Culture pH interacts with corn oil concentration to affect biohydrogenation of unsaturated fatty acids and disappearance of neutral detergent fiber in batch culture

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ABSTRACT

Effects of culture pH and corn oil (CO) concentration on biohydrogenation (BH) of unsaturated fatty acids and disappearance of neutral detergent fiber (NDF) in batch culture were evaluated in a 2×3 factorial design experiment. Culture vessels (100 mL; 4 replicates/treatment per time point) included ground alfalfa hay plus CO at 0, 1, or 2% dry matter inclusion rate and were incubated at pH 5.8 (low pH) or 6.2 (high pH) for 0, 6, 12, 18, or 24 h. Effects of culture pH, CO, time, and their interactions were determined. Adding CO increased total fatty acid concentration in substrates to 1.01, 2.31, and 3.58% dry matter for 0, 1, and 2% CO, respectively. Corn oil concentration interacted with culture pH and resulted in different effects on BH of *cis*-9,*cis*-12 18:2 at low or high culture pH. After 24 h of incubation, low pH, compared with high pH, reduced disappearance of NDF by 35% and BH extent of *cis*-9,*cis*-12 18:2 by 31%. Increasing CO increased disappearance of NDF across pH treatments and decreased BH extent of *cis*-9,*cis*-12 18:2 at low pH and increased it at high pH over 24 h. Compared with high pH, low pH reduced concentrations of 18:0 by 31% and increased concentrations of *trans*-10,*cis*-12 18:2 and *trans*-10 18:1 by 110 and 79% after 24 h, respectively. Adding CO at low pH had greater effect on BH intermediates of *cis*-9,*cis*-12 18:2 compared with adding oil at high pH. In particular, increasing CO to 1 and 2% DM at low pH, compared with at high pH, resulted in a 36 and 46% reduction in the concentration of 18:0, an 84 and 131% increase in the concentration of *trans*-10,*cis*-12 18:2, and an 81 and 129% increase in the concentration of *trans*-10 18:1, respectively. Despite the interactions between culture pH and CO concentration, main effects across time were also significant for the response variables of interest. In conclusion, culture

pH interacted with CO concentration to affect BH of UFA and disappearance of NDF in batch culture, as the effects were greater at low culture pH than at high culture pH.

Key words: batch culture, biohydrogenation, fatty acid

INTRODUCTION

Milk fat yield is one of the major determinants of milk income. Compared with other milk components, fat is the most sensitive to changes in diets and environment. Diet-induced milk fat depression (MFD) is defined as a reduction of up to 50% in milk fat yield with no change in the yield of milk and other milk components (Bauman et al., 2011). Rumen bacteria biohydrogenate UFA in the rumen and produce various fatty acid (FA) intermediates (Shingfield and Wallace, 2014). In most situations, diet-induced MFD is caused by the inhibition of milk fat synthesis in the mammary gland by specific rumen PUFA biohydrogenation (BH) intermediates (Bauman et al., 2011). Among known BH intermediates, *trans*-10,*cis*-12 18:2 has been well characterized as a potent inhibitor of milk fat synthesis (Bauman et al., 2011) and *trans*-10 18:1 in milk is correlated with reductions in milk fat concentration (Bauman et al., 2011).

Linoleic acid (*cis*-9,*cis*-12 18:2) is an abundant dietary FA for dairy cows. Rumen bacteria biohydrogenate *cis*-9,*cis*-12 18:2 with *cis*-9,*trans*-11 18:2 and *trans*-11 18:1 as major intermediates. However, other intermediates are also produced during BH of *cis*-9,*cis*-12 18:2 (Honkanen et al., 2012). Changes in diet and the rumen environment can cause more *cis*-9,*cis*-12 18:2 to be converted to *trans*-10,*cis*-12 18:2 and *trans*-10 18:1 (Bauman et al., 2011). Furthermore, rumen pH and milk fat percentage are highly positively correlated (Allen, 1997), potentially due to differential inhibition of different species of rumen bacteria (Russell and Dombrowski, 1980). Several in vitro studies have tested the effects of pH on BH of UFA and have shown that

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low pH inhibited BH and increased formation of *trans*-10,*cis*-12 18:2 (Troegeler-Meynadier et al., 2003, 2014; Fuentes et al., 2011). Previous in vitro incubation studies have found that increasing *cis*-9,*cis*-12 18:2 inhibits BH of *cis*-9,*cis*-12 18:2 to 18:0 (Troegeler-Meynadier et al., 2003; Honkanen et al., 2012), potentially through an inhibition of *Butyrivibrio fibrisolvens* growth (Kim et al., 2000).

Most published studies, however, do not take into account the interactions between dietary and ruminal factors. Typically, dietary changes will affect the rumen environment, and the combinations of those alterations may increase the risk of MFD. Van Nevel and Demeyer (1996) determined the influence of pH and soybean oil level on triglyceride hydrolysis and BH in vitro and found that compared with BH, hydrolysis was more sensitive to low pH, especially with increased inclusion of soybean oil. However, no nutritive substrate other than soybean oil was included in the cultures, and no specific FA intermediates of BH were reported. The presence of a fibrous substrate is essential to better represent the complex metabolism present in the rumen, and quantification of BH intermediates is necessary to discern possible effects of dietary and rumen factors on milk fat synthesis. Therefore, additional research is necessary to assess the effect of rumen pH and dietary UFA on the production of BH intermediates associated with MFD. The objective of our study was to determine the interactions of pH and corn oil (CO), as a source of *cis*-9,*cis*-12 18:2, at common dietary concentrations on BH and NDF disappearance using an in vitro batch culture method. We hypothesized that rumen pH and CO concentration would interact to affect BH of *cis*-9,*cis*-12 18:2 and disappearance of NDF in batch cultures. Furthermore, we hypothesized that increasing *cis*-9,*cis*-12 18:2 at a low pH would cause greater inhibition of BH compared with a high-pH environment and increase formation of *trans*-10,*cis*-12 18:2 and *trans*-10 18:1.

MATERIALS AND METHODS

Treatment and Incubation

Our experiment used a 2 × 3 factorial arrangement of treatments: 2 culture pH levels (low pH = 5.8; high pH = 6.2) and 3 CO concentrations (0, 1, or 2% DM inclusion rate). All cultures were run in quadruplicate, with 2 analyzed for NDF residue and 2 for FA concentrations. All cultures contained alfalfa hay (41.0% NDF and 1.01% FA, DM basis) as the base substrate, which was dried at 55°C for 48 h and ground through a 1-mm screen using a Wiley mill (Arthur H. Thomas, Philadelphia, PA). The 0% CO treatment was alfalfa

Table 1. Fatty acid (FA) profile and total FA content of alfalfa hay containing corn oil (CO) used in the in vitro batch cultures

Item	CO concentration		
	0%	1%	2%
FA, g/100 g of total FA			
16:0	31.0	19.9	16.7
18:0	5.27	3.30	2.76
<i>cis</i> -9 18:1	4.37	17.9	21.8
<i>cis</i> -9, <i>cis</i> -12 18:2	18.4	39.0	44.8
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 18:3	19.7	9.41	6.49
22:0	2.20	1.03	0.69
24:0	2.03	1.00	0.68
Σ Unknown	5.45	2.48	1.66
Σ Others	11.49	6.00	4.43
Total FA, % of DM	1.01	2.31	3.58

hay containing no CO. Rather than adding oil directly to cultures, alfalfa hay containing 2% CO on a DM basis was prepared by spraying CO (Mazola, ACH Food Companies Inc., Memphis TN) in ethanol onto the corresponding weight of alfalfa hay. The alfalfa hay plus CO was mixed well during spraying, dried at 55°C to evaporate the ethanol, and ground through a 1-mm screen using a Wiley mill (Arthur H. Thomas) for consistency. Alfalfa hay containing 1% CO was prepared by combining alfalfa hay with prepared alfalfa hay containing 2% CO in 1:1 ratio. The FA content and profile were analyzed for alfalfa hay containing CO (0, 1, or 2%) after grinding, and the results are shown in Table 1.

Three rumen-fistulated mid-lactation Holstein dairy cows (185 ± 9 DIM) fed a common TMR (27% corn silage, 14% alfalfa silage, 20% ground corn, 17% soybean meal, 7% high moisture corn, 7% cottonseed, 2% soy hulls, 3% wheat straw, 3% minerals and vitamins) were used as rumen inoculum donors for this study. Rumen fluid and solid digesta from 3 cows were collected 1 h after feeding, pooled and transported in a prewarmed Thermos container, and blended in a 1-gallon Waring blender at low speed for 25 s to detach the bacteria from the feed particles. The blended mixture was then passed through a Buchner funnel lined with nylon mesh and glass wool to trap large particles, which were discarded. The strained rumen fluid was transferred to a 500-mL Brinkman pipette bottle for inoculating individual culture tubes. The rumen fluid was continuously flushed with CO₂ during all procedures.

All cultures were prepared as described by Goering and Van Soest (1970). Sayre and Van Soest (1972) previously reported that using large glass tubes (200 × 25 mm) as incubation vessels yielded similar NDF disappearance results compared with 125-mL Erlenmeyer flask vessels, which are more typical for in vitro artificial rumen procedures. In the present study, we

used Pyrex centrifuge tubes (100 mL) as incubation vessels. Volumes of 1 M citric acid (~0.14 and 0.27 g/culture) were added to the medium solution to adjust pH to 5.8 or 6.2, as described by Grant and Mertens (1992a). Each vessel contained 500 mg of alfalfa hay with 0, 1, or 2% CO, 40 mL of medium solution (pH 5.8 or 6.2), 2 mL of reducing solution, and 10 mL of rumen fluid. All vessels were swirled gently by hand to ensure that substrate and liquid were well mixed after addition of medium solution, reducing solution, and rumen fluid. The estimated time between rumen fluid collection to completion of inoculations was 40 min. The starting pH of low-pH and high-pH cultures was 5.78 ± 0.01 and 6.17 ± 0.02 , respectively. Weight of alfalfa hay and oil mixtures in each culture was recorded for calculations of BH extent of *cis*-9,*cis*-12 18:2 and disappearance of NDF. Culture tubes were flushed with CO₂ and sealed with 5.5-cm rubber stoppers connected to a Bunsen valve to release excessive gas during incubation. All cultures were incubated in the same water bath with no agitation at 39°C and were swirled gently by hand at 6 h of incubation.

Incubation of cultures was stopped at 0, 6, 12, 18, and 24 h. Immediately after rumen fluid inoculation, culture tubes taken at 0 h were placed in an ice bath and dry ice was added to each vessel to stop enzyme activity. At 6, 12, 18, and 24 h, incubations were also terminated as described above. The pH of all culture tubes was tested immediately before incubations were terminated. Culture tubes for NDF residue analysis were placed in a 4°C cooler until analysis (completed within 48 h). Culture tubes for FA composition analysis were stored at -20°C and subsequently freeze-dried.

Chemical Analyses

The NDF residue was analyzed as described by Mertens (2002) to determine disappearance of NDF as described in the subsequent section. To ensure an accurate analysis of FA content, samples were directly freeze-dried and methylated in the culture tubes using a 2-step methylation protocol adapted from Jenkins (2010). Internal standard (17:0, 1:1 mg/mL toluene) was added to cultures after the incubation was terminated and before storage at -20°C. To determine the dry culture content weight, all tubes were preweighed and reweighed after being freeze-dried. Freeze-dried samples were mixed with 8 mL of 0.5 M sodium methoxide solution in methanol and incubated for 10 min in a 50°C water bath. After tubes cooled, 12 mL of 5% methanolic hydrochloric acid solution was added, followed by 10 min of incubation in an 80°C water bath. After tubes were removed from the water bath and cooled, 10 mL of hexane and 30 mL of 6% K₂CO₃

solution was added and the solution was mixed by vortexing. Tubes were centrifuged for 10 min at $6,000 \times g$ at room temperature, and the hexane layer containing FAME was transferred to a 15-mL centrifuge tube containing 2 g of sodium sulfate. The FAME were extracted again by adding 5 mL of hexane to the tubes and repeating the mixing, centrifuging, and transferring steps described above. The 15-mL centrifuge tubes were inverted, and the samples were allowed to settle for 10 min. The solution was filtered through silica gel and charcoal to remove the sodium sulfate, and the solvent was evaporated with nitrogen gas at 37°C. The FAME samples were weighed, and a 1% solution was prepared with hexane based on weight. The 1% FAME solution was transferred to 2-mL GLC vials for analysis.

Fatty acid composition was determined using a GC-2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan) with a split injector (1:100 split ratio) and a flame ionization detector (FID) using a CP-Sill 88 wall-coated open tubular fused-silica column (100 m $\times$ 0.25-mm i.d. $\times$ 0.2- μ m film thickness; Varian Inc., Lake Forest, CA). The carrier gas was hydrogen at a flow rate of 1 mL/min. Hydrogen, purified air, and nitrogen makeup gas were used as the FID gases at flow rates of 40, 400, and 30 mL/min, respectively. Injector and detector temperatures were 270°C. The oven program was as follows: initial temperature held for 0.5 min at 40°C, programmed to increase to 155°C at 25°C/min and held for 30 min, and then increased to 215°C at 4°C/min and held for 35 min. Injection volume was 1 μ L. The FID response was the basis for integration and quantification (GCsolution software version 2.32.00; Shimadzu). Known FAME standards (GLC reference standard 463, GLC reference standard 481-B, and conjugated octadecadienoic mixture no. UC-59-M from Nu-Chek Prep Inc., Elysian, MN; Supelco 37 component FAME mix, *cis*/*trans* FAME mix, bacterial acid methyl ester mix, and PUFA No. 2 mix from Supelco Inc., Bellefonte, PA) were used for determination of individual FAME by comparing retention times. Quantification of FA covered approximately 45 individual FA in the range of 12:0 to 24:0.

Calculations and Statistical Analysis

Disappearance of NDF at each time point was calculated by subtracting the weight of NDF remaining after 6, 12, 18, and 24 h of incubation from the weight at 0 h and dividing by the weight at 0 h. This provided the percentage of NDF disappearance at each time point. Extent of BH was calculated by subtracting the weight of *cis*-9,*cis*-12 18:2 in the cultures at 6, 12, 18, and 24 h from the weight at 0 h and dividing by the weight at 0 h. Similarly, this provided the percentage of *cis*-9,*cis*-12

18:2 disappearance at each time point compared with 0 h.

All data were analyzed using the fit model procedure of JMP (version 10, SAS Institute Inc., Cary, NC). The pH, disappearance of NDF, extent of BH, total FA content, and FA concentrations of in vitro batch cultures over 24 h of incubation were analyzed using a model that contained the main effects of culture pH, CO concentration, time, and the interactions of culture pH $\times$ time, CO $\times$ time, culture pH $\times$ CO, and culture pH $\times$ CO $\times$ time. To test the treatment effect after 24 h of incubation on these variables, results obtained at 24 h were analyzed separately using the same model but without the interactions of culture pH $\times$ time, CO $\times$ time, and culture pH $\times$ CO $\times$ time. Least squares means with standard error are reported. Significance was declared at $P < 0.05$ and tendencies were declared at $P < 0.10$ for main and interaction effects.

RESULTS

FA Profile and Total FA Content of Substrates

Total FA content and FA profile of alfalfa with different CO concentrations are shown in Table 1. Adding 1 and 2% of DM CO into alfalfa hay increased total FA by 1.30 and 2.57% of DM, respectively. As expected, increasing CO increased concentrations of *cis*-9 18:1 and *cis*-9,*cis*-12 18:2 and decreased concentrations of 16:0, 18:0, and *cis*-9,*cis*-12,*cis*-15 18:3.

pH, Disappearance of NDF, and BH Extent of In Vitro Cultures

Overall effects of treatments on pH, disappearance of NDF, BH extent of *cis*-9,*cis*-12 18:2, and total FA content over 24 h of incubation are shown in Table 2; effects of treatments on those variables after 24 h of incubation are shown in Supplemental Table S1 (<https://doi.org/10.3168/jds.2019-16581>). Increasing CO increased total FA content of cultures at both low and high pH ($P < 0.001$) over 24 h of incubation. Although we observed interactions of pH $\times$ CO, pH $\times$ time, and CO $\times$ time on total FA content ($P < 0.05$), the changes were less than 0.25 mg/culture at the different CO inclusions (Table 2). Over 24 h of incubation, culture pH and CO interacted with time to affect pH, disappearance of NDF, and BH extent of *cis*-9,*cis*-12 18:2 (Figures 1, 2, and 3). Overall, the pH of all cultures fluctuated during incubation, and average pH difference between low-pH and high-pH cultures remained over 0.2 unit over time (Table 2; 5.94 vs. 6.19, $P < 0.001$), and after 24 h of incubation the average pH of low-pH and high-pH cultures was 6.06 and 6.21,

respectively ($P < 0.001$). Corn oil concentration and time also interacted to affect culture pH ($P < 0.001$), but the effects were minor (maximum changes in pH were 0.05) and CO had no effect on culture pH after 24 h of incubation (Supplemental Table S1, <https://doi.org/10.3168/jds.2019-16581>). Disappearance of NDF increased throughout incubations (Figure 2) and was higher in high-pH compared with low-pH cultures over time ($P < 0.001$, Table 2). After 24 h of incubation, low pH decreased NDF disappearance compared with high pH (22.7 vs. 34.9%, $P < 0.001$). Increasing CO increased NDF disappearance (25.4, 29.0, and 32.1% for 0, 1, and 2% CO respectively; $P < 0.001$) after 24 h of incubation. Over 24 h of incubation, we observed interactions between pH and CO ($P < 0.001$) on BH extent. At low pH, adding CO decreased BH extent compared with 0% CO ($P < 0.001$); at high pH, adding CO increased BH extent compared with 0% CO ($P < 0.001$). There was no difference between 1 and 2% CO. Similar to disappearance of NDF, BH extent increased throughout incubations (Figure 3) and was higher in high-pH cultures than in low-pH cultures over time ($P < 0.001$, Table 2). After 24 h of incubation, low pH decreased BH extent by 31% ($P < 0.001$) compared with high pH.

Effects of Culture pH and CO on FA Profile of Cultures over 24 h of Incubation

Results followed a similar pattern for FA if reported as grams per 100 grams of FA or milligrams per culture. Therefore, we only report the results for FA profile and focus on individual FA associated with BH of *cis*-9,*cis*-12 18:2. Due to the dynamic changes in FA profile during incubation, we mainly focus on the overall effects on FA over 24 h (Table 3) and temporal changes during 24 h of incubation (Figures 4, 5, and 6). Supplemental Table S2 (<https://doi.org/10.3168/jds.2019-16581>) shows FA profiles after 24 h of incubation.

Culture pH interacted with CO to alter the FA profile of cultures over 24 h of incubation (Table 3). Compared with 0%, CO at 1 and 2% of DM increased *cis*-9,*cis*-12 18:2 by 141 and 217% at low pH ($P < 0.001$), respectively; at high pH, 1 and 2% CO increased *cis*-9,*cis*-12 18:2 by 97 and 164% ($P < 0.001$), respectively. Compared with 0%, CO at 1 and 2% decreased 18:0 by 32 and 48% at low pH ($P < 0.001$), respectively; at high pH, 1 and 2% CO decreased 18:0 by 17 and 33% ($P < 0.001$), respectively. Compared with 0%, 1 and 2% CO increased *trans*-11 18:1 by 40 and 47% at low pH ($P < 0.001$), respectively, and by 102 and 161% at high pH ($P < 0.001$), respectively.

Overall, compared with high pH, low pH increased concentrations of *cis*-9,*cis*-12 18:2 (18.4 vs. 13.8 g/100

Figure 1. The pH of cultures incubated with 0% (●), 1% (▲), and 2% (■) corn oil (CO) at low pH (dashed line) and high pH (solid line) during 24 h of incubation (SEM = 0.01; interaction between pH and time, $P < 0.001$; interaction between CO and time, $P < 0.001$).

g of FA, $P < 0.001$), *trans*-10:*cis*-12 18:2 (0.11 vs. 0.06 g/100 g of FA, $P < 0.001$), and *trans*-10 18:1 (1.04 vs. 0.73 g/100 g of FA, $P < 0.001$) and decreased concentrations of *trans*-11 18:1 (4.73 vs. 6.11 g/100 g of FA, $P = 0.001$), *cis*-9:*trans*-11 18:2 (0.23 vs. 0.26 g/100 g of FA, $P < 0.001$), 18:0 (20.1 vs. 24.9 g/100 g of

Figure 2. Disappearance of NDF in cultures incubated with 0% (●), 1% (▲), and 2% (■) corn oil (CO) at low pH (dashed line) and high pH (solid line) during 24 h of incubation (SEM = 0.69; interaction among pH, CO, and time, $P = 0.06$). The NDF disappearance was calculated by subtracting the weight of NDF residue in the cultures at 6, 12, 18, and 24 h from the weight at 0 h and dividing by the weight at 0 h.

Table 2. Effects of pH and corn oil (CO) concentration on culture pH, disappearance of NDF, biohydrogenation (BH) extent of *cis*-9:*cis*-12 18:2, and total fatty acid (FA) content over 24 h of incubation¹

Variable	Low pH						High pH						P-value			
	0% CO	1% CO	2% CO	0% CO	1% CO	2% CO	0% CO	1% CO	2% CO	pH	CO	pH × CO	pH × time	CO × time	pH × CO × time	
pH	5.95	5.93	5.93	6.20	6.18	6.19	<0.001	<0.001	<0.001	0.40	<0.001	<0.001	<0.001	<0.001	0.85	
NDF disappearance, ² %	9.39	12.4	13.0	19.0	21.3	24.5	<0.001	<0.001	<0.001	0.34	<0.001	<0.001	<0.001	0.04	0.06	
BH extent of <i>cis</i> -9: <i>cis</i> -12 18:2, ³ %	38.4	34.5	30.3	47.6	56.2	55.2	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	
Total FA, mg/culture	10.9	16.8	22.9	11.1	17.0	22.7	<0.001	0.26	<0.001	0.02	0.003	0.003	0.003	0.003	0.94	

¹Values are LSM of 10 replicates for all variables except for pH value, which is the LSM of 20 replicates.

²Disappearance of NDF at each time point was calculated by subtracting the weight of NDF remaining after incubation from the weight at 0 h and dividing by the weight at 0 h.

³The BH extent of *cis*-9:*cis*-12 18:2 was calculated by subtracting the weight of *cis*-9:*cis*-12 18:2 in the cultures at 6, 12, 18, and 24 h from the weight at 0 h and dividing by the weight at 0 h.

Figure 3. Biohydrogenation (BH) extent of *cis-9,cis-12* 18:2 in cultures incubated with 0% (●), 1% (▲), and 2% (■) corn oil (CO) at low pH (dashed line) and high pH (solid line) during 24 h of incubation (SEM = 1.03; interaction among pH, CO, and time, $P < 0.001$). The BH extent was calculated by subtracting the weight of *cis-9,cis-12* 18:2 in the cultures at 6, 12, 18, and 24 h from the weight at 0 h and dividing by the weight at 0 h.

FA, $P < 0.001$), and total odd- and branched-chain FA (OBCFA; 3.78 vs. 4.43 g/100 g of FA, $P < 0.001$). Increasing CO increased concentrations of *cis-9,cis-12* 18:2 (7.88, 17.4, and 23.0 g/100 g of FA, $P < 0.001$), *cis-9,trans-11* 18:2 (0.13, 0.27, and 0.33 g/100 g of FA, $P < 0.001$), *trans-10,cis-12* 18:2 (0.04, 0.09, and 0.13 g/100 g of FA, $P < 0.001$), *trans-11* 18:1 (3.46, 5.85, and 6.95 g/100 g of FA, $P < 0.001$), *trans-10* 18:1 (0.72, 0.92, and 1.03 g/100 g of FA, $P < 0.001$), and OBCFA (5.80, 3.76, and 2.75 g/100 g of FA, $P < 0.001$) and decreased 18:0 (28.7, 21.7, and 17.1 g/100 g of FA, $P < 0.001$) for 0, 1, and 2% CO, respectively.

Changes in FA Associated with BH of *cis-9,cis-12* 18:2 in Cultures During 24 h of Incubation

The effects of culture pH and CO interacted with time to affect FA associated with BH of *cis-9,cis-12* 18:2 ($P < 0.001$), and their changes over time are shown in Figures 4, 5, and 6. *Cis-9,cis-12* 18:2 decreased during incubation in both low- and high-pH cultures but decreased faster at high pH than at low pH, which resulted in 58% lower concentration than low pH after 24 h of incubation. Furthermore, 18:0 increased during incubation in high-pH cultures ($P < 0.001$), and it had minor changes in low-pH cultures only after 18 and 24 h of incubation ($P = 0.003$). *Cis-9,trans-11* 18:2 increased during the first 12 h of incubation and decreased during the second 12 h of incubation in cultures with CO at high pH (Figure 5A, $P < 0.05$). *Cis-9,trans-11* 18:2 in-

creased throughout 24 h of incubation in cultures with CO at low pH ($P < 0.01$) except 18 to 24 h. Compared with 0% CO, addition of CO increased *cis-9,trans-11* 18:2 during incubation at both low and high pH after 6 h of incubation. Generally, *trans-11* 18:1 increased in all cultures but increased at a higher rate in cultures containing CO than in those containing 0% CO from 0 to 24 h of incubation (Figure 5B). Furthermore, *trans-11* 18:1 increased at a higher rate in cultures at high pH than those at low pH from 0 to 24 h of incubation.

Trans-10,cis-12 18:2 increased in cultures with 1 or 2% CO at low pH during 24 h of incubation (Figure 6A). Furthermore, *trans-10,cis-12* 18:2 increased during the first 18 h of incubation and decreased during the last 6 h of incubation in cultures with 1 or 2% CO at high pH. Compared with 0% CO, cultures with CO increased *trans-10,cis-12* 18:2 during incubation. Generally, *trans-10* 18:1 increased in all cultures during incubation, except those containing no CO at high pH, which decreased *trans-10* 18:1 concentration (Figure 6B). Cultures at low pH, regardless of CO concentration, increased *trans-10* 18:1 content faster than cultures at high pH. Cultures containing 2% CO at high pH ended the incubation period with lower *trans-10* 18:1 concentration than cultures containing 1% CO at low pH.

Figure 7 shows the interaction between culture pH and CO on concentrations of *trans-10,cis-12* 18:2 and *trans-10* 18:1 after 24 h of incubation. Cultures containing 2% CO at high pH had similar *trans-10,cis-12* 18:2 compared with those containing 1% CO at low pH. Increasing CO linearly increased concentrations of *trans-10,cis-12* 18:2 and *trans-10* 18:1 at both low and high pH. However, rate of change of *trans-10,cis-12* 18:2 and *trans-10* 18:1 was greater at low pH (0.10 and 0.64, respectively) than at high pH (0.04 and 0.38, respectively) with increasing CO.

DISCUSSION

Dietary PUFA are toxic to rumen bacteria, possibly by influencing membrane integrity or inhibiting bacterial metabolism (Maia et al., 2007). Rumen bacteria biohydrogenate PUFA to SFA through several isomerization and hydrogenation steps, reducing the toxicity of UFA. Numerous FA intermediates are formed during these processes. *Cis-9,cis-12* 18:2 is the most abundant PUFA in most cow diets based on corn and soy. Among all intermediates, *cis-9,trans-11* 18:2 and *trans-11* 18:1 are the major intermediates produced during BH of *cis-9,cis-12* 18:2 (Bauman and Griinari, 2003). However, specific dietary and rumen environment changes may result in more *cis-9,cis-12* 18:2 being biohydrogenated through alternative pathways, producing *trans-*

Table 3. Effects of pH and corn oil (CO) concentration on fatty acid (FA) profile (g/100 g of total FA) of cultures over 24 h of incubation^{1,2}

FA, g/100 g	Low pH						High pH						P-value			
	0%			1%			0%			1%			SEM	pH	CO	pH × CO
	CO	1%	2%	CO	1%	2%	CO	1%	2%	CO	1%	2%				
14:0	1.81	1.19	0.89		2.10	1.39	0.99	0.01	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	0.13
16:0	25.0	20.2	17.7		24.9	19.9	17.6	0.08	0.03	0.03	0.48	0.005		<0.001	<0.001	0.59
18:0	27.4	18.6	14.3		30.0	24.8	20.0	0.19	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001
<i>trans</i> -4 18:1	0.03	0.02	0.02		0.03	0.04	0.04	<0.01	<0.001	<0.001	0.23	<0.001		<0.001	<0.001	0.08
<i>trans</i> -5 18:1	0.02	0.03	0.02		0.04	0.04	0.04	<0.01	<0.001	<0.001	0.40	0.11		0.01	0.05	
<i>trans</i> -6 + <i>trans</i> -7 + <i>trans</i> -8 18:1	0.34	0.33	0.29		0.30	0.39	0.45	<0.01	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001
<i>trans</i> -9 18:1	0.28	0.45	0.48		0.24	0.45	0.55	<0.01	0.02	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001
<i>trans</i>-10 18:1	0.85	1.09	1.18		0.58	0.75	0.87	0.01	<0.001	<0.001	0.03	<0.001		<0.001	<0.001	<0.001
<i>trans</i>-11 18:1	3.67	5.14	5.40		3.26	6.57	8.51	0.09	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001
<i>trans</i> -12 18:1	0.60	0.54	0.46		0.57	0.70	0.76	0.01	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001
<i>cis</i> -9 18:1	5.22	13.3	17.3		4.88	11.0	14.7	0.09	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001
<i>cis</i>-9, <i>trans</i>-11 18:2	0.14	0.26	0.29		0.12	0.28	0.37	0.01	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001
<i>cis</i>-9, <i>cis</i>-12 18:2	8.38	20.2	26.5		7.38	14.5	19.5	0.14	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001
<i>trans</i>-10, <i>cis</i>-12 18:2	0.06	0.12	0.16		0.03	0.07	0.10	0.00	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	0.002
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 18:3	5.47	3.64	2.88		4.43	3.04	2.46	0.07	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	0.61
24:0	0.56	0.39	0.35		0.52	0.48	0.40	0.03	0.23	0.23	0.11	<0.90		<0.001	0.17	0.65
Σ Others	12.4	8.94	7.25		13.2	9.88	8.20	0.03	<0.001	<0.001	0.002	<0.001		<0.001	<0.001	<0.001
Σ Unknown	7.76	5.57	4.49		7.48	5.66	4.57	0.07	0.53	0.07	0.02	0.001		<0.001	<0.001	0.84
SFA	59.0	43.3	35.6		62.0	49.9	41.5	0.19	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001
Total <i>trans</i> 18:1	5.82	7.60	7.85		5.01	8.94	11.21	0.10	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001
MUFA <i>cis</i>	9.26	16.5	20.1		8.89	14.5	17.9	0.09	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001
PUFA <i>cis</i>	14.0	24.0	29.5		12.0	17.7	22.0	0.18	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001
OBCFA ³	5.32	3.45	2.56		6.29	4.07	2.94	0.04	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	0.45

¹Values are LSM of 10 replicates for all variables.

²Fatty acids associated with major biohydrogenation pathways of *cis*-9, *cis*-11 18:2 are in bold.

³Odd- and branched-chain FA.

10, *cis*-12 18:2 and *trans*-10 18:1 as intermediates. It has been well documented that *trans*-10, *cis*-12 18:2 exhibits bioactive functions, including the inhibition of milk fat synthesis (Bauman et al., 2011). Similarly, *trans*-10 18:1 is negatively correlated with milk fat content and is commonly used as a marker for MFD (Bauman et al., 2011). Diet-induced MFD is often caused by the inter-

Figure 4. Concentrations of (A) *cis*-9, *cis*-12 18:2 and (B) 18:0 in cultures incubated with 0% (●), 1% (▲), and 2% (■) corn oil (CO) at low pH (dashed line) and high pH (solid line) during 24 h of incubation (SEM = 0.32 and 0.43, respectively; interaction among pH, CO, and time, both $P < 0.001$). FA = fatty acids.

Figure 5. Concentrations of (A) *cis*-9, *trans*-11 18:2 and (B) *trans*-11 18:1 in cultures incubated with 0% (●), 1% (▲), and 2% (■) corn oil (CO) at low pH (dashed line) and high pH (solid line) during 24 h of incubation (SEM = 0.01 and 0.20, respectively; interaction among pH, CO, and time, both $P < 0.001$). FA = fatty acids.

action of several risk factors rather than a single diet characteristic. Diet-induced MFD requires the presence of both dietary PUFA and changes in rumen microbial populations or rumen environment, including alteration of pH (Bauman and Griinari, 2003). Although lower rumen pH is not a prerequisite for diet-induced MFD (Harvatine and Allen, 2006a,b), a decrease in rumen pH is often observed during diet changes, and rumen microbes have high-sensitivity pH changes (Russell and

Figure 6. Concentrations of (A) *trans*-10,*cis*-12 18:2 and (B) *trans*-10 18:1 in cultures incubated with 0% (●), 1% (▲), and 2% (■) corn oil (CO) at low pH (dashed line) and high pH (solid line) during 24 h of incubation (SEM = 0.01 and 0.03, respectively; interaction among pH, CO, and time, both $P < 0.01$). FA = fatty acids.

Dombrowski, 1980). Therefore, our study used CO as a PUFA source and investigated the effect of the interaction between culture pH and CO concentration on disappearance of NDF and BH of *cis*-9,*cis*-12 18:2. By adding CO to alfalfa hay, we successfully increased concentrations of total FA and *cis*-9,*cis*-12 18:2. The FA profile of alfalfa hay in the current study is similar to

Figure 7. Interaction between pH and corn oil (CO) concentration and its effect on concentrations of (A) *trans*-10,*cis*-12 18:2 [low pH: $\text{trans-10,cis-12 18:2 (g/100 g of total FA)} = 0.10 \times \text{CO (\%)} - 0.07$, $R^2 = 1.00$; high pH: $\text{trans-10,cis-12 18:2 (g/100 g of total FA)} = 0.04 \times \text{CO (\%)} - 0.02$, $R^2 = 0.99$; $P = 0.04$] and (B) *trans*-10 18:1 [low pH: $\text{trans-10 18:1 (g/100 g of total FA)} = 0.64 \times \text{CO (\%)} + 0.44$, $R^2 = 0.96$; high pH: $\text{trans-10 18:1 (g/100 g of total FA)} = 0.38 \times \text{CO (\%)} + 0.20$, $R^2 = 1.00$; $P = 0.003$] in culture after 24 h of incubation. FA = fatty acids.

that reported by Ribeiro et al. (2005). We also noticed a considerable amount of FA with carbon length more than 18 (>C18) in alfalfa hay.

Several in vitro studies have tested the effects of pH on BH of PUFA across a wide range (pH = 5.5–6.78;

AbuGhazaleh et al., 2005; Fuentes et al., 2011; Troegeler-Meynadier et al., 2014). However, the range of tested pH levels was very wide and less suitable for evaluating the sensitivity of BH to typical variations in rumen pH. Low pH inhibits the growth of rumen bacteria and reduces rumen nutrient digestibility (Russell and Dombrowski, 1980; Russell and Wilson, 1996), and pH 5.8 is often chosen as a threshold for subacute acidosis (Beauchemin and Yang, 2005; Dohme et al., 2008; Mohammed et al., 2012). Therefore, we used a small pH range, 5.8 to 6.2, in our study. Although buffer solutions were added to cultures to avoid the influence of accumulated acid on pH (Grant and Mertens, 1992a), we still observed some fluctuations in pH during culture incubation, which was also reported in previous in vitro studies (Troegeler-Meynadier et al., 2003, 2014). Following the incubation period, our low-pH cultures had a pH of 6.06 ± 0.01 and our high-pH cultures had a pH of 6.21 ± 0.01 . Adding CO did not affect the pH of cultures in our study, and Honkanen et al. (2012) reported a similar result with no effect of *cis*-9,*cis*-12 18:2 on culture pH.

Previous in vitro studies used hydrochloric acid and sodium hydroxide solution to adjust pH of buffer solutions (Troegeler-Meynadier et al., 2003; Zened et al., 2011; Lascano et al., 2016). However, this method might introduce excess salt into incubation cultures and influence the growth of bacteria. Citric acid can be metabolized by rumen bacteria (Van Soest, 1994) and was used in our study to minimize negative effects on fermentation. The effectiveness of using citric acid and phosphoric acid to adjust pH of phosphate-bicarbonate buffer has previously been evaluated (Grant and Mertens, 1992b); compared with phosphoric acid, citric acid was better able to maintain pH in rumen cultures during 72 h of fermentation. Compared with phosphoric acid, using citric acid to adjust buffer pH had no negative effect on NDF digestion, and low pH decreased NDF digestion compared with high pH (Grant and Mertens, 1992b). In our study, approximately 0.14 and 0.27 g of citric acid/culture was added to flasks to achieve high- and low-pH cultures, respectively. There are no studies, that we are aware of, that tested the effect of citric acid on BH. However, because rumen microbes are able to use citric acid as an energy source and produce acetic acid (Van Soest, 1994), we cannot rule out the effects of culture pH level on NDF digestion and BH being partially attributable to addition of citric acid.

Disappearance of NDF was determined to measure viability of cultures and effects of treatments. Cultures with 0% CO exhibited similar NDF disappearance as reported in continuous culture trials (Ribeiro et al., 2005). Both culture pH and CO affected NDF disap-

pearance. In our study, compared with high pH, low pH decreased NDF disappearance by 35%. Low rumen pH is detrimental to cellulolytic bacteria (Russell and Wilson, 1996), and its relation to BH of UFA is discussed later. Grant and Mertens (1992b) reported that both rate and lag time of NDF digestion were affected negatively when pH dropped below 6.2. Although we did not measure the microbial biomass in the current study, OBCFA, a group of FA mainly synthesized by rumen microbes, have previously been used as a marker for estimation of rumen microbial mass (Vlaeminck et al., 2005). Low pH and the addition of CO decreased the concentration OBCFA, which suggests a reduction in microbial biomass. It is commonly recognized that UFA decreases NDF digestion. Hristov et al. (2005) found that 5% dietary linoleic acid-rich oil decreased total-tract NDF disappearance of beef cattle. A meta-analysis by Weld and Armentano (2017) showed that free oil supplementation decreased total-tract NDF digestibility but increased NDF digestibility in the rumen. Interestingly, we observed an increase in NDF disappearance with increasing CO concentration. This may be partially explained by the fact that fiber-rich alfalfa hay was the sole substrate in our in vitro culture. Bateman and Jenkins (1998) reported that up to 8% of soybean oil could be added to a high-fiber diet without depressing NDF disappearance. The maximum CO addition in our experiment was 2% of DM, which is lower than in previous in vivo studies (Hristov et al., 2005; Weld and Armentano, 2017). Changes in rumen bacteria populations may be another reason for improved NDF disappearance in cultures with added CO. Although UFA are toxic to cellulolytic bacteria in pure culture (Maia et al., 2007), Hackmann and Firkins (2015) reported that *Butyrivibrios* might be able to synthesize long-chain FA de novo to compensate for the disruption of membrane integrity caused by the addition of *cis*-9,*cis*-12 18:2, which could also explain the interaction between culture pH, CO, and time on total FA content. Furthermore, Ivan et al. (2013) observed an increase in total cellulolytic bacteria in rumen fluid with 6% dietary linoleic acid-enriched oil, and Qiu et al. (2004) reported increases in cellulolytic bacteria when adding 1% linoleic acid in continuous culture. The effect of oil addition on rumen NDF digestion is debatable, and further research is needed to determine the effect of level of oil addition on NDF digestion and its interaction with diet.

We determined BH extent of *cis*-9,*cis*-12 18:2 via the disappearance of *cis*-9,*cis*-12 18:2 during the incubations. Several previous in vitro studies have reported higher BH extent of *cis*-9,*cis*-12 18:2 than our study during short-term incubation (<8 h; Troegeler-Meynadier et al., 2003, 2006; Zened et al., 2011; Honkanen et

al., 2012). The difference may be due to several factors, including (1) higher culture pH (Troegeler-Meynadier et al., 2003, 2006; Honkanen et al., 2012), which increased BH of *cis*-9,*cis*-12 18:2 (Troegeler-Meynadier et al., 2006), and (2) addition of free *cis*-9,*cis*-12 18:2 instead of triglyceride (Zened et al., 2011; Honkanen et al., 2012), which avoided the inhibition of low culture pH on hydrolysis of triglyceride (Van Nevel and Demeyer, 1996). Even though we observed lower BH extents during the initial 12 h of incubation, BH extents after 24 h of incubation were comparable with previous batch culture studies (Troegeler-Meynadier et al., 2003, 2006; Zened et al., 2011). In a previous continuous culture trial with fluid and solid retention times of 10 and 18 h, respectively, Ribeiro et al. (2005) reported 47% BH extent of *cis*-9,*cis*-12 18:2 in alfalfa hay, which was between 45 and 56% at 12 and 18 h of incubation in our study. An in vivo study reported over 80% BH extent of *cis*-9,*cis*-12 18:2 with an approximately 40-h retention time in the rumen, which might have led to the higher BH extent compared with our study (Harvatine and Allen, 2006c).

Dietary PUFA and alteration of rumen pH are often requirements for diet-induced MFD (Bauman and Griinari, 2003; Bauman et al., 2011), and we observed interactions between these factors in our study. Increasing CO increased BH of *cis*-9,*cis*-12 18:2 in cultures with high pH. This suggests that bacteria might be able to increase BH capacity to compensate for increasing PUFA, or a minor increase in dietary PUFA might stimulate or have no negative influence on bacterial growth. Ivan et al. (2013) reported that 6% dietary linoleic acid-enriched oil increased total cellulolytic bacteria in rumen fluid. Martin and Jenkins (2002) observed greater effects of culture pH on BH of UFA compared with other factors tested, including dilution rate and soluble carbohydrate concentration. Qiu et al. (2004) also reported that adding 1% of *cis*-9,*cis*-12 18:2 at culture pH 6.5 increased BH of UFA by 33%, whereas lower pH (5.8 vs. 6.5) decreased BH of UFA by 51%. Previous studies have reported that low pH inhibits BH of *cis*-9,*cis*-12 18:2, decreases *cis*-9,*trans*-11 18:2, and increases formation of *trans*-10,*cis*-12 18:2 after no more than 8 h of incubation (Fuentes et al., 2011; Troegeler-Meynadier et al., 2014). We also observed inhibition on BH of *cis*-9,*cis*-12 18:2 at low pH and accumulation of both *trans*-10,*cis*-12 18:2 and *cis*-9,*trans*-11 18:2 after 24 h of incubation. It is worth noting that the incubation times were only 6 h in previous studies (Van Nevel and Demeyer, 1996; Troegeler-Meynadier et al., 2014). In our 24-h incubation, *cis*-9,*trans*-11 18:2 increased during the first 12 h and decreased during the second 12 h in high-pH cultures containing CO but continued to increase in low-pH cultures containing CO across 24 h.

Despite fluctuations, the proportion of *trans*-10,*cis*-12 18:2 to *cis*-9,*trans*-11 18:2 after 24 h of incubation was higher in low-pH cultures than in high-pH cultures (56 and 35%, respectively). Troegeler-Meynadier et al. (2003) also observed that low pH increased the ratio of *trans*-10,*cis*-12 18:2 to *cis*-9,*trans*-11 18:2. Generally, cellulolytic bacteria are the major group of bacteria producing *cis*-9,*trans*-11 18:2 and *trans*-11 18:1 during BH of *cis*-9,*cis*-12 18:2 (Polan et al., 1964). Cellulolytic bacteria are sensitive to rumen pH; when pH drops below 5.7, cell yields of *Ruminococcus albus*, *Bacteroides succinogenes*, *Ruminococcus flavefaciens*, and *Butyrivibrio fibrisolvens* decrease in pure cultures (Russell and Dombrowski, 1980). However, *Megasphaera elsdenii*, one of the reported producers of *trans*-10,*cis*-12 18:2 in vitro (Kim et al., 2002), is more tolerant to low pH and still grows in cultures until pH reaches 4.9 (Russell and Dombrowski, 1980). Therefore, it is more competitive than cellulolytic bacteria at low pH. Overall, regardless of CO level, differences in culture pH appear to be the main driver of changes in BH extent.

Both in vitro and in vivo studies have shown that shift in *trans*-11 to *trans*-10 BH pathways or MFD can happen with increasing dietary UFA without changes in rumen pH (Harvatine and Allen, 2006a,b; Zened et al., 2013; Ventto et al., 2017). Results of our study are consistent with this observation because we observed that increasing CO resulted in the accumulation of *trans*-10,*cis*-12 18:2 and *trans*-10 18:1 in both high- and low-pH cultures. However, the accumulation of *trans*-10 FA intermediates was greater in cultures with low pH compared with high pH. Previous studies found that *M. elsdenii* is low-pH tolerant and insensitive to PUFA (Russell and Dombrowski, 1980; Maia et al., 2007). This bacterium may be able to isomerize *cis*-9,*cis*-12 18:2 to *trans*-10,*cis*-12 18:2 in culture, whereas growth and metabolism of other bacteria are inhibited by low pH and high PUFA. At high pH, CO increased isomerization of *cis*-9,*cis*-12 18:2 but decreased hydrogenation of *trans* 18:1 to 18:0. This suggests that bacteria associated with the last step of BH might be more sensitive to PUFA than bacteria associated with other steps of BH. *Clostridium proteoclasticum* has a lower tolerance to *cis*-9,*cis*-12 18:2 than *Butyrivibrio*, which transfer *cis*-9,*cis*-12 18:2 to CLA isomers (Jenkins et al., 2008). Maia et al. (2007) observed similar results in a pure bacterial culture study, with butyrate-producing bacteria (e.g., *Butyrivibrio proteoclasticus*) being more sensitive to UFA than other bacteria. Additionally, compared with high pH, increasing CO in cultures with low pH resulted in greater decreases in *iso* 16:0, an OBCFA derived from rumen microbes (Supplemental Table S2, <https://doi.org/10.3168/jds.2019-16581>). Colman et al. (2010) suggested that compared with milk

fat content or *trans*-10 18:1, *iso* 16:0 content in milk is a better indicator of acidosis (Colman et al., 2010). Our study results suggest that dietary UFA level may also need to be considered if using milk *iso* 16:0 as an indicator of acidosis.

CONCLUSIONS

Low culture pH decreased both disappearance of NDF and the BH extent of *cis*-9,*cis*-12 18:2 and increased the accumulation of BH intermediates (*trans* 18:1 and CLA isomers). In particular, low pH resulted in a greater increase in both *trans*-10 18:1 and *trans*-10,*cis*-12 18:2 compared with high pH. Addition of CO increased disappearance of NDF and BH extent of *cis*-9,*cis*-12 18:2. Accumulation of both *trans*-10 18:1 and *trans*-10,*cis*-12 was greater with increasing CO at low pH compared with high pH. This implies a higher risk for MFD when diets contain high *cis*-9,*cis*-12 18:2 concentration, provided by vegetable oil, along with factors that may cause low rumen pH, and it is more important to maintain adequate rumen pH to reduce risk for MFD. Culture pH interacted with CO concentration to affect BH of UFA and disappearance of NDF in batch culture, as the effects were greater at low culture pH than at high culture pH.

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