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In Vitro Fermentation of Mixed Rumen Microorganisms on Molasses-based Substrates

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ABSTRAK

Sejenis alat penapai telah dibentuk untuk mengkaji penapaian mikroorganisma rumen yang diberi molases-urea. Alat penapai ini terdiri daripada dua ruang (luar dan dalam) yang dipisahkan oleh selaput dialisis. Lebih kurang 35 ml bendalir rumen daripada kambing Merino yang diberi 100 g "oaten chaff" dan molases (mengandungi 3% urea, 0.135% campuran mineral) secara ad libitum, *digunakan untuk permulaan penapaian di ruang dalam alat penapai. Substrat molases-urea (8.333% molases dan 0.277% urea) dialirkan (3 ml* h^{-1}*) ke dalam media penapaian dan 'air liur rekaan' ke ruang luar (30 ml* h^{-1}*). Alat-lat penapai diletakkan dalam kukus air tertutup 39°C yang dipenuhi oleh gas* CO_2*. Semasa digunakan, penapaian pH terkawal dalam julat fisiologi dengan penghasilan asid lemak meruap (VFA) pada kadar 30 – 40* μ*mol* ml^{-1} h^{-1}*. Kesan penggantian separa urea dengan tripton sebagai sumber nitrogen dikaji dengan substrat 8.333% molases, 0.208% urea dan 0.202% tripton (w/v). Faktor-faktor yang dilihat ialah penghasilan VFA,* NH_3*, jisim kering dan protein kasar. Hasil kajian tidak menunjukkan kesan positif protein ke atas faktor-faktor di atas melainkan tahap* NH_3 *pada penapai kawalan (molases-urea) didapati lebih tinggi (signifikan,* $P<0.05$*) daripada penapai rawatan (molases-urea-tripton).*

ABSTRACT

A fermentor consisting of two compartments (called inner and outer) separated by a dialysis membrane was developed for studying the digestion pattern in vitro *of rumen microorganisms of sheep fed a molasses-urea based diet. About 35 ml of strained rumen liquor withdrawn from Merino wethers adapted to a molasses-urea diet (100 g oaten chaff and molasses containing 3% urea and 0.135% mineral mix fed* ad libitum*) was used as the initial fermenting inoculum in the inner compartment. Molasses-urea substrate (8.333% molasses and 0.277% urea, w/v) prepared in artificial saliva was infused at 3 ml* h^{-1} *into the fermenting medium and artificial saliva at 30 ml* h^{-1} *into the outer compartment. Incubation was carried out in a specially constructed water bath maintained at 39°C and filled with* CO_2 *gas. With these flow rates, pH of fermenting fluids was maintained within physiological range with volatile fatty acids (VFA) production at 30 – 40* μ*mole* ml^{-1} h^{-1}*. Using the fermentor, the effect of partial substitution of tryptone for urea as a nitrogen source on rumen microorganisms adapted to a molasses-urea based diet was studied with infusion solution consisting of 8.333% molasses, 0.208% urea and 0.202% tryptone (w/v) prepared in artificial saliva. Factors related to cell yield studied were, VFA and ammonia (*NH_3*) productions, dry matter (DM) and CP yields. However, no positive effects of protein supplementation (when compared to molasses-urea substrate) were observed in VFA productions, DM and CP yields. Only* NH_3 *levels of fermentors infused with molasses-urea substrate were significantly higher (*$P<0.05$*) than protein supplemented fermentors.*

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INTRODUCTION

In the post-weaning ruminant the digestion in the abomasum and small intestine of microbial protein synthesized in the fore-stomachs is an important and often the major source of amino acids to the host animal. On diets, in which the animal obtains most of its energy in the form of molasses, with urea as a source of non-protein nitrogen for microbial synthesis, supplemental protein improves animals' food consumption and weight gain (Preston *et al.* 1967, Preston, 1972). It is desirable, therefore, to know whether this type of diet improves microbial fermentation and growth yields.

This paper describes a continuous fermentor which was designed to study the digestion pattern *in vitro* of rumen microorganisms of sheep fed a molasses-urea based diet. Using the fermentor, the effects of protein supplementation on patterns of volatile fatty acids (VFA) formation, ammonia (NH_3) content, dry matter (DM) and crude protein (CP) yields were studied. These parameters are related to microbial fermentation and cell yields.

MATERIALS AND METHODS

Animals

Four matured Merino wethers each with rumen fistulae were housed in single pens and adapted to a molasses-urea based diet, which consisted of molasses (mixed with 3% urea and 0.135% mineral mix) provided *ad libitum* and a minimal amount (100 g) of oaten chaff to ensure normal rumen function. The animals consumed about 0.3 – 1.3 kg of molasses daily and they served as donors for rumen liquor.

Fermentor

The design concept of the fermentor was that of inner and outer compartments separated by a dialysis membrane, both fitted with overflows, one compartment serving as a fermentation chamber with continuous infusion of substrates and continuous mixing and the other serving to remove fatty acid by diffusion and interchange with a continuously flowing artificial saliva (McDougall, 1948). *Figures 1* and *2* illustrate the basic working construction of the fermentor. The materials used in the construction of the fermentor were mostly polythene.

During experiments the fermentors were held within a specially constructed 'Perspex' (about 1.3 cm thickness) thermo-statically controlled water bath (65 × 25 × 21.5 cm internal) fitted with a lid. The lid of the bath had openings fitted with rubber bungs carrying the infusion leads and gas inlet. The gas space in the bath was filled with CO_2 during experiments. Both nutrient and artificial saliva were prewarmed by flowing through stainless steel coils immersed in the liquid phase of the water bath. The bottom of the bath too had openings fitted with rubber bungs that carried the connection between the collecting funnels and the 'anti-gas flow back' device *(Fig. 2)*. Effluents from these 'anti-gas flow back' devices were collected in ice at regular intervals for analysis. Stirring of fermenting fluid in the inner compartment was achieved magnetically by placing magnetic stirrers under the water bath.

Operation

Four fermentors were prepared and warmed in the water bath which was maintained at 39°C. Due to the slow infusion rates (3 ml h^{-1} for substrate and 30 ml h^{-1} for artificial saliva) all connecting parts were first primed with the appropriate infusion solution. About 200 ml of strained rumen liquor was collected from a sheep consuming about 0.8 kg molasses daily and 35 ml was placed in the inner compartment of each fermentor.

Infusion Solutions

All materials and infusion solutions used were heat sterilised where possible. Materials not suitable for autoclaving were washed thoroughly with boiled double-distilled water.

Artificial Saliva. Artificial saliva was freshly prepared according to McDougall (1948) when required and gassed with CO_2 gas for 20 min or

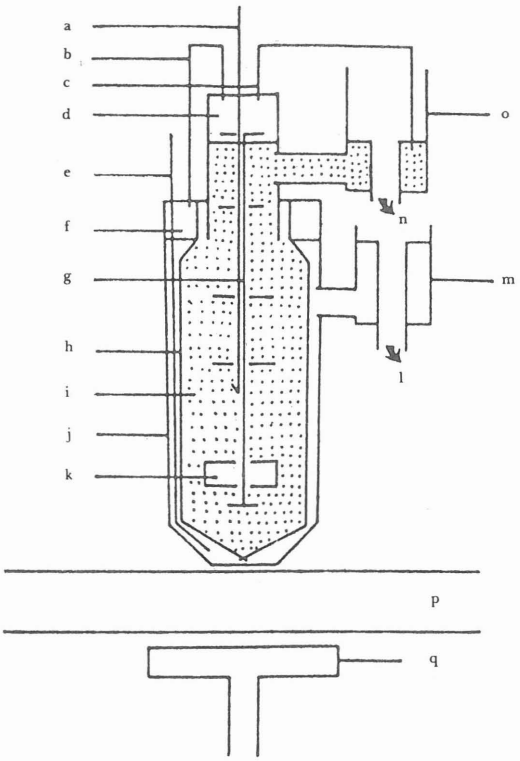

Fig. 1: Diagramatic representation of the fermentor

a. Substrate infusion lead (through the stirrer).
b. Gas space connecting tube (between inner and outer compartments).
c. Gas outlet.
d. Gas space (inner compartment).
e. Infusion lead for artificial saliva into the outer compartment.
f. Gas space for the outer compartment.
g. Magnetically driven stirrer.
h. Dialysis sac (4.2 cm flat width).
i. Rumen contents and substrate (inner compartment).
j. Outer compartment.
k. Magnetic bar (0.6 cm diameter × 1.5 cm length).
l. Spent fermenting medium into collecting cup connected to the 'anti-gas flow back' device *Fig. 2)*.
m. Outflow device for inner compartment.
n. Spent artificial saliva into collecting cup connected to the 'anti-gas flow back' device *(Fig. 2)*.
o. Outflow device for outer compartment (laterally displaced).
p. Bottom of water bath.
q. Magnetic bar (magnetic stirrer 12.7 × 13.8 × 9 cm).

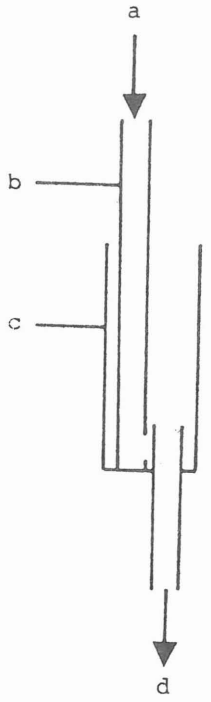

Fig. 2: 'Anti-gas flow back' device

a. Sample from collecting cup
b. Glass tubing (4 mm internal diameter)
c. Glass tubing (1.4 cm internal diameter) with hold-up volume of about 1 ml.
d. Effluent (into collection beaker at 4°C).

longer. Boiled double-distilled water was used for all infused solutions.

Molasses-urea Solution. Molasses-urea solution (control-substrate) contained 8.333% (w/v) molasses and 0.277% (w/v) urea. These two components were first boiled in water, cooled and centrifuged at 17 – 19,000 × g for 30 to 40 min. The supernatant was mixed with 50% (v/v) double strength artificial saliva. The mixture was then filter sterilised using a Millipore filter (R.A. 1.2 μ filter paper).

Molasses-urea-tryptone Solution. Molasses-urea-tryptone solution (treatment-substrate) was prepared similarly as the molasses-urea solution except the concentration of urea was reduced to 0.208% (w/v) and the N-source was made up by

0.202% (w/v) tryptone (Oxoid), which was heat sterilised before adding to the other components, usually prior to filteration. Both infusion solutions were isonitrogenous (129.3 mg N 100 ml^{-1}).

Infusion solutions were placed in suitable containers and continuously gassed with CO_2 gas during the experiment. Polythene tubings (0.2 cm internal diameter) were used as infusion leads.

Chemical Analysis

At collection times the samples were checked for pH and volumes, and then acidified with 5 M H_2SO_4 at 1/10 th sample volume. Samples collected from the inner compartment were centrifuged at $17-19{,}000 \times g$ for 30 – 40 min. The supernatant was analysed for VFA by the method of Geissler *et al.* (1976) with a 'PYE Series 104' Gas Chromatograph. Dry matter was obtained by solvent drying the wet residues and nitrogen (N) content was determined by semi-micro Kjeldahl method. Crude protein was calculated as 6.25 × N. Acidified samples from the outer compartments were analysed for VFA without further treatment. Ammonia – N (NH_3-N) was determined in both effluents by distillation and titration.

The effect of partial substitution of tryptone for urea as a nitrogen source was carried out with duplicate fermentors. Treated fermentors were infused with molasses urea tryptone substrates. Effluents were successively collected from the inner and outer outflows at 3 h interval. The results reported were from two repeated studies.

RESULTS

Preliminary testing of the fermentors using rumen contents as inoculum and molasses-urea solution as substrates in 6 – 10 h duration showed that there was a rapid and satisfactory removal of VFA by the artificial saliva from the fermenting fluid in the inner chamber, maintaining the pH within physiological range with VFA productions at 30 – 40 μmol $ml^{-1}h^{-1}$.

Fig. 3 shows the molar percentage distribution of acetate, propionate and butyrate in successive collection of inner and outer compartments of control and treated fermentors. The other acids (isobutyric, isovaleric and valeric) made up a small percentage of the total acids, about 5% or less. The average molar percentage of acetate, propionate and butyrate in the initial rumen liquor is given on the ordinate at zero time. The first 3 h was considered a settling-down period in the fermentor. Analysis of covariance on the molar percentages showed no significant difference between control and treated fermentors for any of these acids.

However, with time, significant ($P<0.01$) changes in fermentation pattern were observed, with a decrease in molar proportions of acetate and an increase in molar proportions of propionate.

The various fermentation parameters are as shown in Table 1. Analysis of variance on the data from various sampling times showed no significant effect of tryptone supplementation on all the parameters.

Total mg NH_3-N detected in the effluents of control (22.6 ± 1.5, $n = 4$) was significantly ($P<0.05$) higher than those detected from treated fermentors (18.3 ± 3.0, $n = 4$) after 9 h incubation. However, in terms of NH_3-N concentration in the fermenting fluids, the amount ranges from 8 to 20 g NH_3-N 100 ml^{-1}.

DISCUSSION

Although the quantities of VFA produced and the pH of the fermenting chambers is well maintained, there is a marked shift in fermentation pattern towards increased propionate production. In the present experiments, there was no allowance for solid addition. Whether the regular addition of solid material would prevent the drift of fermentation pattern needs to be examined. The cause of changes in VFA proportion is not really known, but the replacement of the hydrogen producing fermentation giving acetate by the hydrogen consuming reaction of forming propionate suggests that methano-

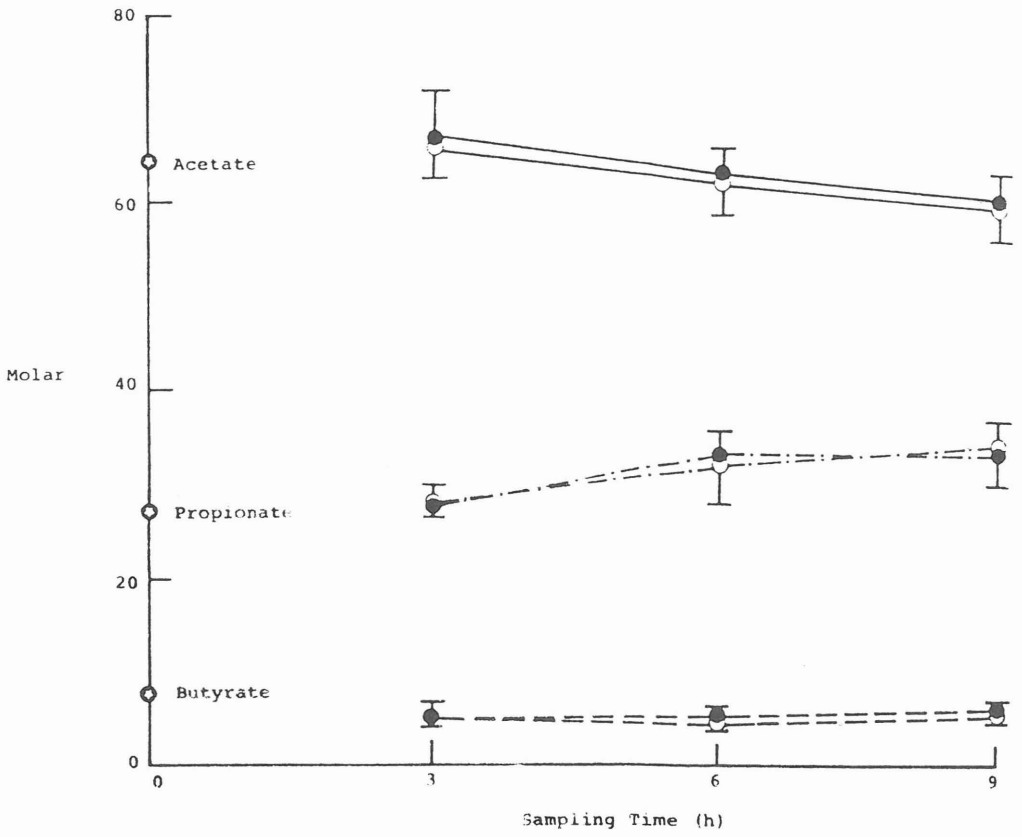

Fig. 3: Mean (± standard error, n = 4) molar percentage distribution of acetate, propionate and butyrate in succesive collections of control ● and treated O fermentors.

Regression equations and correlations

y (molar % acetate, control)
= 69.6 − 1.1X (h)
r = −0.7 (n = 12, P<0.01)

Y (molar % acetate, treated)
= 68.8 − 1.1X (h)
r = −0.7 (n = 12, P < 0.01)

Y (molar % propionate, control)
= 25.5 + 0.9X (h)
r = 0.7 (n = 12, P<0.01)

Y (molar % propionate, treated)
= 24.7 + 1.1X (h)
r = 0.7 (n = 12, P<0.01)

Y (molar % butyrate, control)
= 4.6 + 0.1X (h)
r = 0.2 (n = 12, not significant)

Y (molar % butyrate, treated)
= 5.1 − 0.1X (h)
r = −0.1 (n = 12, not significant)

genesis may be decreasing as the fermentation continues.

The *in vitro* experiments do not indicate any significant effect of preformed amino acid (as tryptone) in VFA pattern, VFA production, DM and CP content. The absence of an effect of supply of proteolysate in the *in vitro* experiments on factors related to cell yield is so much in contrast with the findings of Maeng *et al.* (1976) where drastic gains in cell yield were obtained when 25% of ammonia nitrogen was replaced by preformed amino acids. However, differences in the source of the microorganisms used and the nature of inoculum many contribute to the differences in the results. Maeng *et al.* (1976) used microorganisms from animals fed alfalfa cubes *ad libitum* and 1.4 kg of concentrate twice daily. Washed cell suspensions (free of protozoa) was used so that the starting material would be depleted of preformed amino acids and thus responses to added amino acids would be maximised.

TABLE 1
Fermentation parameters of control (infused with molasses-urea solution) and treated (infused with molasses-urea-tryptone solution) fermentors after 9 h incubation.

Sample	pH	DM produced [A] (mg μ mole^{-1} hexose infused)	CP produced (mg μ mole^{-1} hexose infused)	CP produced (mg μmole^{-1} VFA)	Total VFA produced (μ mole μmole^{-1} hexose infused)	Rate of VFA produced (μ mole ml^{-1} h^{-1})
Control	6.7 ± 0.1	55.4 ± 4.5	6.0 ± 2.9	4.1 ± 2.0	1.48 ± 0.04	34.3 ± 2.3
Treated	6.8 ± 0.1	55.2 ± 8.2	7.2 ± 3.3	4.7 ± 2.1	1.55 ± 0.08	34.4 ± 1.7

Each value in the table represents a mean ± S.E. (n = 4)

Average pH of rumen liquor was 6.9 (n = 2)

Molasses was found to contain 60.6% hexose by the glucose oxidase assay (Bergmeyer and Berut, 1974).

Total hexose infused was 6.8 ± 0.4 mmole for control and 6.7 ± 0.4 mmole for treated fermentors (n = 4)

[A] DM was obtained by solvent drying.

The results obtained in the experiments reported here appeared to be concordant with the findings of Ramirez (1972) on the negative effects of added protein (as fish meal) on bacterial synthesis in the rumen of molasses fed cattle. In the cattle on molasses based diets, Ramirez and Kowalczyk (1971) observed very high levels of ruminal α-amino N even when there was no protein supplementation of the diets. With this type of diet rumen fluid turnover is extremely slow (Marty and Sutherland, 1970) and the relatively high density rumen protozoa (Elias *et al.*, 1967) may result in bacterial digestion and amino acid release (Owen and Coleman, 1977).

It was reported by Ramirez and Kowalczyk (1971) that the quantity of microbial N synthesized in the rumen of calves fed a diet based on molasses-urea, as 2.5 g of microbial N per each 100 g of readily fermentable carbohydrates (or 4.05 g N mole^{-1} carbohydrate). This could be recalculated to give 25.3 g microbial protein or approximately 40 g DM mole^{-1} carbohydrate or 13.2 g microbial protein mole^{-1} VFA, assuming 1.18 mole VFA is produced by 100 g carbohydrate (Walker and Nader, 1970). The values obtained in the present experiment of about 55 g DM mole^{-1} hexose infused (both for control and treated fermentors) are higher than the estimates of Ramirez and Kowalczyk (1971), but within the range (40 – 60 g DM mole^{-1} carbohydrate) calculated from the studies on microbial protein synthesis *in vitro* carried out by Bucholtz and Bergen (1973). However, CP production (expressed as mg CP mole^{-1} hexose or mg CP mole^{-1} VFA, Table 1) are very much lower than the estimates quoted above. The low values is an indication of greater deposition of sugar as storage polysaccharide. Also, the occurrence of secondary fermentation (a sludge type fermentation) as observed by Rowe *et al.* (1979) in sheep fed a diet based on molasses may play a part in lowering N content. The yields of VFA of 1.48 and 1.56 mole mole^{-1} hexose infused at 0.1 h^{-1} dilution rate are close to the value of 1.53 at 0.12 h^{-1} dilution rate obtained by Isaacson *et al.* (1975). However, these values would be slightly lower than 1.9 if 1.18 mole VFA is produced by 100 g carbohydrate (Walker and Nader, 1970).

As might be expected, the production of NH_3 – N was significantly ($P < 0.05$) higher in the controls where urea was the only source of N. The NH_3 – N concentrations (mg 100 ml^{-1}) in these experiments were generally higher than the upper limit (5 mg 100 ml^{-1}) observed by Satter and Slyter (1974) above which there was no effect on microbial protein in their *in vitro* studies using a protein free purified diet. So it is unlikely that deficiency of NH_3 itself was limiting growth.

CONCLUSION

The results emerging from these *in vitro* studies indicate that when mixed microorganisms from the rumen of sheep fed molasses diet are studied, preformed amino acids supplementation seems not to show any effect on improving cell yields.

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