Effects of Patterns of Suboptimal pH on Rumen Fermentation in a Dual-Flow Continuous Culture System

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ABSTRACT

Low ruminal pH affects rumen fermentation, with the effects being larger as the time at suboptimal pH increases. Eight 1,325-mL dual-flow continuous culture fermenters were used to examine the hypothesis that the negative effects of a single cycle of 12 h (experiment 1) or 8 h (experiment 2) at pH 5.5 can be reduced by splitting it into several cycles. Temperature (39°C), diet (97 g/d of a 60:40 forage:concentrate diet), and solid (5%/h) and liquid (10%/h) dilution rates were kept constant. In experiment 1, treatments were a constant pH 6.4 (H); 1 cycle of 12 h at pH 5.5 (L12); 2 cycles of 6 h at pH 5.5; and 3 cycles of 4 h at pH 5.5. In experiment 2, treatments were a constant pH 6.4 (H); 1 cycle of 4 h at pH 5.5 (L4); 1 cycle of 8 h at pH 5.5 (L8); or 2 cycles of 4 h at pH 5.5. During the rest of the day, pH was maintained at 6.4. Each experiment consisted of 2 replicated periods of 8 d (5 d for adaptation and 3 d for sampling). Within period, treatments were randomly assigned to fermenters. Data were analyzed as a randomized complete block using PROC MIXED of SAS and differences declared at $P < 0.05$ using the Tukey's test. In experiment 1, L12 reduced neutral detergent fiber (NDF) digestion, acetate proportion, and the acetate:propionate ratio, increased propionate proportion, and tended to reduce ammonia N concentration, compared with H, but had no effect on the flow of dietary or microbial N, crude protein degradation, efficiency of microbial protein synthesis, or the flow of total, essential, and individual amino acids. Dividing the 12 h at suboptimal pH into 2 or 3 cycles reduced true organic matter (OM) degradation compared with H, and did not alleviate the negative effects on NDF digestion and volatile fatty acid profile observed in L12. In experiment 2, L4 tended to reduce true OM digestion, ammonia N concentration, and bacterial N flow, reduced CP degradation, and increased dietary N flow. Treatment L8

reduced OM and NDF digestion, and ammonia N concentration, compared with H. Treatments L4 and L8 also reduced acetate proportion and the acetate:propionate ratio, and increased propionate proportion and the flow of total, essential, and most individual amino acids, but had no effect on efficiency of microbial protein synthesis compared with the H treatment. When the 8 h at suboptimal pH was divided into 2 cycles of 4 h the effects were not different from L8. Results suggest that the effects of low pH are dependent on the total amount of time that pH is suboptimal and are not reduced by splitting it into various cycles.

Key words: acidosis, pH, rumen fermentation

INTRODUCTION

Low pH has negative effects on rumen fermentation (Hoover, 1986; de Veth and Kolver, 2001a). Thus, mean daily pH has been used to predict nutrient digestion in rumen steady-state mathematical models (Pitt et al., 1996). However, ruminal pH varies considerably during the course of the day (French and Kennelly, 1990; Robinson and McQueen, 1994) and researchers have recently become interested in evaluating the effects of pH fluctuations on microbial fermentation (de Veth and Kolver, 2001a,b; Calsamiglia et al., 2002; Wales et al., 2004). It appears that the impact of low pH on rumen microbial fermentation is dependent not only on the mean pH, but also on the time that pH is suboptimal (Sauvant et al., 1999; de Veth and Kolver, 2001b; Cerrato et al., 2007). In fact, the time during which pH is suboptimal has been used more recently to define acidosis (Cooper et al., 1999; Beauchemin et al., 2003; Erickson et al., 2003). Previous studies in our laboratory identified pH 6.4 and 5.5 as optimal and suboptimal, respectively (Cardozo et al., 2000). Cerrato et al. (2007) evaluated the effects of time at this suboptimal pH and concluded that changes in rumen fermentation started immediately after the pH started to decrease, and that the largest effects occurred within the first 12 h of suboptimal pH. However, Calsamiglia et al. (2002) suggested that bacteria may resist short periods of suboptimal pH, and it is unclear if the negative effects of

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a period of low pH can be reduced by splitting this time in 2 or more cycles. The hypothesis tested in the present experiment was that the negative effects of a period of suboptimal pH on rumen microbial fermentation can be reduced by splitting the total time that pH is suboptimal into various cycles.

MATERIALS AND METHODS

Apparatus, Experimental Design, and Diets

Eight 1,320-mL dual-flow continuous culture fermenters (Hoover et al., 1976) were used in 2 experiments each consisting of 2 repeated periods of 8 d each (5 d for adaptation, 3 d for sampling). Within each period, fermenters were inoculated with ruminal fluid obtained from a rumen-fistulated lactating dairy cow fed a 60% forage, 40% concentrate diet. Temperature (38.5°C) and liquid (10 %/h) and solid (5 %/h) dilution rates were controlled and monitored using a personal computer and LabView Software (FieldPoint, National Instruments, Austin, TX). Anaerobic conditions were maintained by the infusion of N₂ at a rate of 40 mL/min. Artificial saliva (Weller and Pilgrim, 1974) was continuously infused into flasks and contained 0.4 g/L of urea to simulate recycled N.

In both experiments, fermenters were fed continuously 97 g of DM/d automatically (0.67 g/10 min) of a diet that consisted of (DM basis) 38.0% alfalfa hay, 20.4% ground corn grain, 17.5% ground corn silage, 14.6% soybean meal, 8.8% ground barley grain, 0.22% white salt, and 0.48% of a vitamin and mineral mix. The vitamin and mineral mix contained, per kilogram, 7 mg of Co, 167 mg of Cu, 33 mg of I, 2,660 mg of Mn, 27 mg of Se, 660 mg of Zn, 1,000 kIU of vitamin A, 200 kIU of vitamin D₃, 1,330 mg of vitamin E, 2.67 g of urea, 67 g of NaCl, 33 g of sulfur, and 300 g of MgO. The diet was designed to meet or exceed current nutrient recommendations for a Holstein cow (650 kg of BW) producing 30 kg of milk (NRC, 2001) and contained (DM basis) 18.9% CP, 30.8% NDF, and 19.7% ADF in experiment 1, and 18.2% CP, 33.0% NDF, and 21.0% ADF in experiment 2.

Treatments

Ruminal pH was controlled from the start of each period at either optimal pH (6.4 ± 0.05) or suboptimal pH (5.5 ± 0.05) by automatic infusion of 3 N HCl or 5 N NaOH. The change in pH from optimal to suboptimal or vice versa due to the programmed fluctuations was achieved within 15 min.

Experiment 1. Treatments were a) a constant pH of 6.4 (H); b) 1 cycle of 12 h at pH 5.5 (L12); c) 2 cycles of 6 h at pH 5.5 (L6×2); and d) 3 cycles of 4 h at pH 5.5

(L4×3). In L12, the pH was low from 2000 to 0800 h. In L6×2, the pH was low from 1400 to 2000 h and from 0200 to 0800 h. In L4×3, the pH was low from 1200 to 1600 h, from 2000 to 2400 h, and from 0400 to 0800 h. Therefore, at 0800 h (time 0) all treatments were returning from a suboptimal pH cycle into a pH 6.4 cycle. The fermenters were maintained at pH 6.4 the rest of the time.

Experiment 2. Treatments were a) a constant pH of 6.4 (H); b) 1 cycle of 4 h at pH 5.5 (L4); c) 1 cycle of 8 h at pH 5.5 (L8); and d) 2 cycles of 4 h at pH 5.5 (L4×2). The period of low pH started at 2200 h in L4 and at 1800 h in L8. In L4×2, the pH was low from 1000 to 1600 h and from 2200 to 0200 h. Therefore, at 0200 h (time 0) all treatments were returning from a suboptimal pH cycle into a pH 6.4 cycle.

Sample Collection and Processing

Each experimental period consisted of 5 d for adaptation and 3 d for sampling. During the 3 d for sampling, 8 mL of filtered fermenter fluid were taken at time 0 (0800 h, when suboptimal pH returned to optimal pH, representing the least favorable time) in both experiments, time 4 (1200 h) in experiment 1, and time 8 (1600 h) in experiment 2 (4 and 8 h after the pH returned to optimal) to determine the osmolality of the incubation fluid as well as VFA and ammonia N concentrations. In addition, during sampling days, liquid and solid effluent collection vessels were maintained at 4°C to impede microbial action, their contents were mixed and homogenized for 1 min, and a 500-mL sample was removed via aspiration. Upon completion of each period, effluents from the 3 days of sampling were composited and mixed within fermenter and homogenized for 2 min. Samples were taken for total N, ammonia N, and VFA analyses. The remainder of the sample was lyophilized. Dry samples were analyzed for DM, ash, NDF, and purine contents. Bacterial cells were isolated from fermenter flasks on the last day of each period by a combination of several procedures selected to obtain the maximum detachment without affecting microbial cell integrity (Whitehouse et al., 1994). One hundred milliliters of a 0.2% methylcellulose solution and small beads (30 of 2 mm diameter and 15 of 4 mm diameter) were added to each fermenter, incubated in the same fermenter flask at 39°C for 1 h to remove attached bacteria, and refrigerated at 4°C for 24 h. After refrigeration, fermenter contents were agitated for 1 h to dislodge loosely attached bacteria. The contents were filtered through cheesecloth and washed with saline solution. Bacterial cells were isolated by differential centrifugation at 1,000 × g for 15 min to eliminate feed particles, and at 20,000 × g for 20 min to isolate the

bacterial pellet. Pellets were rinsed twice with saline solution and centrifuged at $20,000 \times g$ for 20 min. The last rinse was done with distilled water to prevent contamination of bacteria with ash. Bacterial cells were lyophilized and analyzed for DM, ash, N, and purine contents. Digestion of OM, fiber, and CP, and flows of total, nonammonia, microbial, and dietary N were calculated as described by Stern and Hoover (1990).

Chemical Analyses

Effluent DM was determined by lyophilizing 200-mL aliquots in triplicate, and a subsample was dried at 103°C in a forced-air oven for 24 h to determine the final DM content. The DM content of diets and bacterial samples were determined by drying samples for 24 h in a 103°C forced-air oven according to AOAC (1990). Dry samples were ashed overnight at 550°C in a muffle furnace. Fiber components of diets and effluents were analyzed by the detergent system (Van Soest et al., 1991) sequentially using thermostable α -amylase and sodium sulfite. Total N in feed, effluents, and bacterial samples were determined by the Kjeldahl method (AOAC, 1990). Ammonia-N was analyzed in a 4-mL subsample of filtered fluid that was acidified with 4 mL of 0.2 N HCl and frozen. Samples were centrifuged at $25,000 \times g$ for 20 min, and the supernatant was analyzed for ammonia N by colorimetry (Chaney and Marbach, 1962). Samples for VFA were prepared as described by Jouany (1982) using 4-methylvaleric acid as internal standard. The analysis was performed by GLC (model 6890, Hewlett Packard, Palo Alto, CA) using a polyethylene glycol nitroterephthalic acid-treated capillary column (BP21, SGE, Europe Ltd., Buckinghamshire, UK). Osmolality was determined with a MicroOsmometer (Type 13/13DR, Hermann Roebling Messtechnik, Berlin, Germany). Dry effluent and mixed bacterial cells were analyzed for purine base content by HPLC (Beckman Instruments, Palo Alto, CA) according to the procedure of Balcells et al. (1992). Amino acids were analyzed by hydrolyzing samples (25 mg) with 1,000 μL of 6 N HCl containing 0.5 $\mu\text{L}/\text{mL}$ mercaptoethanol at 110°C for 22 h 45 min in sealed evacuated tubes. Derivatization was conducted using the AccQ-Tag Amino Acid Analysis method (Waters Co., Milford, MA). This involved the derivatization of samples with 30 $\mu\text{L}/\text{tube}$ of the Waters AccQFluor reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) at 55°C for 9 min 20 s. Amino acid analysis was performed by reversed-phase HPLC (HP1100 Agilent, AccQ-Tag Amino Acid Analysis Column, 3.9 mm $\times$ 150 mm silica base bonded with C18) with UV-visible detection using modified procedures of the Waters method to ensure separation of derivatized AA. Norleucine was used as the internal standard.

Statistical Analysis

The experiment, planned as a randomized complete block design, was analyzed using PROC MIXED of SAS (version 9.1, SAS Institute, Inc., Cary, NC). Differences between treatments ($n = 4$ for each treatment) were tested using Tukey's multiple comparison test and declared at $P < 0.05$ unless otherwise indicated. The statistical model used was $Y_i = \mu + A_i + e_i$, where Y_i is the observations for dependent variable, μ is the population mean, A_i is the mean effect of treatment, and e_i is the residual error. The period was considered a random effect. Results are reported as least squares means. The effects of treatments on VFA and ammonia N concentrations from the fermenter flask at 0 and 4 h (experiment 1) or 8 h (experiment 2) after returning to optimal pH were analyzed using PROC MIXED for repeated measures (Littell et al., 1998). The model accounted for the effects of treatments and time of sampling, and the interaction of treatments with time. The statistical model used was $Y_{ij} = \mu + A_i + B_j + (A \times B)_{ij} + e_{ij}$, where Y_{ij} is the observation for dependent variables; μ is the population mean; A_i is the mean effect of treatment; B_j is the mean effect of time of sampling; $(A \times B)_{ij}$ is the effect of interaction between treatment i and time of sampling j ; and e_{ij} is the residual error. Period was considered a random effect. The VFA and ammonia N concentrations were subjected to 3 covariance structures: compound symmetric, autoregressive order one, and unstructured covariance. The covariance structure that yielded the largest Schwarz's Bayesian criterion was considered the most desirable analysis, and the least squares means for treatments are reported. Differences between treatments were declared at $P < 0.05$ using the Bonferroni comparison test.

RESULTS

Experiment 1

Treatment L12 did not affect true OM digestion compared with H. In contrast, when the 12-h cycle at suboptimal pH was divided in 2 or 3 cycles (treatments L6 $\times$ 2 and L4 $\times$ 3), true OM digestion was lower compared with H (Table 1). There were no differences in true OM digestion between L4 $\times$ 3 and L12, and L4 $\times$ 3 and L6 $\times$ 2, but was lower in L6 $\times$ 2 than in L12. For the digestion of NDF, all treatments differed from H, and there were no differences among L12, L6 $\times$ 2, and L4 $\times$ 3.

The maintenance and fluctuations of pH require the addition of NaOH and HCl. Because pH was different in each treatment, daily consumption of acid (7.21, 36.0, 53.3, and 50.0 mL/d for H, L12, L6 $\times$ 2, and L4 $\times$ 3, respectively) and base (10.3, 12.4, 18.5, and 22.6 mL/d for H, L12, L6 $\times$ 2, and L4 $\times$ 3, respectively) differed between

Table 1. Effect of the number and duration of cycles at suboptimal pH (5.5) on true OM and NDF digestion in continuous culture

Item	Treatment ¹				SEM ²
	H	L12	L6×2	L4×3	
True OM digestion, %	51.7 ^a	47.7 ^{ab}	42.6 ^c	46.4 ^{bc}	2.06
NDF digestion, %	32.7 ^a	21.5 ^b	17.3 ^b	19.5 ^b	2.09

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

¹H = constant pH 6.4; L12 = 1 cycle of 12 h at suboptimal pH 5.5; L6×2 = 2 cycles of 6 h at suboptimal pH 5.5; L4×3 = 3 cycles of 4 h at suboptimal pH 5.5. The rest of the day the pH was at 6.4.

²SEM = standard error of the mean.

treatments. As a result, osmolality was increased as the total amount of acid and base increased, as expected (Table 2). Mean total VFA concentration did not vary among treatments, but when measured at 0 and 4 h after recovery from a suboptimal pH cycle, it was lower

in L12, L6×2, and L4×3 compared with H (Table 2). Compared with H, L12 increased the mean molar proportion of propionate and decreased the mean molar proportion of acetate and branched-chain VFA and the mean acetate to propionate ratio. Dividing the 12-h suboptimal pH cycle into 2 or 3 cycles had no effect on these estimates, which were similar in L6×2 and L4×3 compared with L12, but different from H. Similar trends were observed in the samples obtained from the fermenter flask at 0 and 4 h after returning from a suboptimal pH cycle, although differences were smaller (Table 2). Mean molar proportions of valerate and butyrate did not vary among treatments, and, although differences at 0 and 4 h after returning from a suboptimal pH cycle tended to be significant ($P < 0.10$), they were small (Table 2).

Mean ammonia N concentration was not affected by treatments, but tended ($P = 0.09$) to be lower in L12

Table 2. Effect of the number and duration of cycles at suboptimal pH (5.5) on total VFA concentration and proportions in continuous culture

Item	Treatment ¹				SEM ²
	H	L12	L6×2	L4×3	
Osmolality, mosmol/kg					
time 0	325 ^b	329 ^{ab}	343 ^a	351 ^a	9.6
time 4	304 ^b	316 ^b	354 ^b	348 ^a	8.7
Average	314 ^b	322 ^{ab}	348 ^a	349 ^a	6.9
Total VFA, mM					
time 0	115.4 ^a	94.7 ^b	93.1 ^b	95.0 ^b	5.17
time 4	112.1 ^a	100.1 ^b	92.2 ^{c†}	97.0 ^{bc}	5.44
Daily composite	101.8	97.5	86.0	88.9	9.01
Acetate, mol/100 mol					
time 0	68.2 ^a	53.5 ^c	57.6 ^b	57.6 ^b	0.88
time 4	68.2 ^a	53.9 ^c	58.4 ^b	58.5 ^b	0.91
Daily composite	67.7 ^a	55.4 ^b	57.6 ^b	58.0 ^b	1.14
Propionate, mol/100 mol					
time 0	14.3 ^c	27.3 ^a	24.4 ^{ab}	23.1 ^b	1.24
time 4	14.1 ^c	27.1 ^a	24.1 ^{ab}	22.8 ^b	1.22
Daily composite	14.7 ^b	26.6 ^a	25.2 ^a	24.7 ^a	1.61
Butyrate, mol/100 mol					
time 0	10.2 ^b	10.8 ^{ab}	12.2 ^{a*}	12.2 ^{a*}	0.72
time 4	10.3	10.5	11.6	11.7	0.78
Daily composite	11.0	12.9	12.5	12.5	0.82
Valerate, mol/100 mol					
time 0	2.5 ^b	3.1 ^{a*}	3.0 ^{ab}	3.0 ^{ab}	0.21
time 4	2.6 ^b	3.2 ^a	3.1 ^{a*}	2.9 ^{ab}	0.19
Daily composite	2.8	3.2	2.4	3.2	0.28
BCVFA, ³ mol/100 mol					
time 0	3.5 ^a	0.8 ^b	1.0 ^b	1.1 ^b	0.22
time 4	3.5 ^a	0.9 ^b	1.0 ^b	1.1 ^b	0.21
Average	3.8 ^a	1.9 ^b	1.4 ^b	1.5 ^b	0.29
Acetate:Propionate					
time 0	4.5 ^a	1.9 ^c	2.1 ^{bc}	2.3 ^{b†}	0.16
time 4	4.5 ^a	1.9 ^c	2.3 ^{bc}	2.4 ^{b†}	0.17
Daily composite	4.6 ^a	2.1 ^b	2.4 ^b	2.4 ^b	0.20

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

¹H = constant pH 6.4; L12 = 1 cycle of 12 h at suboptimal pH 5.5; L6×2 = 2 cycles of 6 h at suboptimal pH 5.5; L4×3 = 3 cycles of 4 h at suboptimal pH 5.5. The rest of the day the pH was at 6.4.

²SEM = standard error of the mean.

³Branched-chain VFA includes isobutyrate and isovalerate.

*Indicates values different from H ($P < 0.10$); † indicates values different from L12 ($P < 0.10$).

Table 3. Effect of the number and duration of cycles at suboptimal pH (5.5) on N metabolism of rumen microbes in continuous culture

Item	Treatment ¹				SEM ²
	H	L12	L6×2	L4×3	
N-NH ₃ , mg/dL	14.0	12.3	13.0	11.1	1.95
time 0	14.7 ^a	8.2 ^b	9.4 ^b	8.2 ^b	1.95
time 4	10.2 ^a	6.8 ^{b*}	8.6 ^{ab}	7.6 ^{ab}	0.90
Daily composite					
N flow, g/d					
Total	3.40	3.35	3.40	3.24	0.05
Ammonia	0.31 ^a	0.21 ^{b*}	0.27 ^{ab}	0.23 ^{ab}	0.03
Nonammonia	3.07	3.14	3.13	3.01	0.05
Bacterial	1.68	1.51	1.44	1.55	0.16
Dietary	1.39	1.62	1.68	1.47	0.14
EMPS ³	36.2	35.3	37.8	37.2	4.60
CPd, ⁴ %	52.2	44.3	42.2	49.8	4.80

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

¹H = constant pH 6.4; L12 = 1 cycle of 12 h at suboptimal pH 5.5; L6×2 = 2 cycles of 6 h at suboptimal pH 5.5; L4×3 = 3 cycles of 4 h at suboptimal pH 5.5. The rest of the day the pH was at 6.4.

²SEM = standard error of the mean.

³Efficiency of microbial protein synthesis; g of N/kg of OM truly digested.

⁴CP degradation.

*Indicates values different from H ($P < 0.10$).

compared with H (Table 3). When samples were taken from the fermenter flask, ammonia N concentration was not affected by treatments at time 0, but was lower in treatments L12, L6×2, and L4×3 compared with H at time 4. There were no significant differences among treatments in the flow of total, nonammonia, bacterial, and dietary N, efficiency of microbial protein synthesis (EMPS), CP degradation, and the flow of total, essential, nonessential, and individual AA (Tables 3 and 4).

Experiment 2

Relative to H, treatments L8 and L4 tended ($P = 0.10$) to decrease true OM digestion, but dividing the 8 h at suboptimal pH into 2 cycles resulted in no changes in digestibility compared with H, L4, or L8 (Table 5). Digestion of NDF was similar in L8 and L4×2 and lower compared with H (Table 5). There were no significant differences between L4 and the other treatments.

The maintenance and fluctuations of pH require the addition of NaOH and HCl. Because pH was different in each treatment, daily consumption of acid (5.1, 27.9, 32.5, and 40.2 mL/d for H, L4, L8, and L4×2, respectively) and base (6.4, 15.9, 18.0, and 22.9 mL/d for H, L4, L8, and L4×2, respectively) was different between treatments. As a result, osmolality was higher in L4×2 compared with H, where total amount of acid and based added was also higher (Table 6). Mean total VFA concentration was not affected by treatment (Table 6), but at time 0, just after recovering from a cycle of subopti-

mal pH, total VFA was lower in all treatments compared with H, and tended ($P < 0.10$) to be lower in L8 compared with L4×2. Compared with H, L4 decreased the mean molar proportion of acetate and the acetate to propionate ratio, and increased the mean proportion of propionate. Compared with H, L8 decreased the mean molar proportion of acetate and branched-chain VFA, and the acetate to propionate ratio, and increased the mean molar proportion of propionate. Dividing the 8-h suboptimal pH cycle into 2 cycles of 4 h had no effect on these estimates, which were similar in L4×2 compared with L8, but different from H. Similar trends were observed in the samples obtained from the fermenter flask at times 0 and 8. Treatments had no effect on mean valerate and butyrate concentrations.

Ammonia N concentration was lower in L8 and tended ($P < 0.10$) to be lower in L4×2 and L4 compared with H (Table 7), but there were no differences among L4, L8, and L4×2. When samples were taken from the fermenter flask, ammonia N concentration did not vary among treatments at time 0, but at time 8 it was lower in L4, L8, and L4×2 compared with H, and in L8 compared with L4 and L4×2 (Table 6).

There were no differences among H, L8, and L4×2 in CP degradation and the flows of total, nonammonia, and dietary N. Compared with H, L4 resulted in lower CP degradation and higher dietary N flow, and tended ($P < 0.10$) to decrease the flow of bacterial and ammonia N. Treatments had no effect on EMPS.

The flows of Ser, Arg, Pro, Val, Leu, Phe, essential, nonessential, and total AA was similar in L8 and L4×2 and higher than in H (Table 8). Treatment L4×2 resulted in higher flows of Thr, Met, Ile, Lys, and His, and Tyr than in H, but similar compared with L4 and L8. There were no differences in the flows of these AA between L4 and L8. Compared with H, L4×2 tended ($P < 0.10$) to increase the flow of Gly and Ala, and L4 tended ($P < 0.10$) to increase the flow of Val.

DISCUSSION

Although the addition of NaOH and HCl required to control pH were different among treatments and osmolality was affected, changes were within the physiological range (from 260 to 340 mosmol/kg; Hoover and Miller, 1992). The effects of pH fluctuations are confounded with the increase in osmolality, but such changes also occur in vivo where, as a result of an increase in VFA, pH decreases and osmolality increases at the same time (Hoover and Miller, 1992). Although the changes in osmolality due to the addition of VFA or acid and base are likely different, it is not possible to control all variables, and although the results from this experiment should be interpreted with caution,

Table 4. Effect of the number and duration of cycles at suboptimal pH (5.5) on AA flow in continuous culture of rumen contents

AA flow, mg/d	Treatment ¹				SEM ²
	H	L12	L6×2	L4×3	
Ser	697	756	765	778	40.3
Thr	728	748	757	774	45.1
Gly	645	675	682	704	36.5
Ala	880	921	930	941	57.7
Arg	654	748	757	766	40.4
Pro	627	706	735	732	31.1
Val	836	886	896	906	65.1
Met	222	226	235	232	19.9
Ile	792	836	942	842	50.0
Leu	1,144	1,273	1,285	1,270	83.8
Phe	755	827	841	820	48.7
Lys	748	825	839	877	37.8
His	255	282	285	289	22.0
Tyr	510	523	547	546	21.7
Essential, ³ g/d	6.12	6.65	6.74	6.78	0.40
Nonessential, ³ g/d	3.36	3.59	3.66	3.70	0.17
Total, g/d	9.42	10.24	10.40	10.48	0.57

¹H = constant pH 6.4; L12 = 1 cycle of 12 h at suboptimal pH 5.5; L6×2 = 2 cycles of 6 h at suboptimal pH 5.5; L4×3 = 3 cycles of 4 h at suboptimal pH 5.5. The rest of the day the pH was at 6.4.

²SEM = standard error of the mean.

³Essential AA: Thr, Arg, Val, Met, Ile, Leu, Phe, Lys, His; nonessential AA: Ser, Gly, Ala, Pro, Tyr.

they provide data relevant to the effect of pH fluctuation on rumen microbial fermentation within a physiological range of osmolality.

It is generally accepted that digestion of OM is reduced by low ruminal pH (Hoover et al., 1984; Shriver et al., 1986; Cerrato et al., 2007). In the present experiment, however, the numerical decrease in true OM digestion (a reduction of 4.0 and 6.5 percentage units after 12 and 8 h at suboptimal pH, respectively) did not reach significance. Previous studies in our laboratory (Cerrato et al., 2007) observed that increasing the time at suboptimal pH between 0 and 24 h resulted in a cubic reduction in true OM digestion, with a reduction of 9.6 and 9.0 percentage units when pH was suboptimal for 8 and 12 h, respectively. de Veth and Kolver (2001b) also found a moderate reduction in true OM digestion when pH was maintained low (5.4) for 8 h (a reduction

of 6.5 percentage units) and 12 h (a reduction of 7.7 percentage units). The lack of significance in the present trials appears to be due to a slightly lower reduction in true OM digestion and slightly higher standard error, but trends were similar. Changes in OM digestion were paralleled by changes in NDF digestion, which were lower after 12 (experiment 1) and 8 (experiment 2) h at suboptimal pH compared with H, and were in agreement with previous studies (de Veth and Kolver, 2001b; Cerrato et al., 2007).

Treatments L12 (experiment 1) and L8 (experiment 2) did not affect mean total VFA concentration, increased the mean molar proportion of propionate, and decreased the mean molar proportion of acetate and branched-chain VFA compared with H. The changes in the VFA profiles in L12 and L8 agree with previous *in vitro* studies in which pH was controlled for 8 and 12 h at suboptimal pH (de Veth and Kolver, 2001b; Cerrato et al., 2007). The changes in acetate and propionate proportions in these treatments are likely related to the reduction in fiber digestion. The reduction of branched-chain VFA may be the result of a decreased deamination of branched-chain AA due to the low pH (Lana et al., 1998). In fact, mean ammonia N concentration tended ($P < 0.10$) to be lower in L12 and was lower in L8 compared with H. When samples were taken from the fermenter flask, L12 and L8 had no effects on ammonia N concentration at time 0, just after recovering from a cycle of suboptimal pH, but ammonia N concentration was reduced 4 h (in experiment 1) and 8 h (in experi-

Table 5. Effect of the number and duration of cycles at suboptimal pH (5.5) on true OM and NDF digestion in continuous culture

Item	Treatment ¹				SEM ²
	H	L4	L8	L4×2	
True OM digestion, %	51.1 ^a	44.4 ^{b*}	44.6 ^{b*}	46.5 ^{ab}	1.63
NDF digestibility, %	31.6 ^a	27.0 ^{ab}	23.5 ^b	25.4 ^b	2.60

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

¹H = constant pH 6.4; L4 = 1 cycle of 4 h at suboptimal pH 5.5; L8 = 1 cycle of 8 h at suboptimal pH 5.5; L4×2 = 2 cycles of 4 h at suboptimal pH 5.5. The rest of the day the H was at 6.4.

²SEM = standard error of the mean.

*Indicates values different from H ($P < 0.07$).

Table 6. Effect of the number and duration of cycles at suboptimal pH (5.5) on total VFA concentration and proportions in continuous culture

Item	Treatment ¹				SEM ²
	H	L4	L8	L4×2	
Osmolality, mosmol/kg					
time 0	294 ^b	317 ^{ab}	328 ^{ab}	332 ^a	11.4
time 8	298 ^b	330 ^a	331 ^a	340 ^a	6.4
Average	296 ^b	323 ^{ab}	329 ^a	336 ^a	9.1
Total VFA, mM					
time 0	111.2 ^a	98.8 ^{bc}	94.3 ^{c*}	101.1 ^b	2.20
time 8	115.0	114.7	110.5	108.8	2.66
Daily composite	100.6	95.2	92.8	92.7	6.06
Acetate, mol/100 mol					
time 0	61.8 ^a	55.9 ^b	53.3 ^b	53.9 ^b	2.54
time 8	62.5 ^a	55.6 ^b	53.7 ^b	53.7 ^b	2.51
Daily composite	62.5 ^a	55.8 ^b	54.0 ^b	55.4 ^b	2.61
Propionate, mol/100 mol					
time 0	15.6 ^b	25.8 ^a	25.6 ^a	24.9 ^a	0.95
time 8	14.7 ^b	25.2 ^a	25.5 ^a	25.3 ^a	0.90
Daily composite	16.9 ^b	23.4 ^a	24.6 ^a	24.0 ^a	1.28
Butyrate, mol/100 mol					
time 0	14.6 ^b	15.4 ^{b†}	17.4 ^a	14.7 ^b	1.85
time 8	14.6 ^{b†}	15.4 ^{ab}	16.6 ^a	13.6 ^b	1.86
Daily composite	15.1	15.6	16.4	16.0	2.00
Valerate, mol/100 mol					
time 0	3.2	3.4	3.6	3.3	0.47
time 8	3.2	3.6	3.5	3.2	0.47
Daily composite	2.7	3.4	3.9	3.4	0.67
BCVFA, ³ mol/100 mol					
time 0	2.1 ^a	1.8 ^{ab}	1.4 ^b	1.2 ^b	0.22
time 8	2.0 ^a	1.8 ^a	1.3 ^b	1.3 ^b	0.24
Daily composite	2.8 ^a	1.7 ^{ab}	1.0 ^b	1.1 ^b	0.39
Acetate:Propionate					
time 0	3.9 ^a	2.2 ^b	2.1 ^b	2.3 ^b	0.20
time 8	4.1 ^a	2.3 ^b	2.0 ^b	2.4 ^b	0.19
Daily composite	3.7 ^a	2.4 ^b	2.2 ^b	2.3 ^b	0.23

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

¹H = constant pH 6.4; L4 = 1 cycle of 4 h at suboptimal pH 5.5; L8 = 1 cycle of 8 h at suboptimal pH 5.5; L4×2 = 2 cycles of 4 h at suboptimal pH 5.5. The rest of the day the H was at 6.4.

²SEM = standard error of the mean.

³Branched-chain VFA, includes isobutyrate and isovalerate.

*Indicates values different from L4×2 ($P < 0.10$); † indicates values different from L8 ($P < 0.10$).

ment 2) after recovering from a cycle of suboptimal pH. These treatment differences in ammonia N concentration taken at different times from the fermenter flask may be attributed to differences in ammonia N production or utilization by bacteria during periods of low pH compared with periods of optimal pH. We speculate that bacteria do not die during periods of low pH, but temporarily reduce their growth, reducing the utilization of ammonia N, which then accumulates. When pH returns to optimal, bacteria are able to recover and regrow, increasing the uptake of ammonia N, which then results in a lower ammonia N concentration.

It is generally accepted that CP degradation is reduced by low ruminal pH (de Veth and Kolver, 2001b; Calsamiglia et al., 2002; Wales et al., 2004). Although changes were not significant, L12 and L8 decreased CP degradation by 7.9 and 16.7 percentage units, respec-

tively. This trend is consistent with the reduction in ammonia N concentration and branched-chain VFA proportions observed. However, CP degradation in L4 was lower than in H and numerically lower than in L4×2, which has no biological explanation. Changes observed in ammonia N concentration and branched-chain VFA proportions suggest that degradation was not different between L4 and L4×2. It is likely that CP degradation in L4 may be the result of an underestimation of microbial N flow.

Treatments L8 and L12 did not affect EMPS. The effect of pH on the EMPS has been controversial. Although some in vitro studies have shown no effect (Hoover and Miller, 1992; Cardozo et al., 2000; Calsamiglia et al., 2002), others reported a reduction in EMPS (Wales et al., 2004). When fermentation has been maintained for only a part of the day at low pH, a reduction

Table 7. Effect of the number and duration of cycles at suboptimal pH (5.5) on N metabolism of rumen microbes in continuous culture

Item	Treatment ¹				SEM ²
	H	L4	L8	L4×2	
N-NH ₃ , mg/dL					
time 0	13.9	14.4	13.3	11.2	1.54
time 8	12.0 ^a	5.6 ^b	4.0 ^c	7.0 ^b	1.54
Daily composite	10.4 ^a	6.6 ^{b*}	5.6 ^b	6.9 ^{b*}	0.90
N flow, g/d					
Total	3.25	3.21	3.25	3.24	0.03
Ammonia	0.33 ^a	0.21 ^{b*}	0.18 ^b	0.22 ^{b*}	0.03
Nonammonia	2.92	3.00	3.07	3.02	0.04
Bacterial	1.43 ^a	0.82 ^{b*}	1.11 ^{ab}	1.31 ^{ab}	0.16
Dietary	1.49 ^a	2.17 ^b	1.96 ^{ab}	1.71 ^{ab}	0.15
EMPS ³	30.3	20.4	26.9	30.7	2.87
CPd, ⁴ %	47.3 ^a	23.3 ^b	30.6 ^{ab}	39.6 ^{ab}	5.29

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

¹H = constant pH 6.4; L4 = 1 cycle of 4 h at suboptimal pH 5.5; L8 = 1 cycle of 8 h at suboptimal pH 5.5; L4×2 = 2 cycles of 4 h at suboptimal pH 5.5. The rest of the day the H was at 6.4.

²SEM = standard error of the mean.

³Efficiency of microbial protein synthesis; g of N/kg of OM truly digested.

⁴CP degradation.

*Indicates values different from H ($P < 0.10$).

in the EMPS has been reported (de Veth and Kolver, 2001b; Cerrato et al., 2007).

The flow of most AA increased after 8 h of suboptimal pH in experiment 2, in agreement with Calsamiglia et

al. (2002). Trends were similar in L12, but the lack of significance can be attributed to the relatively high standard error in experiment 1 (mean of 42.9) compared with experiment 2 (mean of 15.5). Few other studies have evaluated the effect of suboptimal pH on the flow of AA and, in particular, the impact of diurnal variation in ruminal pH. Erfle et al. (1982) suggested that the flow of AA should increase when fermentation is conducted at low pH. Calsamiglia et al. (2002) also reported a higher flow of total and essential AA, Gly, Lys, Leu, His, and Arg when fermentation was conducted at constant pH of 5.7 compared with constant pH of 6.4, and intermediate flows of these AA when pH fluctuated with a total of 12 h at suboptimal pH. They attributed this to the higher flow of dietary N and the change in dietary to bacterial N ratio in the total N flow. Cerrato et al. (2007) reported a linear increase in the flow of total and individual AA (except Met) with increasing time at suboptimal pH. These results would support our previous suggestion that a transitory acidosis may be beneficial from the AA nutrition point of view (Calsamiglia et al., 2002; Cerrato et al., 2007).

Overall, reducing pH to 5.5 for 8 and 12 h resulted in the expected changes in rumen microbial fermentation. Therefore, treatments L8 and L12 became adequate controls for testing the hypothesis that splitting the time at suboptimal pH in various cycles may alleviate these negative effects. Dividing the 12-h period at sub-

Table 8. Effect of the number and duration of cycles at suboptimal (5.5) on AA flow in continuous culture of rumen contents

Item	Treatment ¹				SEM ²
	H	L4	L8	L4×2	
Flow, mg/d					
Ser	701 ^b	768 ^a	796 ^a	782 ^a	12.67
Thr	735 ^b	768 ^{ab}	774 ^{ab}	794 ^a	19.3
Gly	670 ^b	697 ^{ab}	707 ^{ab}	715 ^{a*}	14.9
Ala	897 ^b	939 ^{ab}	935 ^{ab}	949 ^{a*}	20.1
Arg	657 ^b	756 ^a	764 ^a	767 ^a	17.0
Pro	662 ^b	706 ^{ab}	723 ^a	723 ^a	12.8
Val	865 ^b	916 ^{a*}	919 ^a	937 ^a	12.8
Met	207 ^b	227 ^{ab}	224 ^{ab}	237 ^a	6.7
Ile	817 ^b	853 ^{ab}	856 ^{ab}	882 ^a	18.4
Leu	1,166 ^b	1,267 ^a	1,286 ^a	1,301 ^a	18.9
Phe	747 ^b	807 ^a	814 ^a	823 ^a	15.5
Lys	764 ^b	822 ^{ab}	830 ^{ab}	865 ^a	22.3
His	246 ^b	273 ^{ab}	289 ^{ab}	298 ^a	12.3
Tyr	524 ^b	558 ^{ab}	557 ^{ab}	576 ^a	13.5
Essential, ³ g/d	6.21 ^b	6.68 ^a	6.75 ^a	6.90 ^a	0.10
Nonessential, ³ g/d	3.45 ^b	3.67 ^a	3.72 ^a	3.74 ^a	0.05
Total, g/d	9.66 ^b	10.40 ^a	10.48 ^a	10.65 ^a	0.15

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

¹H = constant pH 6.4; L4 = 1 cycle of 4 h at suboptimal pH 5.5; L8 = 1 cycle of 8 h at suboptimal pH 5.5; L4×2 = 2 cycles of 4 h at suboptimal pH 5.5. The rest of the day the H was at 6.4.

²SEM = standard error of the mean.

³Essential AA: Thr, Arg, Val, Met, Ile, Leu, Phe, Lys, His; nonessential AA: Ser, Gly, Ala, Pro, Tyr.

*Indicates values different from H ($P < 0.10$).

optimal pH in 2 or 3 cycles (treatments L6×2 and L4×3) resulted in a reduction in true OM digestion that reached significance compared with H (experiment 1) but did not improve true OM digestion compared with L12. Similarly, dividing L8 into two 4-h cycles (L4×2) had no effect on true OM digestion (experiment 2). Similar trends were observed for NDF digestion, in which splitting the 12- and 8-h cycles at low pH in various cycles (L4×3 and L6×2 in experiment 1, and L4×2 in experiment 2) reduced NDF digestion compared with H but were not different from L12 and L8, respectively.

Dividing 12 h into 2 or 3 cycles of suboptimal pH (experiment 1) and 8 h into 2 cycles of suboptimal pH (experiment 2) had small effects on total VFA, acetate, propionate, and branched-chain VFA proportions, ammonia N concentration, and N metabolism compared with L12 and L8. Only one previous report tested the effect of equal times at low pH with different number of cycles, and results were similar (Calsamiglia et al., 2002). These results suggest that the negative effects of low pH were dependent on the total amount of time that pH was suboptimal and were not reduced by dividing it into various short periods.

CONCLUSIONS

Periods of 12 h and 8 h at suboptimal pH had moderate effects on true OM digestion, reduced NDF digestion, acetate proportion, acetate to propionate ratio, and ammonia N concentration, and increased propionate proportion, as expected. Splitting the time at suboptimal pH in various cycles did not alleviate these effects on rumen microbial fermentation. Overall, it appears that the effects of low pH are dependent on the total amount of time that pH is suboptimal and are not reduced by splitting it into various cycles.

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REFERENCES

- AOAC. 1990. Official Methods of Analysis. 15th ed. Association of Official Analytical Chemists, Arlington, VA.
- Balcells, J., J. A. Guada, J. M. Peiró, and D. S. Parker. 1992. Simultaneous determination of allantoin and oxypurines in biological fluids by high performance liquid chromatography. *J. Chromatogr.* 575:153–157.
- Beauchemin, K. A., W. Z. Yang, D. P. Morgavi, G. R. Ghorbani, W. Kautz, and J. A. Z. Leedle. 2003. Effects of bacterial direct-fed microbials and yeast on site and extent of digestion, blood chemistry, and subclinical ruminal acidosis in feedlot cattle. *J. Anim. Sci.* 81:1628–1640.
- Calsamiglia, S., A. Ferret, and M. Devant. 2002. Effects of pH and pH fluctuations on microbial fermentation and nutrient flow from a dual-flow continuous culture system. *J. Dairy Sci.* 85:574–579.
- Cardozo, P. W., S. Calsamiglia, and A. Ferret. 2000. Effects of pH on microbial fermentation and nutrient flow in a dual flow continuous culture system. *J. Dairy Sci.* 83(Suppl. 1):265. (Abstr.)
- Cerrato, M., S. Calsamiglia, and A. Ferret. 2007. Effects of time at suboptimal pH on nutrient digestion and rumen microbial fermentation on a dual flow continuous culture system. *J. Dairy Sci.* 90:1486–1492.
- Chaney, A. L., and E. P. Marbach. 1962. Modified reagents for determination of urea and ammonia. *Clin. Chem.* 8:130–132.
- Cooper, R. J., T. J. Klopfenstein, R. A. Stock, C. T. Milton, D. W. Herold, and J. C. Parrott. 1999. Effects of imposed feed intake variation on acidosis and performance of finishing steers. *J. Anim. Sci.* 77:1093–1099.
- de Veth, M. J., and E. S. Kolver. 2001a. Digestion of ryegrass pasture in response to change in pH in continuous culture. *J. Dairy Sci.* 84:1449–1457.
- de Veth, M. J., and E. S. Kolver. 2001b. Diurnal variation in pH reduces digestion and synthesis of microbial protein when pasture is fermented in continuous culture. *J. Dairy Sci.* 84:2066–2072.
- Erfle, J. D., R. J. Boila, R. M. Teather, S. Mahadevan, and F. D. Sauer. 1982. Effect of pH on fermentation characteristics and protein degradation by rumen microorganisms in vitro. *J. Dairy Sci.* 65:1457–1464.
- Erickson, G. E., C. T. Milton, K. C. Fanning, R. J. Cooper, R. S. Swingle, J. C. Parrott, G. Vogel, and T. J. Klopfenstein. 2003. Interaction between bunk management and monensin concentration on finishing performance, feeding behavior, and ruminal metabolism during an acidosis challenge with feedlot cattle. *J. Anim. Sci.* 81:2869–2879.
- French, N., and J. J. Kennelly. 1990. Effects of feeding frequency on ruminal parameters, plasma insulin, milk yield, and milk composition in Holstein cows. *J. Dairy Sci.* 73:1857–1863.
- Hoover, W. H. 1986. Chemical factors involved in ruminal fiber digestion. *J. Dairy Sci.* 69:2755–2766.
- Hoover, W. H., B. A. Crooker, and C. J. Sniffen. 1976. Effects of differential solid-liquid removal rates on protozoa numbers in continuous cultures of rumen contents. *J. Anim. Sci.* 43:528–534.
- Hoover, W. H., C. R. Kincaid, G. A. Varga, W. V. Thayne, and L. L. Junkins. 1984. Effects of solids and liquid flows on fermentation in continuous cultures. IV. pH and dilution rate. *J. Anim. Sci.* 58:692–699.
- Hoover, W. H., and T. K. Miller. 1992. Rumen digestive physiology and microbial ecology. *Bull. 708T, Agric. Forestry Exp. Sta., West Virginia University, Morgantown.*
- Jouany, J. P. 1982. Volatile fatty acids and alcohol determination in digestive contents, silage juice, bacterial cultures and anaerobic fermentor contents. *Sci. Aliments* 2:131–144.
- Lana, R. P., J. B. Russell, and M. E. Van Amburgh. 1998. The role of pH in regulating ruminal methane and ammonia production. *J. Anim. Sci.* 76:2190–2196.
- Littell, R. C., P. R. Henry, and C. B. Ammerman. 1998. Statistical analysis of repeated measures data using SAS procedures. *J. Anim. Sci.* 75:1216–1231.
- NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th ed. Natl. Acad. Press, Washington, DC.
- Pitt, R. E., J. S. Van Kessel, D. G. Fox, A. N. Pell, M. C. Barry, and P. J. Van Soest. 1996. Prediction of ruminal volatile fatty acids and pH within the net carbohydrate and protein system. *J. Anim. Sci.* 74:226–244.
- Robinson, P. H., and R. E. McQueen. 1994. Influence of supplemental protein source and feeding frequency on rumen fermentation and performance in dairy cows. *J. Dairy Sci.* 77:1340–1353.
- Sauvant, D., F. Meschy, and D. R. Mertens. 1999. Les composantes de l'acidose ruminale et les effets acidogènes des rations. *INRA Prod. Anim.* 12:49–60.
- Shriver, B. J., W. H. Hoover, J. P. Sargent, R. J. Crawford, Jr., and W. V. Thayne. 1986. Fermentation of a high concentrate diet as affected by ruminal pH and digesta flow. *J. Dairy Sci.* 69:413–419.

- Stern, M. D., and W. H. Hoover. 1990. The dual flow continuous culture system. Pages 17–32 in *Proc. Continuous Culture Fermenters: Frustration or Fermentation*. Northwest ADSA-ASAS Regional Meeting, Chazy, NY.
- Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74:3583–3597.
- Wales, W. J., E. S. Kolver, P. L. Thorne, and A. R. Egan. 2004. Diurnal variation in ruminal pH on the digestibility of highly digestible perennial ryegrass during continuous culture fermentation. *J. Dairy Sci.* 87:1864–1871.
- Weller, R. A., and A. F. Pilgrim. 1974. Passage of protozoa and volatile fatty acids from the rumen of a sheep and from a continuous in vitro fermentation system. *Br. J. Nutr.* 32:341–351.
- Whitehouse, N. L., V. M. Olson, C. G. Schwab, W. R. Chesbro, K. D. Cunningham, and K. D. Lycos. 1994. Improved techniques for dissociating particle-associated mixed ruminal microorganisms from ruminal digesta solids. *J. Anim. Sci.* 72:1335–1343.