes from the samples of rumen contents and from particulate fractions was performed according to the procedure of Huhtanen and Khalili (1992) while CMC-ase and xylanase activities in the extracts were measured as described by Gorleau and Forsberg (1981) and Huhtanen and Khalili (1992). Reducing sugars released from carboxymethylcellulose (Sigma, No. C 5678) and xylan (Sigma No. X 0502) during incubation of the both substrates with the extracts mentioned above were determined quantitatively with the use of 3,5-dinitrosalicilic acid reagent (Miller et al., 1960). Substrates without extracts and extracts alone, incubated simultaneously, were used as controles and D-glucose was used as a standard. Both CMC-ase and xylanase activities were expressed as μmol reducing sugars released/g DM/min.

Dry matter (DM) of rumen contents and particulate fractions was determined by drying at 105°C for 24 h.The determination of NDF, ADF and ADL was described in the preceding paper (Pająk et al., 1997). DAPA in the bacteria and in the rumen digesta was measured according to Czerkawski (1974). Total protozoa were counted with an use of a Fuchs-Rosenthal chamber, while specific counts were performed according to Michałowski (1975). The ciliates were identified after Dogiel (1927) and Grain (1966).

Statistical analysis

Statistical calculations were made according to Ruszczyc (1970).

RESULTS

The composition of rumen fauna was similar in all three cows. The following species were identified: *Entodinium exiguum, Entodinium rostratum, Entodinium caudatum, Entodinium longinucleatum, Entodinium bursa, Anoplodinium denticulatum, Eudiplodinium rostratum, Polyplastron multivesiculatum, Ostracodinium gracile, Isotricha prostoma, Isotricha intestinalis* and *Dasytricha ruminantium*. The total number of ciliates and contribution of three main morphological groups of protozoa to the total counts is presented in Table 1. The diet affected the number of ciliates and significant differences were found before the morning feeding and 4 h thereafter. Most numerous group was *Entodinium* followed by *Holotricha* and *Diplodinia*. The significant influence of the diet on the percentage of particular groups of ciliates was found only at 4 h after feeding.

TABLE 1

Total number of ciliates (x 10^3/g) and percentage of particular groups in the rumen contents of cows fed two different diets

Time after feeding h	Ciliates	Diets			Significance of difference
		R	F	SD	
0	Total	123.4	192.0	40.07	0.1
	Entodinium	83.8	85.4	2.56	N.S.
	Diplodinium	4.1	4.0	1.30	N.S.
	Holotricha	12.1	10.6	3.74	N.S.
4	Total	121.3	223.2	58.43	0.01
	Entodinium	85.8	93.0	5.33	0.05
	Diplodinium	5.8	2.7	2.72	0.05
	Holotricha	8.4	4.3	3.08	0.05
8	Total	127.6	159.6	32.33	N.S.
	Entodinium	88.0	88.0	1.78	N.S.
	Diplodinium	6.4	6.7	2.09	N.S.
	Holotricha	5.6	5.3	3.04	N.S.

Protozoal mass varied in the rumen from about 208 to 358 g DM. It contributed to 12.3-31.8% of the total microbial mass and in the majority of cases was lower than 20%. The biomass of ciliates was calculated on the basis of DM content in single cells of protozoa separated into three groups (Table 2), and their number in the rumen. „Small *Ciliates*" consisted of *Eudiplodinium rostratum, Dasytricha ruminantium* and *Entodinia* with the exception of *Entodinium bursa*. This last species as well as *Anoplodinium denticulatum* and

TABLE 2

Dry matter content (ng) in the single cell of ciliates isolated from the rumen of cows

Protozoa group	Dry matter
„Small *Ciliates*"	13.1 ± 0.38
„Medium *Ciliates*"	69.1 ± 32.18
„Large *Ciliates*"	326.2 ± 153.20

Ostracodinium gracile are listed in „Medium *Ciliates*". *Polylastron multivesiculatum* and both species of *Isotricha* formed ther group of „Large *Ciliates*".

The density of the population of bacteria expressed as mg/g of rumen digesta was 11-14.9 and was not influenced by the diet. Bacterial mass in the rumen varied from about 938 to over 1520 g DM and contributed to 68-88% of the total biomass of microorganisms. Mean values are given in Table 3. The biomass of bacteria, expressed as DM, was calculated on the basis of DAPA concentration in the bacteria cells and in the rumen digesta. The mean concentration of 2,6-diaminopimelic acid in bacteria varied from 1.9 to 2.6 mg/g DM. Diet significantly influenced the bacterial mass in the rumen only before the morning feeding.

TABLE 3

Biomass of protozoa and bacteria (g DM/rumen) in the rumen of cows fed diets supplemented with rapeseed meal (R) or fish meal (F)

Time after feeding h	Ciliates	Diets			Significance of difference
		R	F	SD	
0	Bacteria	986.3	1241.3	194.26	0.05
	Protozoa	208.2	340.6	70.40	0.01
4	Bacteria	1335.9	1391.6	138.19	N.S.
	Protozoa	233.0	358.6	121.06	0.05
8	Bacteria	1237.9	1284.7	130.77	N.S.
	Protozoa	224.2	249.1	33.89	N.S.

Xylanase activity in the rumen digesta varied from 68.4 to 96.0 μmol reducing sugars released from xylan/g DM/min (Table 4) and was significantly affected by the diet. The diet did not influence the activity of CMC-ase.

The activity of xylanase extracted from three particulate fractions varied from 34.0 to 63.4 while this of CMC-ase from 1.9 to 4.6 μmol reducing sugars released from appropriate substrates/g DM/min (Table 5). The both activities extracted from particulate fractions were significantly lower ($P < 0.05$) than these found for the whole digesta (see Tables 4 and 5). Diet influenced the activity of the both enzymes attached to feed particles irrespective of their dimensions. A higher

TABLE 4

The activity of xylanase and CMC-ase (μmol reducing sugars released from xylan or CMC/g DM/min) in the rumen contents of cows fed diets supplemented with rapeseed meal (R) or fish meal (F)

Time after feeding	Diets			Significance of difference
h	R	F	SD	
		Xylanase		
0	72.7	72.1	6.54	N.S.
4	82.1	68.4	9.99	0.05
8	96.0	78.0	14.42	0.05
		CMC-ase		
0	6.5	6.8	1.00	N.S.
4	6.2	6.7	0.70	N.S.
8	6.9	6.8	0.64	N.S.

activity was in majority of cases found when cows were fed the diet F. However, the activity of xylanolytic enzymes extracted form the largest particles was higher when the animals received the diet R. No relationship was found between enzyme activities and neither ADF nor NDF contents in particulate fractions. There was, however, a negative correlation between CMC-ase activity and ADL content (Figure 1).

TABLE 5

The activity of xylanase and CMC-ase (μmol reducing sugars released from xylan or CMC/g DM/min) associated with particles of three different size separated from the rumen contents of cows fed diets supplemented with rapeseed meal (R) or fish meal (F)

Time after feeding	Xylanase Diets			Significance of	CMC-ase Diets			Significance of
h	R	F	SD	difference	R	F	SD	difference
				>5 mm				
0	49.2	41.8	4.05	0.5	3.8	3.3	0.71	N.S.
4	34.0	38.5	4.94	N.S.	2.8	3.4	0.44	0.05
8	50.4	48.4	3.46	N.S.	3.6	4.1	0.32	0.05
				1-5 mm				
0	38.8	48.6	8.23	0.05	2.9	3.7	0.56	0.05
4	43.8	50.9	5.14	0.05	3.5	4.1	0.51	N.S.
8	47.7	63.4	9.25	0.01	3.3	4.6	0.65	0.01
				0.25-1 mm				
0	43.3	44.6	3.59	N.S.	2.8	3.0	0.39	N.S.
4	34.6	42.8	5.21	0.05	1.9	2.8	0.31	0.01
8	44.7	50.1	7.15	N.S.	2.9	3.2	0.56	N.S.

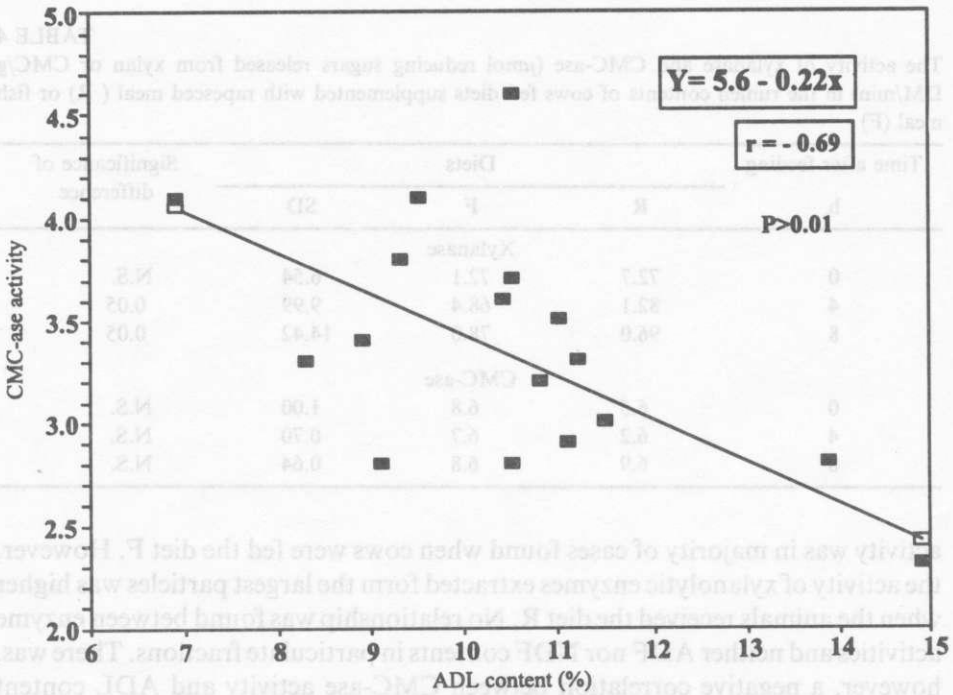

Figure 1. Straight line correlation between the ADL content in particulate fractions of rumen contents and the activity of particle-associated CMC-ase in the rumen of cows

Total activity of xylanase and CMC-ase in the rumen is presented in Table 6. The activity extracted from the all three particulate fractions contributed only to 26.5 to 35.4 % of the activity of the whole digesta in the rumen. This was calculated from the activity extracted from all three fractions and the percentage of these fractions in the total DM of rumen contents.

The changes in activity of xylanase and CMC-ase extracted from the particles incubated in the nylon bags are presented in Figure 2. An increase in the activity

TABLE 6

The activity of xylanase and CMC-ase in whole rumen digesta and its particulate fractions mmol reducing sugars released from xylan and CMC/rumen/min) in cows fed diets supplemented with rapeseed meal (R) or fish meal (F)

Activity	Diet R		Diet F	
	rumen content	particulate fractions	rumen content	particulate fractions
Xylanase	742 ± 126.9	222 ± 28.64	677 ± 84.5	240 ± 35.6
CMC-ase	60 ± 5.7	16 ± 2.7	63 ± 9.0	18 ± 2.6

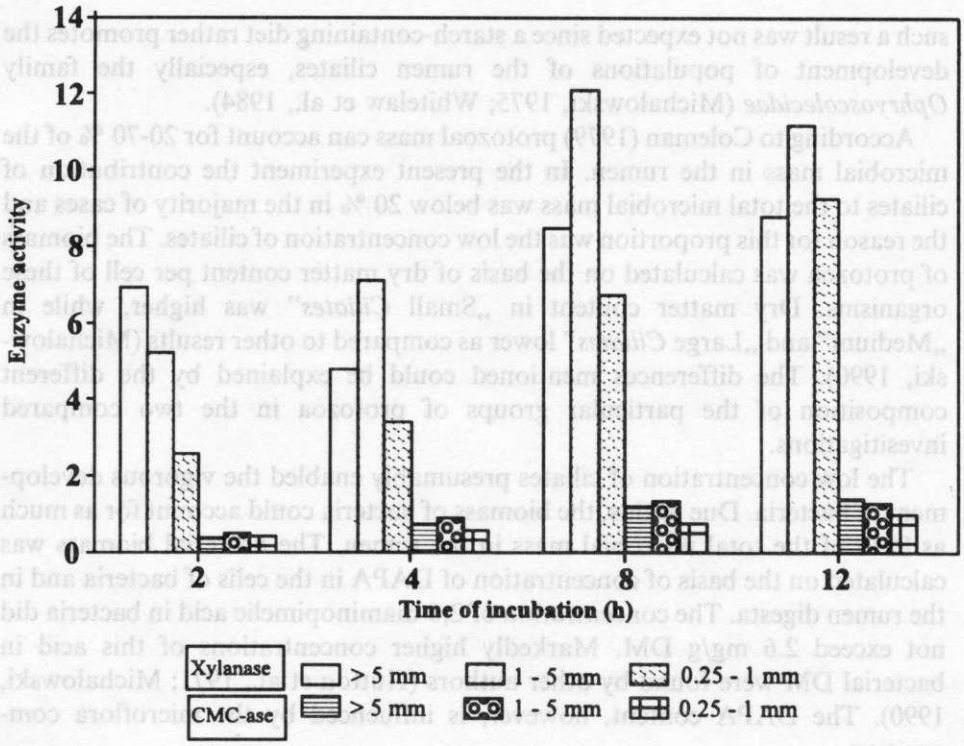

Figure 2. Changes in the activity of particle-associated enzymes during incubation of particles of hay of the length of ⩾5 mm, 1-5 mm and 0.25-1 mm in nylon bags in the rumen of cows

of the both enzymes was observed with the time of incubation. The highest activity found in this experiment was, however, significantly lower ($P<0.01$) than the activity extracted from the particulate fractions of rumen digesta (see Table 5).

DISCUSSION

The diets used were isonitrogenous and isoenergetic but they differed from each other in protein supplement and proportion of barley grain. The higher contents of barley grain in diet F seems to be the main cause of the more numerous populations of ciliates in the rumen when cows were fed this diet, as they consumed more starch which is the main component of the cereal grain (Czerkawski, 1986). The influence of protein source can not, however, be precluded. In general, the number of protozoa in the rumen of cows was low and

such a result was not expected since a starch-containing diet rather promotes the development of populations of the rumen ciliates, especially the family *Ophryoscolecidae* (Michałowski, 1975; Whitelaw et al., 1984).

According to Coleman (1979) protozoal mass can account for 20-70 % of the microbial mass in the rumen. In the present experiment the contribution of ciliates to the total microbial mass was below 20 % in the majority of cases and the reason for this proportion was the low concentration of ciliates. The biomass of protozoa was calculated on the basis of dry matter content per cell of these organisms. Dry matter content in „Small *Ciliates*" was higher, while in „Medium" and „Large *Ciliates*" lower as compared to other results (Michałowski, 1990). The differences mentioned could be explained by the different composition of the particular groups of protozoa in the two compared invesitigations.

The low concentration of ciliates presumably enabled the vigorous development of bacteria. Due to this, the biomass of bacteria could account for as much as 88% of the total microbial mass in the rumen. The bacterial biomass was calculated on the basis of concentration of DAPA in the cells of bacteria and in the rumen digesta. The concentration of 2,6-diaminopimelic acid in bacteria did not exceed 2.6 mg/g DM. Markedly higher concentrations of this acid in bacterial DM were found by other authors (Hutton et al., 1971; Michałowski, 1990). The DAPA content, however, is influenced by the microflora composition.

The activity of structural polysaccharide degrading enzymes was measured in whole rumen digesta and in particulate matter separated into particle sizes of over 5 mm, from 1 to 5 mm and from 0.25 to 1 mm. The results obtained showed that the xylanase and CMC-ase activities associated with all three fractions of particles accounted only for 26.5-35.4% of the total activity, despite these fractions contributing over 50% of the dry matter of rumen digesta (Pająk et al., 1997). This suggests that the major part of both activities originated from the fraction which can be referred to as a strained rumen fluid and consisting of the smallest feed particles, unattached microorganisms and rumen liquor. There are data showing very low xylanolytic and cellulolytic activity of bacteria of the liquid phase (Huthanen and Khalili, 1992) and these findings are confirmed by our own observations (unpublished). This suggests that the majority of the xylanase and CMC-ase activity in the rumen of cows originated from the bacteria associated with the smallest feed particles and perhaps from protozoa. Of the ciliates presented in the rumen of cows, species from the genus *Diplodinium* should be taken into consideration. Very high activity extracted from the smallest particulate fractions was also found by Huhtanen et al. (1992). In contrast, however, to the these findings, no negative relationship between activity of particle-associated enzymes and particle size was observed here.

Moreover the CMC-ase activity extracted from the particles sized 0.25-1 mm tended to be lower than those extracted from larger particles. No such phenomenon was observed when hay particles of different size were incubated in the nylon bags in the rumen. This suggests that the surface area can be an important factor influencing the colonization of particulate matter by bacteria as supposed by Huhtanen et al. (1992). Other factors, however, should also be taken into consideration and one of them seems to be the chemical composition of the colonized particles. Such a possibility is suggested by the correlation between ADL content in the particulate fractions and CMC-ase activity extracted from this material. It is possible that the high lignin content in the feed particles diminishees their attractiveness for the colonizing bacteria.

The diet did not influenced the concentration of bacterial matter in the rumen digesta. The activity of xylanase, however, in the rumen contents as well as the activity of the both enzymes associated with the particulate fractions were significantly affected by the diet. The higher activity of xylanase and CMC-ase could result from more intense colonization of this material by xylanolytic and cellulolytic bacteria, from an increase in the activity of enzymes produced by these microorganisms, or from the both (Silva et al., 1987; Huthanen and Khalili, 1992). This question, however, remains unknown as the biomass of the bacteria attached to the feed particles was not determined in this experiment. The activity of the particle-associated enzymes was higher when the cows were fed the fish-meal supplemented diet. This may suggest that protein quality can affect the colonization of feed particles by fibrolytic bacteria (Silva et al., 1987) influencing, perhaps, in this way fibre digestion in the rumen (Pająk et al., 1997).

Weimer (1996) is of the opinion that attachment of the predominant cellulolytic bacteria to the feed particles protects them from protozoal engulfment. This opinion seems to be a good explanation for our results showing that an increase in protozoal number had no detrimental effect on the activity of the particle-associated enzymes. If it is the case, then the higher fibrolytic activity observed in faunated animals (Jouany, 1989) could be explained by the synergetic effects of bacteria colonizing the feed particles and protozoa species able to digest the structural polysaccharides.

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STRESZCZENIE

Wpływ dawki na biomasę drobnoustrojów oraz aktywność ksylanazy i karboksymetylcelulazy w treści żwacza i jej frakcjach u krów

Oznaczono aktywność enzymów trawiących składniki włókna w żwaczu trzech krów, z trwałymi przetokami żwacza, żywionych dawkami różniącymi się rodzajem paszy białkowej: śrutą poekstrakcyjną rzepakową lub mączką rybną. Próby treści do analizy pobierano po ewakuacji zawartości żwacza i dokładnym jej wymieszaniu.

Ogólna liczba orzęsków wahała się od $121{,}3x10^3$ do $223{,}2x10^3$/g treści żwacza i była istotnie większa w okresie podawania mączki rybnej. Gęstość populacji bakterii, mierzona zawartością ich suchej masy, wynosiła 11,0-14,9 mg/g treści. Biomasa pierwotniaków w żwaczu wahała się od 208,2 do 358,6 g, a biomasa bakterii od 986,3 do 1391,6 g suchej masy. Aktywność ksylanazy w pełnej treści żwacza, wyrażona jako ilość cukrów redukujących uwalnianych z ksylanu/g suchej masy/min, wahała się od 68,4 do 96,0 μmol i była większa u krów żywionych paszą z dodatkiem śruty poektrakcyjnej rzepakowej. Aktywność CMC-azy była niezależna od rodzaju diety i wynosiła 6,2-6,9 μmol cukrów redukujących uwalnianych z CMC/g suchej masy/min. Nie stwierdzono zależności między aktywnością enzymów a biomasą bakterii w treści żwacza. Aktywność ksylanazy i CMC-azy związanych z cząstkami stałymi treści żwacza wahała się odpowiednio od 34,0 do 63,3 i od 1,9 do 4,6 μmol cukrów redukujących, uwalnianych z substratu/g suchej masy/min. Wyższą aktywność stwierdzono u zwierząt żywionych dietą uzupełnianą mączką rybną. Wyższej aktywności enzymów towarzyszyła większa liczebność populacji orzęsków. Stwierdzono istnienie negatywnej korelacji między aktywnością CMC-azy ekstrachowanej z fragmentów stałych treści żwacza a zawartością ADL w tym materiale.