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VERIFICATION OF A CONTINUOUS FLOW FERMENTATION SYSTEM AND EFFECT OF DILUTION RATE ON RUMEN FERMENTATION AND MICROBIAL NUMBERS

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Summary

A study was conducted to assess the ability of a continuous flow fermentation system to simulate in vivo fermentation. Two dilution rates (D) were imposed on four fermenters during one of two periods in a crossover design. Effects of a slower D (D1, 4.1%/h) on rumen fermentation were compared with those of a higher D (D2, 5.7%/h). A medium concentrate pelleted diet was provided to each fermenter twice daily. Each period consisted of a five-d adaptation followed by a five-d collection. Data from each fermenter were averaged for the five-d collection prior to statistical analysis, since preliminary analysis had detected only two significant day by treatment interactions, neither of which would interfere with interpretation of main effects. Fermenters with D1 exhibited reduced pH ($P<.01$), a marked depression in protozoal numbers ($P<.01$) and greater bacterial numbers ($P<.05$) when compared with D2. Slower D also resulted in lower ammonia concentration ($P<.01$), decreased molar proportion of acetate ($P<.01$), increased propionate ($P<.05$), increased valerate ($P<.05$) and higher total VFA concentration ($P<.05$) at four h postfeeding. No significant effect of D was observed for molar proportion of butyrate, isobutyrate or isovalerate. Organic matter digestibility (OMD) tended to be reduced at the faster D. A day effect ($P<.05$) was observed for pH and propionate, which indicates a longer adjustment period might have been desirable. Values and responses to D alteration were generally consistent with reported values, suggesting normal fermentation was achieved. However, pH and protozoal counts were abnormally low at the slow D, although the protozoal population was effectively maintained with D2.

Introduction

Continuous flow fermentation systems have become a popular tool for the study of rumen fermentation and microbiology. Applications for such an in vitro system include study of diet quality, nutrient bypass, microbial populations and protein synthesis, etc., with less labor, facilities or expense than with animal trials. Furthermore, treatments and experimental conditions are quite easily controlled, and ruminal effects are readily isolated from those of the animal.

This experiment was conducted to verify that a continuous flow system recently constructed at New Mexico State University was able to maintain fermentation patterns and microbial numbers comparable to those typically observed in ruminants. Secondly, dilution rate (D) was manipulated to substantiate that this system simulated characteristic in vivo changes when D was altered.

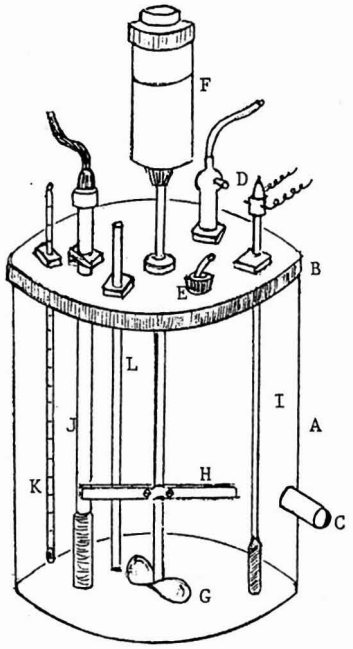

FIGURE 1. CONTINUOUS FERMENTATION APPARATUS.

Experimental Procedures

Culture Apparatus. The continuous flow culture system (figure 1) consisted of a cylindrical plexiglass container (A; height 23 cm, id 22.2 cm) and cover (B). An overflow port (C; id 1.3 cm) was situated 8.2 cm from the base to establish a liquid and gas volume of 2905 ± 107.2 ml and 5447.5 ± 105.3 ml, respectively. Buffer was stored in a 15 liter flask and infused at port D via flexible plastic tubing (id .5 cm). Flow rate was regulated by a variable speed liquid pump.[2] The outflow port was connected to a four liter effluent flask by a plastic hose (id 1.5 cm) to collect fermenter contents displaced by buffer influx. Port E (id 2.3 cm) served as an access for addition of substrate and was sealed with a rubber stopper. A heavy duty stirring motor[3] (F) was mounted above the fermenter and the shaft (length 30.5 cm, od .9 cm) extended into the center of the container. A swivelled blade (G; length 7 cm) was located approximately 2.5 cm from the fermenter base to prevent excessive sedimentation. A plexiglass blade (H; length 10.3 cm) was attached to the shaft at the liquid surface to serve as a foam disrupter. A mercury thermoregulater[4] (I; length 38.6 cm, od .9 cm) and relay[5] and an immersion heater (J; length 29.4 cm, od 1.6 cm) were

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[1] Scientific paper 165, Agr. Exp. Sta., New Mexico State University, Las Cruces, NM 88003. The authors acknowledge the invaluable assistance of R. C. Chabot and M. E. Hubbert in design and construction of the fermenters.

[2] Model RPG-20, Fluid Metering, Inc., Oyster Bay, NY.

[3] Model 5VB; LFE Corp., Fluids Control Divison, Hamden, CT.

[4] Model CE-712, PSG Industries, Perkasie, PA.

[5] Model PRSS-11, PSG Industries, Perkasie, PA.

mounted on the fermenter to regulate culture temperature. A mercury thermometer (K) was attached to monitor temperature. A glass tube (L; length 29 cm, id .7 cm) was inserted to provide a sampling port for fermenter contents. A plastic hose (id .5 cm) connected this tube to a sampling syringe and was clamped when not in use.

Experimental Protocol. A crossover design consisting of four fermenters, two periods and two treatments was employed. The two treatments imposed were two dilution rates (D1 = 4.1 $\pm$ .2%/h, D2 = 5.7 $\pm$ .2%/h). Each period consisted of five d to attain steady-state conditions followed by five d for sampling. Between periods, fermenters were disassembled and cleaned.

Three mature, rumen-cannulated donor steers ($\bar{x}$ average wt of 453.4 kg) were maintained on a diet of 40% ground milo (IFN 4-04-444) and 60% alfalfa hay (IFN 1-00-063). Each steer received 8.1 kg (dry matter basis) of this diet daily in two equal portions (0800 h and 1700 h) for 10 d prior to collecting rumen fluid.

On inoculation day, 2.5 liters of rumen fluid were obtained from each steer four h postfeeding and strained through two layers of cheesecloth. A 1.5 liter mixture of the three sources of fluid was placed in each fermenter. Also, one liter of buffer (Weller and Pilgrim, 1974; urea omitted) was preheated to 39 C and added to each fermenter. A rubber gasket was placed between the fermenter vessel and cover and o-rings were inserted around all ports where various attachments entered the fermenter to prevent O_2 seepage into the system. Fermenters were then flooded with CO_2 to purge existing O_2 from the gas phase. This culture was then allowed to batch ferment overnight. The following morning (0700 h), precalibrated liquid pumps were switched on to provide continuous infusion of buffer at two predetermined flow rates. Buffer was stored in a sealed glass flask at a pH of 7.2, which was achieved by bubbling CO_2 through the solution.

Fermenter contents displaced by buffer influx flowed into the effluent flask. Each morning (0700 h) the effluent flask was removed and replaced with a clean flask, which had been flooded with CO_2 to maintain anaerobic conditions. Each flask contained 60 ml (D1) or 80 ml (D2) of 2N H_2SO_4 to inhibit microbial growth. Total volume displaced during each previous 24 h period was recorded and dilution rate was calculated as the mean percentage of liquid volume (corrected for acid content) displaced/h for the entire 10 d period. Throughout the experiment, fermenters were maintained at 39 C with a stir rate of 150 rpm and were covered with black cloth to prevent light exposure.

Fermentation substrate was a commercially pelleted ($\bar{x}$ pellet length .8 cm, od .4 cm) rabbit chow (table 1). A 47-g (dry matter basis) portion was placed in each fermenter at 1900 h on the d of inoculation, and twice daily (0700 h and 1900 h) for the remainder of the study period. During each feeding, the port was flooded with CO_2 to prevent O_2 contamination.

Analytical Methods. During each collection period, daily 200 ml subsamples (in duplicate) were frozen for subsequent DM and ash determination. Daily apparent OMD (uncorrected for microbial contribution) was calculated for each fermenter during the collection period by the difference in organic matter (OM) input and OM in the effluent.

A 25 ml sample of fermenter contents was withdrawn from each fermenter daily at 1100 h throughout the entire experiment. An equivalent amount of buffer was backflushed through the sampling tube to rinse the tube and to prevent a change in D. A combination electrode[6] was used to determine pH of the fermenter contents. One milliliter of this sample was diluted 10-fold and 1000-fold with a .85% saline and 10% formalin solution prior for enumeration of protozoa and bacteria, respectively. Daily bacterial and protozoal counts were conducted in duplicate with phase contrast microscopy.[7] Protozoal concentration was determined at 100 power in Sedgewick-Rafter chambers (1 mm depth) while bacterial counts were obtained at 600 power with Petroff-Hausser counting chambers. The remainder of the fermenter contents sample was acidified with .5 ml H_2SO_4 and frozen. These samples were subsequently thawed and centrifuged (10,000 x g) for 10 min. Ammonia concentration was determined on the supernatant (Broderick and Kang, 1980). Also, five ml of supernatant was combined with one ml of 25% metaphosphoric acid containing 2-ethyl butyric acid as an internal standard. This solution was recentrifuged as before and subjected to gas chromatography for VFA analysis. Column[8] temperature was 135 C, inlet and detector temperatures were 170 C and flow rates were 40, 400 and 25 ml/min for H, air and N, respectively. A subsample of rabbit chow was ground in a Wiley mill through a 2 mm screen. Analysis of DM, ash, CP, ADF and ADL were as described by A.O.A.C. (1980).

Analysis of variance using General Linear Models Procedures of Statistical Analysis System (Helwig and Council, 1979) was conducted for each measured variable. Initially, a model was used which included effects for fermenter, period, treatment, day and all possible interactions. A day x treatment interaction was detected for molar proportion of butyrate and total VFA concentration; however, the nature of the interaction did not preclude examination of main effects. Therefore, values for each variable for a given fermenter were averaged for each five d collection period and these means were reanalyzed to determine treatment differences, using only fermenter, period and treatment main effects in the model.

Results and Discussion

In general, values for criteria measured (table 2) were within normally reported ranges (Hungate, 1966). Exceptions included pH and protozoal numbers at the slow D.

Protozoal numbers at four h postfeeding were abnormally low for D1. Slyter et al. (1966) observed that protozoa populations were diminished when pH declined below 5.5. The lower pH ($P<.01$) at D1 (table 2) was expected in view of the reduced buffer entry and slower flow rate of acidic end products from the fermenter. However, at D2, reasonable protozoal concentrations suggest that this system exhibits a capacity to sequester protozoa and prevent washout. Also, a day effect ($P<.05$) was observed for pH, which indicates that the pH had not completely stabilized

[6]Orion Ionalyzer Model 399 A meter; Orion Research, Inc., Cambridge, MA.

[7]Nikon Model Optiphot; Allen Associates, Inc., Scottsdale, AZ.

[8]Column - 10% SP120/1% H_3PO_4 on 80/100 Chromasorb W; Supelco, Inc., Bellefonte, PA.

when initial sampling occurred.

Bacterial numbers (table 2) were elevated at the slower D (P<.05), probably in response to reduced protozoal levels. Bacteria might be expected to proliferate in the absence of protozoa due to reduced competition for nutrients, lack of protozoal predation or an increase in growing space. Also, the slow flow rate should enable bacterial concentration to increase due to a slower removal rate. Bacterial mass is inversely related to rate of bacterial growth (Owens and Isaacson, 1977), thus bacterial numbers should be lower at higher D.

A greater VFA concentration (P<.05) was noted for D1 (table 2). As indicated previously, a day x treatment interaction (P<.05) was detected for VFA concentration, due to depressed VFA levels for D1 on the 4th collection d. A lower VFA concentration would be expected with a more rapid flow rate since existing VFA would be diluted faster. Lower bacterial concentration with D2 might also cause some decrease in VFA levels. Also, apparent OMD tended to be depressed at the higher D (table 2). This effect might partly be due to decreased OM residence time at the faster D, although it is unknown whether the difference would occur if OMD were corrected for microbial content. Kennedy and Milligan (1978) observed decreased ruminal CWC digestibility in vivo with accelerated D. Lower bacterial numbers and reduced residence time might produce some decline in OMD and VFA concentration, although increased efficiency of bacterial growth at elevated D (Owens and Isaacson, 1977) might counteract these effects to some extent.

Increased D promoted an increased (P<.01) proportion of acetate (Ac) and decreased (P<.05) propionate (Pr) (table 2). These fermentation shifts with D alteration are similar to those observed by Harrison et al. (1975), Hodgson and Thomas (1975) and Thomson et al. (1978). Fermentation reverts to a more efficient acetate fermentation when D is elevated (Owens and Isaacson, 1977). Propionate production is also higher at depressed pH (Slyter et al., 1966) which could partially explain higher Pr proportions at slower D. A pH fluctuation may initiate changes in bacterial growth and species (Russell et al., 1979) which might account for changes in end product ratios at differing D. Furthermore, Males and Purser (1970) observed that defaunated animals exhibited lower proportions of Ac and butyrate (Bu). Thus, lower protozoa numbers with D1 may have contributed to the reduced molar proportion of Ac observed in this study. A day effect was observed for Pr (P<.05), indicating that a longer stabilization period might have been beneficial. Examination of daily measurements suggested that the decline in Pr with D2 and increase with D1 was complete by the third d of sampling.

Butyrate appeared to increase slightly at elevated D (table 2). As mentioned previously, a day x treatment interaction (P<.05) was detected but was ignored due to the nature of the interaction. Values for both treatments were similar until the final two d of collection, at which time D2 exhibited greater Bu proportion than did D1. The trend for increased Bu at faster D is in contrast to in vitro studies by Isaacson et al. (1975) and Crawford et al. (1980), who observed a negative relationship between Bu and D. Harrison et al. (1975) suggested that Bu should increase as D increased, assuming that Ac and Bu interconversion occurs in the rumen, since greater Ac is characteristic of faster D. Alternatively, the response could be attributed to a lack of protozoa at D1 since Males and Purser (1970) observed greater Bu proportions in faunated animals.

Decreased proportions of valerate (P<.05) were observed for D2 (table 2). Increased efficiency at higher D might divert bacterial metabolism to pathways which do not produce valerate, or cause shifts to species which produce less valerate. No significant differences in isobutyrate or isovalerate proportions were observed at the two dilution rates

Increased NH_3 concentration (P<.01) was noted (table 2) for the faster D, which agrees with a reported inverse relationship between ruminal NH_3 levels and Pr proportion when D was altered (Hodgson and Thomas 1975). More efficient growth and reduced residence time would suggest an increased NH_3 assimilation and decreased feed deamination by bacteria and consequently decreased NH_3 at faster D. However, suboptimal at D1 may have decreased deamination, or more efficient deaminase systems may be present in bacteria at higher D. Also, more ruminal NH_3 is present in faunated sheep (Males and Purser, 1970), thus loss protozoa at D1 might have increased NH_3 uptake due increased bacterial numbers.

This study suggests that the New Mexico State University continuous fermentation system produces fermentation patterns similar to those reported in vivo. Moreover, the system responded to alteration in dilution rate in a manner consistent with publis reports. Further investigations are needed to evaluate microbial protein production in this continuous fermentation system.

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TABLE 1. CHEMICAL COMPOSITION OF RABBIT CHOW USED AS A FERMENTATION SUBSTRATE

Item	%
Dry matter	94.1
Ash[a]	12.8
Crude protein[a]	17.6
Acid detergent fiber[a]	22.1
Acid detergent lignin[a]	5.6

[a]Percentage of dry matter.

TABLE 2. MEAN CULTURE CHARACTERISTICS AT TWO DILUTION RATES

	Treatment[a]		
Item	D1	D2	SEM
Protozoa (x 10^4/ml)	.3[b]	6.2[c]	.26
Bacteria (x 10^{10}/ml)	17.0[d]	11.3[e]	.83
pH	5.4[b]	5.9[c]	.003
Acetate, mol/100 mol	54.4[b]	61.5[c]	.14
Propionate, mol/100 mol	25.7[d]	18.6[e]	.54
Butyrate, mol/100 mol	12.4	14.3	.47
Isobutyrate, mol/100 mol	.47	.63	.06
Valerate, mol/100 mol	5.5[d]	2.8[e]	.35
Isovalerate, mol/100 mol	1.5	2.2	.14
VFA, mmol/liter	76.2[d]	66.7[e]	.71
NH_3, mg/100 ml	4.3[b]	10.7[c]	.02
Apparent OMD, %	56.0	49.7	3.24

[a] Each treatment mean represents an average of four fermenters over five d. D1 = 4.1%/h, D2 = 5.7%/h.

[b,c]Means with different superscripts differ ($P<.01$).

[d,e]Means with different superscripts differ ($P<.05$).