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EFFECT OF FEEDING FREQUENCY ON FERMENTATION PATTERN AND MICROBIAL ACTIVITY IN THE BOVINE RUMEN*

By

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JENSEN, KURT and JENS WOLSTRUP: *Effect of feeding frequency on fermentation pattern and microbial activity in the bovine rumen.* Acta vet. scand. 1977, *18*, 108—121. — The influence of the feeding frequency on the fermentation in the bovine rumen was investigated by a single reversal trial with restricted supply of feed. The study comprised 6 rumen-fistulated dairy heifers fed a complete diet, low in crude fibre, at 2 frequencies. The effect of the treatment was assessed by the parameters: Concentration of microbial metabolites, total counts of microorganism, concentration of adenosine triphosphate (ATP), and fermentative activity in the rumen.

Frequent feeding compared to feeding twice daily resulted in marked reduction of the diurnal variation in the concentration of ruminal metabolites without significant influence on the daily means of total VFA and pH, but the molar composition of the VFA mixture and the production rate of VFA were highly affected. The implications of these observations for improved feed utilization to milk production and fattening are discussed.

Pronounced increment of the concentration of ATP was found by frequent feeding, but the total counts of microorganisms were found almost unaffected. Since the rate of fermentation was highly correlated with the concentration of ATP, the ATP pool may be an indicator of the fermentative activity of rumen microorganisms.

feeding frequency; cows; complete feed; ruminal metabolites; total cell count; ATP concentration; VFA production rate.

In feeding experiments, increased frequency of feeding in connection with optimized management have been found to improve the milk yield of dairy cows (*Larsen & Eskedal* 1952). A positive effect of high frequency of feeding, in terms of milk

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yield and milk fat percentage, has been observed (*Campbell & Merilan* 1961, *Rohr & Daenicke* 1973), particularly with rations low in crude fibre. Similar positive effects on feed utilization and growth rate were seen also in performance trials with dairy heifers fed frequently with hay (*Rakes et al.* 1957) or with hay and concentrates (*Campbell et al.* 1963), whereas *Clark & Keener* (1962) did not find any appreciable advantage of frequent feeding.

According to the literature, frequency of feeding appears to affect the ruminal fermentation pattern in terms of molar composition of the VFA mixture and the diurnal variations of the concentrations of the microbial metabolites (*Kaufmann & Hagemeister* 1973). The total count of bacteria seems unaffected, but increases resulting from frequent feeding have been observed in numbers of protozoa (*Moir & Somers* 1957, *Putnam et al.* 1961), and in microbial activity in terms of production rate of propionic acid (*Mercer et al.* 1974). Recently, *Wolstrup & Jensen* (1976) found that the content of adenosine triphosphate (ATP) in the rumen fluid was highly affected by the level of nutrient supply.

Further investigations on the effects of feeding frequency on microbial activity in the bovine rumen may prove useful, particularly when feeding rations high in easily fermentable carbohydrates. In the present study an examination was made of the microbial activity in the bovine rumen under influence of different feeding frequencies with a complete feed low in crude fibre.

MATERIALS AND METHODS

Experimental plan

The investigation was made as a single reversal trial with equal daily intake of feed offered at 2 frequencies, 2 and 12 times (×) per day. The animals were 6 non-pregnant Jersey heifers (A-F), average weight 263 kg, fitted with rumen cannulae. A pelleted compound diet was used as the complete feed. The animals were individually fed and had free access to water. In the first period, A, C and E were offered 2.55 kg of air-dry feed by hand every 12th hr., while B, D and F were fed from an automatic feeder, supplying 0.43 kg of the feed at 2-hr. intervals.

To ensure adaptation to the diet the feeding regime was unchanged for at least 1 month before sampling. After sampling in

the first period, the treatment was abruptly reversed and feeding continued for another 2 weeks. Samples of total rumen content and rumen liquid-small particle phase were taken every 4th hr. during a 12-hr. period from the animals fed 12 times daily, while samples were taken 7 times at regular intervals throughout a feeding cycle from the animals fed twice a day.

Composition of feed

The feed was composed of 14.0 % cotton seed meal, 5.5 % soybean meal, 36.5 % ground barley, 30.0 % rolled oats, 10.0 % sodium hydroxide-treated straw, 2.0 % molasses and 2.0 % mineral and vitamin mixtures. The ingredients were mixed and pressed into 9 mm pellets. Bulk samples of the feed were collected and analyzed by conventional methods (*Anon.* 1965). The content of dry matter was 87.4 % and the composition of dry matter was 20.0 % crude protein, 2.0 % crude fat, 10.6 % crude fibre, 60.9 % N-free extracts, and 6.5 % crude ash. The content of structural carbohydrates determined according to the method of *Goering & Van Soest* (1970) was, per kg dry matter: 345 g cell wall matter, consisting of 193 g hemicellulose, 115 g cellulose and 37 g lignin.

Analytical methods

Sampling and preparation of rumen fluid and determination of pH was performed as described by *Jensen* (1975). The content of volatile base in the rumen fluid was determined titremetrically after microdiffusion (*Conway* 1957). Rumen fluid total VFA concentration and the molar proportion of the individual acids were determined by gas-solid absorption chromatography (*Jensen* 1974). The ratio non-glucogenic/glucogenic acids (NGGR) in the VFA mixture was calculated using the formula given by *Ørskov* (1975).

Total counts of microorganisms in the rumen liquid-small particle phase were carried out on only 2 samples per animal during each experimental period. The samples were preserved according to *Hungate et al.* (1971), and counts and calculations of biomass were performed as described by *Wolstrup & Jensen* (1976). Determinations of ATP were made by the luciferin-luciferase method originally mentioned by *McElroy* (1947), following the procedure of *Wolstrup & Jensen.*

Fermentative activity was determined by comparison of the contents of VFA and ammonia in samples of rumen content before and after incubation in vitro for 45 min. directly in the rumen. The incubation took place in 100 ml plast bottles, and after incubation microbial activity was stopped immediately by cooling on ice-bath and by addition of mercuric chloride (30 p.p.m.) followed by thorough mixing for 30 sec. by a MSE® laboratory mixer.

RESULTS

The concentrations of microbial fermentation end-products in rumen fluid are shown as daily means in Table 1. The mean pH and the mean concentration of total VFA were not significantly affected by the treatment, but diurnal variations related to time of feeding were observed for both parameters in the animals fed twice daily. Within the sampling period in the 2 × group, the total VFA concentration increased to a maximum level 2—4 hrs. after feeding, and then declined slowly and reached the initial level just before next feeding. The average difference between minimum and maximum was 61 mmol/l. The pH showed an inverse variation with a minimum period coincident with maximum level of VFA. In the frequently fed animals, the VFA concentration and pH varied only slightly during the sampling period.

Frequent feeding resulted in a significantly higher molar percentage of acetic acid than feeding twice daily ($P < 0.001$), whereas the concentrations of propionic and valeric acids were reduced ($P < 0.001$) (Table 1). The molar percentages of butyric, isobutyric and isovaleric acids were found almost unaffected by feeding frequency. As a consequence of the changed molar composition of the VFA mixture, the NGGR was increased significantly from 2.89 to 3.76 by frequent feeding ($P < 0.001$).

The ammonia concentration was, on an average, highly increased by frequent feeding ($P < 0.001$), although marked individual variation occurred (Table 1). Pronounced diurnal variation related to time of feeding was seen in the 2 × group. The concentration of ammonia immediately before feeding was 11.3 mmol/l, increasing 1 hr. after feeding to a peak value of 12.9 mmol/l. One hr. later the concentration had decreased to 9.1 mmol/l, and declined further to a minimum level of 5.5 mmol/l,

Table 1. Influence of feeding frequency on daily mean concentrations of fermentation end-products and on composition of volatile fatty acid mixtures in rumen fluid.

Frequency of feeding per 24 hrs.	Heifer	Ammonia mmol/l	pH	Total VFA mmol/l	Molar percentage of total VFA						NGGR
					acetic acid	propionic acid	butyric acid	valeric acid	isobutyric acid	isovaleric acid	
12 ×	A	18.47	6.52	118.3	60.4	18.5	15.0	1.75	1.50	2.93	4.56
	B	20.60	5.80	173.0	53.3	30.5	9.2	2.97	0.94	3.10	2.23
	C	13.67	6.42	123.2	61.9	18.2	16.0	1.51	0.92	1.49	4.85
	D	11.91	6.65	94.2	64.3	18.8	12.7	1.31	1.13	1.91	4.55
	E	8.00	6.30	108.2	59.2	26.8	8.8	1.64	1.48	2.01	2.76
	F	5.31	5.98	138.7	66.5	22.1	7.9	1.20	0.99	1.32	3.58
	Overall mean	12.99	6.28	125.9	60.9	22.5	11.6	1.73	1.16	2.13	3.76
	± s.e.m.	1.411	0.073	6.73	1.03	1.15	0.78	0.142	0.089	0.170	0.243
2 ×	A	2.97	5.73	133.6	47.9	34.7	12.7	4.27	0.35	0.48	2.00
	B	10.82	6.04	139.5	58.4	24.0	12.0	2.27	0.76	2.59	3.24
	C	10.83	6.43	110.8	58.3	26.9	10.8	1.60	0.97	1.43	2.94
	D	7.25	6.38	102.8	52.2	28.6	12.8	4.00	1.01	1.41	2.55
	E	4.37	6.19	121.7	46.7	22.5	24.2	3.47	1.25	1.87	3.80
	F	10.93	6.36	125.8	56.1	25.3	8.5	1.81	1.35	7.05	2.78
	Overall mean	7.86	6.19	122.4	53.3	27.0	13.5	2.90	0.95	2.47	2.89
	± s.e.m.	0.720	0.065	4.67	0.81	0.73	0.83	0.201	0.084	0.349	0.100

Table 2. Adenosine triphosphate (ATP), microbial counts, calculated amounts of biomass and ratio of biomass/ATP in the liquid-small particle phase of rumen contents. The figures are expressed per volume water phase.

Frequency of feeding per 24 hrs.	Heifer	ATP mg/l	Total count of bacteria $\times 10^{10}$/ml	Volume of protozoa mm³/ml	Biomass dry wt. g/l	Biomass dry wt./ ATP
12 ×	A	54.0	4.4	32	16.7	310
	B	9.4	2.5	47	15.8	1672
	C	55.2	5.1	57	23.7	429
	D	32.6	3.9	24	13.8	423
	E	5.6	2.3	29	11.4	2036
	F	9.1	2.1	16	8.1	889
	Overall mean	27.7	3.4	34	14.9	960
	± s.e.m.	5.42	0.42	6.7	1.97	298
2 ×	A	3.9	3.2	31	14.0	3595
	B	7.0	3.4	19	11.7	1666
	C	29.3	3.3	38	15.7	534
	D	5.5	5.8	17	16.7	3049
	E	10.1	3.7	40	17.0	1680
	F	14.7	4.6	42	19.4	1322
	Overall mean	11.7	4.0	31	15.7	1975
	± s.e.m.	1.61	0.35	5.1	1.33	464

remaining low for the next 4 hrs., after which time the concentration increased slowly towards the initial level.

The daily means obtained by analyzing the rumen liquid-small particle phase for content of ATP are shown in Table 2. Frequent feeding resulted in 2.5 times higher mean concentration of ATP than feeding twice daily. The effect was highly significant ($P < 0.001$), in spite of marked variation between animals. The diurnal variation during the sampling period was quite small and irregular without relation to time of feeding in both groups.

The ratio biomass dry matter/ATP (w/w) was calculated (Table 2). Increased feeding frequency changed the ratio from 1975 to 960, but this effect was only slightly significant ($P<0.05$), mainly because of individual differences in the response of the animals with regard to concentration of ATP.

The microbial activity was characterized by the production rates of VFA and ammonia determined by in vitro incubation of total rumen content. In Table 3 are shown the daily means

Table 3. Influence of feeding frequency on production rates of volatile fatty acids and of ammonia in total rumen contents.

Frequency of feeding per 24 hrs.	Heifer	VFA		Ammonia	
		mmol/l water phase/min.$^{-1}$	µmol/g dry matter/min.$^{-1}$	mmol/l water phase/min.$^{-1}$	µmol/g dry matter/min.$^{-1}$
12 ×	A	0.929	6.68	1.67	0.233
	B	0.630	2.66	0.65	0.152
	C	0.768	6.13	0.98	0.124
	D	0.856	7.69	2.49	0.279
	E	0.525	5.08	0.29	0.029
	F	0.459	2.79	0.11	0.019
	Overall mean	0.695	5.17	1.03	0.139
	± s.e.m.	0.0521	0.483	0.205	0.0245
2 ×	A	0.438	2.76	0.34	0.054
	B	0.534	3.04	0.52	0.088
	C	0.675	4.35	1.04	0.156
	D	0.499	4.76	0.82	0.079
	E	0.456	3.63	0.83	0.100
	F	0.519	3.18	0.37	0.056
	Overall mean	0.520	3.62	0.65	0.089
	± s.e.m.	0.0310	0.213	0.061	0.0073

obtained for both treatments. The increment of total VFA in rumen fluid was significantly increased (about 34 %) by frequent feeding ($P < 0.01$), and after correction for content of dry matter in the samples, the production rate was found increased by an average of 43 % in frequent feeding ($P < 0.001$). The production rate of ammonia responded similarly to the treatment per 1 water phase as well as per g dry matter ($P < 0.05$).

DISCUSSION

The diurnal variation of the concentration of microbial metabolites in the rumen fluid was minimized by frequent feeding, which confirms the observations made by *Kaufmann & Hagemeister* (1973) and *Rohr & Daenicke* (1973). This implicates a more uniform fermentation rate contributing to the prevention of rumen acidosis.

The pronounced diurnal variation of pH and concentration of total VFA related to time of feeding, as observed in animals fed twice daily, equalled the pattern of variation found in examinations of easily fermentable straight feeds (*Jensen* 1977). The opposite directed systematic variations of these 2 parameters

were examined statistically, and the regression equation calculated from the mean values at different sampling times was: pH = 7.53 — 0.0104 mmol VFA/l, $S_{x.y}$ = 0.17, r = —0.86 ($P < 0.001$). The high correlation of pH to the concentration of total VFA within the range measured, as well as the buffering capacity of the rumen content, expressed by the regression coefficient, agreed with the results found by *Jensen* (1977).

The daily means of pH and of total VFA concentration were not influenced by the frequency of feeding, while the molar composition of the VFA mixture was highly affected. This makes it possible to control the ratio non-glucogenic/glucogenic acids in the VFA mixture and, consequently, to influence the efficiency of the energetic utilization of the VFA produced. A NGGR in the range 3.5—4.0 facilitates an efficient utilization of energy to milk production (*Ørskov* 1975). Presently, the mean NGGR was increased from 2.89 to 3.76 by frequent feeding. This may contribute to explain the positive effect on milk production and milk fat percentage seen in the investigations of *Campbell & Merilan* (1961) and *Rohr & Daenicke*. In experiments with complete feed as well as roughage supplemented with concentrates according to milk yield, these authors increased the efficiency of feed conversion to fat corrected milk about 10 % by offering the feed in at least 4 equal daily rations.

Feeding twice a day resulted presently in a mean NGGR of 2.89, which indicates an efficient energetic utilization of the VFA for fattening purposes, according to the range 2.25—3.0 proposed by *Ørskov*. Performance trials with growing heifers have shown somewhat conflicting results (e.g. *Clark & Keener* 1962, *Campbell et al.* 1963), which may partly be explained by a reduction in the VFA production when feeding infrequently. Generally, the interaction between feeding frequency, level of intake, fermentability and site of digestion of the feed should be studied before discussing the feasibility of changing a particular feeding regime.

The fermentation activity was more uniform throughout the day, and the mean VFA production rate increased by 43 % in the group fed 12 times, compared with the 2 times group. The total production of VFA was increased correspondingly by frequent feeding, since the average quantity of dry matter in the reticulorumen was almost unchanged by the treatment (to be published). Similar effect of feeding frequency was obtained by *Mercer et al.*

(1974), as the production rate of propionic acid was increased by 30 %, when silage was fed to dairy cows in 12 rations instead of 2 with equal daily intake. Contrary to these observations, *Gray et al.* (1967) found little effect on total VFA production rates of feeding sheep roughage diets at frequencies of 1, 2, or 12 times daily.

The total counts of bacteria agreed well with the level found by *Bryant & Burkey* (1953) and *Maki & Foster* (1957) in examinations of a similar feeding regime. The bacterial counts were not affected by the treatment, which agreed with the observations made by *Moir & Somers* (1957) in experiments with different feeding frequency to sheep, but like *Putnam et al.* (1961), they found the number of protozoa decreased by infrequent feeding. Apparently, the volume of protozoa was not affected by the treatment in the present experiment. Consequently, the total amount of calculated biomass dry matter was unaffected by the frequency of feeding, although it might have been expected that the increased rate of fermentation would have resulted in an increased amount of biomass present in the rumen. But the production of biomass was increased, since the flow rate of water phase through the reticulo-rumen was increased about 30 % by frequent feeding (to be published).

In a previous experiment (*Wolstrup & Jensen* 1976), the concentration of ATP in rumen fluid was found considerably reduced in dairy heifers fasting for 12—24 hrs. compared with frequent feeding. Presently, frequent feeding resulted in a much higher daily mean concentration of ATP than feeding twice a day with equal daily nutrient supply. These results show that the ATP pool size in rumen fluid instantly reflects the nutrient supply of the microorganisms.

The pool size of ATP and the production rate of VFA varied proportionally within a broad range, indicating a close relationship of these parameters. As shown in Fig. 1, the correlation coefficient of the VFA production rate to the ATP concentration, both expressed per l water phase, was 0.89 ($P < 0.001$) with the regression equation: mmol VFA/l/min.$^{-9}$ = 0.0078 mg ATP/l + 0.45 ($S_{x \cdot y} = 0.081$). The correlation between the fermentation rate and the ATP pool in rumen fluid has not been reported previously, and further investigations are needed to interpret this result. But it suggests that the pool size of ATP is a sensitive indicator of the activity of the rumen microorganisms.

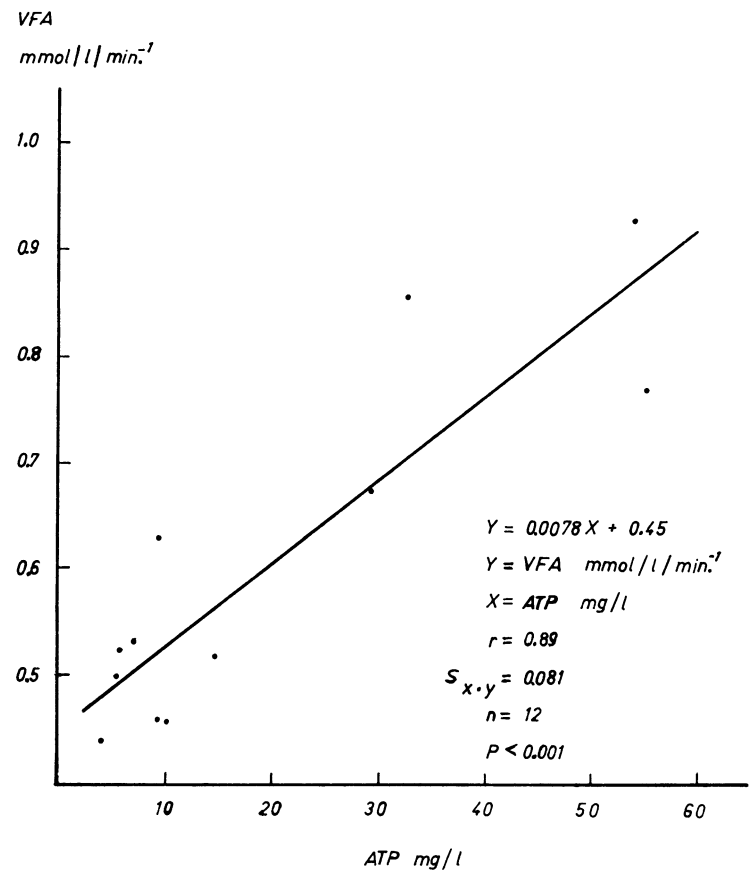

Figure 1. Production rate of total VFA as related to concentration of ATP in rumen fluid. The points represent daily means for the individual animals during both treatments.

As the calculated amount of biomass did not show any significant change according to the treatment, the ratio biomass/ATP varied inversely to the continuity of the nutrient supply. This ratio has been determined in pure cultures of different species of bacteria by several authors (e.g. *Forrest* 1965, *Holm-Hansen & Booth* 1966, *Hobson & Summers* 1972, *Ausmus* 1973), who found ratios in the range of 500—1600, indicating that no fixed proportion between biomass and ATP exists. Great variations in the ATP pool have also been observed within pure cultures in connection with changes in growth conditions or related to different growth phases. Generally, the ATP concentration in

bacterial cells remains unchanged during the exponential growth phase (*Cole et al.* 1967, *Hobson & Summers*), whereas an increase may occur at the beginning of the stationary phase (*Forrest & Walker* 1965). In addition, the ATP pool in the cells usually increases under conditions, where other factors than energy supply are limiting to growth (*Forrest* 1967, *Lazdunski & Belaich* 1972).

Concerning protozoa, *Plesner* (1964) showed an increase of the ATP pool just prior to cell division in Tetrahymena pyriformis. The size of the ATP pool has not yet been studied in rumen protozoa.

The growth phases and growth conditions of microorganisms in the rumen are less well defined than those in pure cultures. Therefore, results from pure cultures and from our experiments are not direct comparable. But the increased concentration of ATP in the microbial cells due to frequent feeding, coincident with higher fermentation rate, may well be explained by other limiting growth factors than energy.

CONCLUSION

The experiments have demonstrated significant responses to increased feeding frequency for several of the parameters used, despite considerable differences between animals.

The results of the experiments strongly indicate that frequent feeding 1) eliminates fluctuations of microbial metabolites in rumen fluid, 2) increases the NGGR in favour of milk fat production, 3) increases the fermentative activity resulting in improved ruminal digestion, and 4) increases the microbial ATP pool.

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SAMMENDRAG

Fodringshyppighedens indflydelse på fermentationsmønstret og den mikrobielle aktivitet i kvægets vom.

Fodringshyppighedens indflydelse på forgæringen i kvægets vom blev undersøgt i et 2-faktorielt forsøg med restriktiv fodertildeling. Undersøgelsen omfattede 6 vom-fistulerede kvier fodret 2 og 12 gange daglig med et fuldfoder med lavt indhold af træstof. Effekten af forsøgsbehandlingen blev målt ved hjælp af følgende parametre: Koncentrationen af mikrobielle stofskifteprodukter, total antal bakterier og protozoer og koncentrationen af adenosin trifosfat (ATP) i vomvæsken samt fermentationsaktiviteten i vomindholdet udtrykt ved produktionshastigheden af flygtige fedtsyrer (VFA) og ammoniak.

Gennemsnitskoncentrationen af total VFA samt pH fandtes ikke påvirket af fodringsfrekvensen, men den betydelige daglige variation af begge parametre, fundet ved 2 gange daglig fodring, kunne elimineres ved kontinuerlig fodring. Fodringshyppigheden påvirkede i betydelig grad produktionshastigheden og den molære sammensætning af de flygtige fedtsyrer. Betydningen af disse resultater for øget foderudnyttelse til mælkeproduktion og fedning diskuteres.

Endvidere medførte hyppig fodring, at vomvæskens indhold af ATP blev væsentligt forøget uden en tilsvarende ændring i antallet af mikroorganismer. Da koncentrationen af ATP samt forgæringshastigheden fandtes stærkt korreleret, formodes koncentrationen af ATP at være egnet til karakterisering af den mikrobielle aktivitet i vommen.

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