The effect of the feed-to-buffer ratio on bacterial diversity and ruminal fermentation in single-flow continuous-culture fermenters

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ABSTRACT

Eight single-flow continuous-culture fermenters were used in a completely randomized block design with a 2×4 factorial arrangement of treatments to investigate the effects of the feed-to-buffer ratio (F/B) on ruminal fermentation, the diversity and community structure of bacteria, nutrient digestibility, and N metabolism. Four diets with forage-to-concentrate ratios of 70:30 or 30:70 with alfalfa or grass hay as forage were supplied to fermenters twice per day at 2 different F/B (23.5 and 35 g of DM/L). The dilution rate was kept constant (5.3%) among all fermenters by infusing the same volume of buffer. An increase in the total volatile fatty acid (VFA) concentration and a decrease in the average pH were observed with an increased F/B. In addition, the molar proportions of all individual VFA found in fermenters differed, depending on the F/B. A terminal restriction fragment length polymorphism analysis showed that the community structure and diversity of bacteria were highly influenced by the F/B. Both diversity and the number of peaks in the electropherograms were lower in most fermenters receiving diets at a high F/B, whereas the similarity percentage of the bacterial communities across diets was higher as the F/B increased. Moreover, the high reduction of neutral detergent fiber digestibility ($15.3\% \pm 3.65$) in fermenters with high F/B suggested a pH-related decrease in the cellulolytic bacterial community as the F/B increased. The crude protein degradation found in fermenters receiving diets with a high F/B was lower compared with that from fermenters with a low F/B. The VFA concentration and purine bases flow response patterns to diets were similar to in vivo conditions only in the case of fermenters with a low F/B. The results suggested that the community structure and diversity of bacteria, as well as the in vitro fermentation parameters, may be affected by the F/B that is used, most likely through a pH effect. In addition, several fermentation parameters

showed different response patterns to diets according to the F/B used. Therefore, the amount of feed supplied to single-flow continuous-culture fermenters in which pH is not under control should be carefully chosen according to the volume of buffer infused for the purpose of simulating ruminal fermentation.

Key words: continuous-culture fermenter, feed/buffer, ruminal fermentation, bacterial diversity

INTRODUCTION

Continuous-culture fermenters (CCF) have been widely used in nutritional research to study ruminal fermentation under carefully controlled conditions (Griswold et al., 2003; Castillejos et al., 2005; Molina-Alcaide et al., 2009). In particular, studies with CCF have recently shown that the average pH (Calsamiglia et al., 2002), the time during which pH values are sub-optimal (Cerrato-Sánchez et al., 2008), and the extent of pH variation (Wales et al., 2004) are important factors influencing ruminal fermentation. In addition, the pH in the fermenters has been thought to be responsible for shifts in the rumen bacterial population (Yang et al., 2002; Calsamiglia et al., 2008; Carro et al., 2009). These findings underline the need to maintain in vitro pH values across time that are as close as possible to those in the rumen of animals consuming similar diets to sustain similar compositions and structures of microbial community and, thus, to accurately simulate ruminal fermentation (Devant et al., 2001; Colombatto et al., 2003). However, in most of the studies carried out with CCF, the pH was set to be constant or within a narrow range (Dahlberg et al., 1988; Mansfield et al., 1995; Griswold et al., 2003). Fixed pH values may reduce shifts in microbial species as the diet composition changes and could explain some differences found between the rumen and fermenters in direct comparative studies (Hannah et al., 1986; Mansfield et al., 1995).

The results of some studies (Hoover et al., 1976; Crawford et al., 1980; Carro et al., 2009; Muetzel et al., 2009) seem to indicate an effect of the feed-to-buffer ratio (F/B; g of DM feed/L of buffer) on the pH reached in CCF, and although this ratio has been

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proposed as being responsible for pH changes (Carro et al., 2009), to our knowledge, no studies have analyzed the effect of different F/B. Because many discrepancies in fermentation between rumens and CCF have been clearly attributed to differences in pH values (Mansfield et al., 1995; Molina-Alcaide et al., 2009), the F/B should be carefully chosen in fermenters in which pH is not under control. However, important variations do exist among CCF studies, with F/B ranging from 10 g of DM/L (Muetzel et al., 2009) to more than 65 g of DM/L (Molina-Alcaide et al., 2009). This lack of uniformity could be due to differences in the diet quality, the saliva composition and the CCF model used, but even using similar conditions, the F/B varies among studies [Tapia et al., 2002 (F/B = 50.6); Carro et al., 2009 (F/B = 31.8)].

The objective of this study was to determine to what extent 2 different F/B (23.5 and 35 g of DM/L) would affect the bacterial diversity and structure together with the in vitro fermentation pattern and N metabolism. Additionally, the present study aimed to determine the ability of single-flow continuous-culture fermenters to discriminate between different dietary treatments similarly to goat rumen. Details of an in vivo study with nonlactating dairy goats receiving the same experimental diets used in the present study have been previously reported (Cantalapiedra-Hijar et al., 2009).

MATERIALS AND METHODS

Apparatus, Diets, and Animals

Four 12-d incubation runs were carried out using 8 single-flow CCF, following the model of Miettinen and Setälä (1989), with an effective volume of 1,000 mL. The experimental diets were grass hay-high forage (**GF**), composed of 70% of grass hay (**G**) and 30% of concentrate (**C**); grass hay-high concentrate (**GC**), composed of 30% of G and 70% of C; alfalfa hay-high forage (**AF**), composed of 70% of alfalfa hay (**A**) and 30% of C; and alfalfa hay-high concentrate (**AC**), composed of 30% of A and 70% of C on a DM basis. Each diet was tested at 2 different F/B levels, a low (23.5 g of DM/L) and a high (35 g of DM/L) level, resulting in 8 treatments allocated to each of the 8 fermenters used in every incubation run. The fermenters were inoculated with 700 mL of rumen fluid from goats previously adapted for 21 d to the same diets provided to the corresponding fermenter (Cantalapiedra-Hijar et al., 2009). Each of the 4 in vitro periods in this work was run concurrently with its respective in vivo period (Cantalapiedra-Hijar et al., 2009). The rumen contents from goats were collected 2 h after feeding, immediately transported to the laboratory in thermal bottles, and strained through 4

layers of cheesecloth. Rumen fluid from each of the 4 rumen-fistulated goats was inoculated within 30 min after collection into 2 fermenters, each one receiving the same diet that the inoculum donor received (Mansfield et al., 1995) at one of the 2 studied F/B. Because the 4 goats were adapted to diets according to a 4 × 4 Latin square design (Cantalapiedra-Hijar et al., 2009), none of the 4 single-flow CCF receiving the same treatment across the 4 incubation runs shared the same donor of rumen fluids. The hay was chopped (theoretical length of cut was 1 cm), and the concentrate was given as small pellets (12 × 4 mm). The management of the animals and the experimental procedures were conducted by trained personnel in strict accordance with Spanish guidelines (Act No. 1201/2005 of 10 October 2005) regarding the protection of experimental animals.

Experimental Procedure and Sampling

Treatments were arranged as a 2 × 4 factorial with F/B and diet as the main effects. Each combination of F/B × diet was used once in each of the 4 incubation runs. The diet was supplied daily in 2 equal portions at 0900 and 1500 h. The flow through all fermenters was maintained by continuous infusion of McDougall (1948) artificial saliva (approximately 53 mL/h) to achieve an outflow rate (5.3%) similar to that of other studies (Tapia et al., 2002; Carro et al., 2009; Molina-Alcaide et al., 2009), and CO₂ was continuously infused into the fermentation flask to maintain anaerobic conditions. Two different F/B were achieved by changing the feed input while keeping constant the volume of artificial saliva infused. Effluent from each fermenter was collected in a vessel maintained in a 3°C bath to prevent microbial growth.

The duration of each incubation run lasted 7 d for adaptation and 5 d for sampling. Every day, effluents were removed, and their weights and volumes were recorded. From d 8 through 11, effluents were collected and filtered through 4 layers of cheesecloth, and two 5-mL aliquots were taken and stored at -20°C for NH₃-N and VFA analyses. At the end of the trial, composite subsamples from 3 sampling days were made, and 1 aliquot was collected for the total N determination. The remainder was freeze-dried before analysis for DM, OM, NDF, ADF, ether extract (**EE**), and purine base (**PB**) contents.

Twice per day, the pH in the fermentation flask was measured immediately before feeding. On d 11, the pH of the content of the fermenters was measured at 0, 2, 4, 6, 8, 10, and 12 h after the morning feeding. In addition, 2 h after the morning feeding, a 2-mL aliquot was taken from the content of the fermenters for the subsequent analysis of the community structure and

diversity of the bacteria. The molecular analysis was limited to samples collected from the 2 first incubation runs ($n = 16$) because of the cost of the terminal RFLP (**T-RFLP**) analysis. On d 12, the total effluent was collected for bacterial pellet isolation following the procedure described by Martín-Orúe et al. (1998), with minor modifications. The effluent was squeezed through 4 layers of cheesecloth and a 50-mm nylon filter was used to trap particulate matter and let the liquid phase pass through (step 1). The solid residue obtained was resuspended in its weight of a 0.85% saline solution at 39°C. After shaking, the suspension was filtered again (step 2). Solid-associated bacteria were separated from the residue obtained in step 2 by diluting the residue in the 0.85% saline solution with 0.1% of methylcellulose at 39°C, shaking it for 30 min and filtering it (step 3). The solid residue obtained in step 3 was finally diluted in a 0.85% saline solution and kept at 4°C for 24 h before its homogenization with a Waring Blender (Waring, New Hartford, CT) and its filtration (step 4). Filtrates obtained in Steps 1, 2, 3, and 4 were pooled together and subjected to a differential centrifugation ($500 \times g$ for 5 min followed by 2 consecutive centrifugations of the supernatant fractions at $20,000 \times g$ for 20 min at 4°C). The bacterial pellets isolated (bacteria from effluent; **EB**) were freeze-dried and then ground to a fine powder with a mortar and pestle before their analysis for DM, OM, N, and PB contents.

Chemical Analyses

Dry matter (method ID 934.01), ash (method ID 942.05), N (method ID 984.13), and EE (method ID 7.045) contents were determined according to the AOAC (2005). The analyses of NDF and ADF were carried out according to Van Soest et al. (1991) using an Ankom²²⁰ fiber analyzer unit (Ankom Technology Corp., Macedon, NY). A heat-stable α -amylase was used for NDF analysis, and both NDF and ADF were expressed exclusive of residual ash. The ADL was determined by the solubilization of cellulose with 75% of sulfuric acid. The in vitro DM, OM, and CP digestibility of the diet ingredients was determined according to the procedure described by Tilley and Terry (1963), using an Ankom Daisy Incubator (Ankom Technology Corp.).

Aliquots of the strained effluents were thawed in the refrigerator overnight and centrifuged at 4°C and $20,000 \times g$ for 20 min, and the supernatant was analyzed for VFA and $\text{NH}_3\text{-N}$. Individual VFA were analyzed (Isac et al., 1994) by GC. The $\text{NH}_3\text{-N}$ concentration was determined by a colorimetric method (Weatherburn, 1967).

Purine bases (adenine and guanine) in effluents and bacterial pellets were determined following the method

described by Balcells et al. (1992) using HPLC, which consisted of a multisolvent delivery system (model 510; Waters, Millipore, Billerica, MA), an injector (WatersTM 717 plus Autosampler, Waters), a multiwavelength detector (model 481; Lambda-Max, LC spectrophotometer, Waters, set to 205 nm) and a double 4.0 mm $\times$ 250 mm C18 ODS-2 analytical column (Waters Spherisorb). The purine bases were quantified by peak integration using the Waters HPLC systems software Millennium32.

Terminal RFLP Analysis

In order to analyze the similarity between bacterial communities, samples of the fermenter contents were freeze-dried and kept at -80°C for 5 min before being beaten at maximum speed in a Minibeadbeater (Biospec Products Inc., Bartlesville, OK) for 1 min. The total DNA was then extracted using a Qiagen QIAmp DNA stool mini kit (Qiagen Ltd., West Sussex, UK), following the manufacturer's instructions but using a high temperature (95°C) for the step of lysis incubation. The PCR was used to amplify approximately 1,300 bp of the 16S rRNA gene before T-RFLP analysis. The amplification reactions contained 0.05 U/ μL Taq DNA polymerase (Promega Corporation, Madison, WI), $1 \times$ reaction buffer as supplied by the manufacturer, 1.5 mM MgCl_2 , 0.25 μmol of each primer, 0.2 mmol of each deoxyribonucleotide triphosphate (dNTP), and 1 μL of extracted DNA in a final volume of 25 μL . The bacterial-specific primer pairs used (Hongoh et al., 2005) were cyanine-labeled 27F (5'-AGA GTT TGA TCC TGG CTG AG-3') and unlabeled 1389R (5'-AGG GGG GGT GTG TAG AAG-3'). Reactions were conducted in a Biorad MyCycler thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA) with an initial denaturation at 94°C for 5 min, followed by 25 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension carried out at 72°C for 2 min. The PCR product was purified on a Millipore MultiScreen PCR _{μ 96} plate (with a 20-inch Hg vacuum). To standardize the digestion of DNA, the concentration of DNA in each sample was determined by spectrophotometry (Nanodrop ND-1000 spectrophotometer, NanoDrop Technologies, Rockland, DE) and then diluted to a concentration of 20 ng/ μL (Osborn et al., 2000). The restriction enzyme digestion was performed using *Hae*III, *Hha*I, *Msp*I, and *Rsa*I (Promega Corp., Madison, WI) in single-enzyme digestions at 0.25 U/ μL (*Hae*III) and 0.15 U/ μL (*Hha*I, *Msp*I, and *Rsa*I) for 5 h at 37°C . The digestion product was then purified and precipitated by washing once with 95% of ethanol at -80°C and twice with 70% ethanol at -20°C with successive centrifugations ($3,000 \times g$ for 15 min at 4°C) and decantations. The purified product

was dried at room temperature until the ethanol was completely removed, resuspended in 35 μ L of a sodium lauryl sulfate buffer solution (Beckman Coulter Inc., Fullerton, CA) and added to 0.2 μ L of DNA standard 600. Terminal restriction fragments (**TRF**) were separated by capillary gel electrophoresis according to their size. The TRF sizes were estimated by comparing their retention times with those of the DNA internal standard, using the local southern algorithm, and aligned by the amplified fragment length polymorphism facility in the CEQ 8000 software. To retrieve the maximum peaks number, data were exported with minimum values of 1% for slope threshold and 0% for relative peak height filtering values within the CEQ 8000 software. Because the differential retrieval of DNA concentration from sample washing and loading inaccuracies may occur, peak areas were converted to relative values, minimizing the correlation between the total peak area and the number of peaks (Sait et al., 2003). In order to remove most of the noisy peaks as well as uncertain peaks, such as those possibly produced by PCR or enzyme digestion, and to retain all major peaks, a percentage threshold (cut-off value) for a relative area of 0.5% was applied (Zhang et al., 2008). The analysis of TRF profiles generated from the 4 single-enzyme digestions was performed with Bray Curtis distances, and dendrograms were constructed with the Ward method (SAS Institute, Inc.) in the CAP 4 software (Pisces Conservation Ltd., Lymington, Hampshire, UK). A similarity percentage (SIMPER) was used to compare the homology in bacterial communities within samples of low- and high-F/B fermenters. A Shannon diversity index, which accounts for both the abundance and the evenness of the species present, was calculated on every sample (Shannon and Weaver, 1963).

Calculations and Statistical Analysis

True DM and OM digestibility was calculated by correcting the apparent digestibility values with microbial DM and OM content, respectively. The apparent DM digestibility in fermenters was calculated as described by Crawford et al. (1980) with corrections for buffer salts lost during freeze-drying. The true NDF and ADF digestibility as well as the apparent digestibility of OM and NFC were calculated from the input and output data. The NFC was calculated as $NFC = OM - (NDF + CP + EE)$. The microbial N flow from the fermenter was estimated by multiplying the total non-ammonia N in the effluent by the ratio of PB/N in the effluent and in EB pellets. The efficiency of microbial protein synthesis was calculated as grams of estimated microbial N flow per 1 kg of OM that is truly digested.

Data were analyzed according to the general linear model procedure of SPSS for Windows, version 15.0 (2007; SPSS Inc., Chicago, IL), with a 2×4 factorial arrangement of treatments in a completely randomized block design. The period (block) was designated as a random effect, whereas F/B, diet, and the interaction between F/B and diet were set as fixed effects. The pH values across time were analyzed, while taking the correlation among repeated measures from the same rumen fluid inoculum into account, and accordingly, a linear mixed model (Littell et al., 1998) was used. The linear mixed model included the effect of time after feeding as well as its interaction with diet and F/B. The time after feeding was considered the repeated factor, and each CCF within the diet was considered the subject. For each variable analyzed, data were subjected to 3 different covariance structures: variance components, compound symmetry, and unstructure. The covariance structure that yielded the largest Schwarz Bayesian was considered to be the most desirable for analysis (Littell et al., 1998). Significance was determined at $P < 0.05$. When significant differences were detected, differences among means were tested using the Tukey comparison test. The Shannon indices, as well as the average pH values, were tested for significant differences between the 2 main detected clusters using one-way ANOVA.

RESULTS AND DISCUSSION

The pH in the rumen depends on the amount of incoming saliva and the accumulation of organic acids produced, particularly VFA and lactic acid (Church, 1976). In CCF, the pH values rely on similar factors. However, the volume of buffer as artificial saliva entering a fermenter usually does not show a response to diet composition as would occur in vivo (Colombatto et al., 2003). The volume of buffer is normally chosen to promote a particular liquid dilution rate (Meng et al., 1999; Martínez et al., 2009). The F/B used in CCF could have a significant effect on pH values (Hoover et al., 1976; Muetzel et al., 2009). The F/B expresses the quotient of 2 of the main factors determining the concentration of the end products of fermentation for a given diet: the amount of fermentable OM supplied per unit of a fermenter's effective volume and the buffering capacity of the system due to the continuous infusion of artificial saliva. Because the aim of this work was to study the effect of the F/B on ruminal fermentation while keeping other operating variables constant, 2 different F/B were achieved by changing only the amount of feed supplied to the fermenters, avoiding differences in responses due to effects linked to the liquid dilution rate. The experimental diets (Table 1) used in

Table 1. Chemical composition (g/kg of DM) and in vitro digestibility (g/g) of the experimental diets

Item	Diet ¹			
	GF	GC	AF	AC
DM, g/kg of fresh matter	903	901	896	898
OM	925	913	897	901
CP	126	168	194	197
Ether extract	21.2	28.0	20.4	27.6
NDF	453	382	398	359
ADF	230	174	263	188
Acid detergent lignin	35.5	30.7	59.2	40.8
Gross energy, MJ/kg of DM	18.6	18.4	18.3	18.2
In vitro DM digestibility	0.65	0.73	0.70	0.74
In vitro OM digestibility	0.65	0.73	0.69	0.74
In vitro CP digestibility	0.72	0.78	0.80	0.81

¹Grass hay-high forage (GF) = 70% of grass hay and 30% of concentrate; grass hay-high concentrate (GC) = 30% of grass hay and 70% of concentrate; alfalfa hay-high forage (AF) = 70% of alfalfa hay and 30% of concentrate; alfalfa hay-high concentrate (AC) = 30% of alfalfa hay and 70% of concentrate (DM basis).

the present experiment were the same as those used in a previous experiment with goats (Cantalapiedra-Hijar et al., 2009), which allowed us to not only compare the fermentation parameters among fermenters receiving different diets at 2 F/B, but also to state the ability of fermenters with different F/B to show response patterns to diets that are similar to what occur in the rumen of goats. Therefore, the effect of diet will be specifically discussed concerning the ability of CCF to discriminate diets as is done in vivo, as well as regarding its possible interaction with the F/B used.

Because no pH control was conducted in the present experiment, the pH values (Figure 1) were the result of fermentable OM input and continuous infusion of artificial saliva. The pH values for each diet were similar across time, regardless the F/B used ($F/B \times \text{diet} \times \text{time} = 0.83$). An increase ($P < 0.001$) in the total VFA concentration and a decrease ($P < 0.001$) in the average pH (Table 2) were observed with increased F/B, in accordance with other studies using stratified (Muetzel et al., 2009), single-flow (Meng et al., 1999), and dual-flow (Hoover et al., 1976) CCF. Diet affected both the pH and VFA concentration ($P \leq 0.002$), but the $F/B \times \text{diet}$ interaction ($P \leq 0.03$) indicated that the response of pH and VFA concentration to diet varied according to the F/B used. The results from Meng et al. (1999) obtained in single-flow CCF also showed that the pH and VFA concentration values for the experimental diets were ranked differently according to the F/B used. However, the different dilution rates achieved by infusing different volumes of buffer into the fermenters can explain the differences found in that study among the different F/B. A higher VFA concentration in CCF compared with that in the rumen is usually explained by the lack of absorption in vitro (Hannah et al., 1986; Mansfield et al., 1995; Devant

et al., 2001), but it ultimately depends on the F/B used in fermenters, as shown in this study. The mean pH values obtained in this in vitro experiment (6.22 to 6.53) were within the range of those found in the rumen of goats (6.21 to 6.43) that were fed the same 4 diets (Cantalapiedra-Hijar et al., 2009), provided that fermenters were supplied diets at a low F/B (23.5 g of DM/L). In addition, pH values and the total daily VFA concentration showed a response pattern to diets similar to that observed in vivo (Cantalapiedra-Hijar et al., 2009), but this was only the case with fermenters with a low F/B. Therefore, the ability of fermenters to detect differences in VFA concentration among diets similar to those of the rumen seemed to depend on the F/B used. These findings support the hypothesis that F/B affects the incubation pH and suggest that some discrepancies found between rumen and CCF by direct in vivo-in vitro comparisons in some studies (Carro et al., 2009; Molina-Alcaide et al., 2009) could be minimized by adjusting the F/B in the fermenters to attain pH values more comparable with those in the rumen.

Pronounced differences ($P < 0.001$) were found in the molar proportions of all individual VFA found in fermenters with high and low F/B. Because individual VFA molar proportions depend on the pH reached in CCF (Calsamiglia et al., 2002; Yang et al., 2002), it is not surprising that the interaction between the main effects on pH values was also found on all individual VFA molar proportions, with the exception of isoacids. Although diets supplied to fermenters at low F/B promoted mean pH values close to those found in vivo (Cantalapiedra-Hijar et al., 2009), the acetate-to-propionate ratios (**C2/C3**) were not within the same range (2.85–3.94 vs. 3.47–4.45 for in vitro and in vivo, respectively). These results may indicate that although the pH values in the fermenter may play an important

Figure 1. Variation of pH values with time after feeding ($\uparrow$) in single-flow continuous-culture fermenters (CCF) receiving the experimental diets at 2 different feed-to-buffer ratios (F/B): low (23.5 g of DM/L) and high (35 g of DM/L); grass hay-high forage (GF) diet = 70% of grass hay and 30% of concentrate; grass hay-high concentrate (GC) diet = 30% of grass hay and 70% of concentrate; alfalfa hay-high forage (AF) diet = 70% of alfalfa hay and 30% of concentrate; alfalfa hay-high concentrate (AC) diet = 30% of alfalfa hay and 70% of concentrate (DM basis). Arrows show the feeding times. The pH values across time in fermenters with low and high F/B were not different (F/B $\times$ diet $\times$ time = 0.83).

role in the in vitro fermentation profile (Calsamiglia et al., 2008), other in vitro-related factors such as the dilution rate (Crawford et al., 1980), content stratification (Muetzel et al., 2009), or the harvest and processing of fermenter inoculum (Ziemer et al., 2000) are also involved in the in vivo-in vitro discrepancies. The C2/C3 values found in this experiment were affected ($P < 0.001$) by both diet and F/B, but the experimental design did not allow us to clarify whether diet (Russell, 1998; de Veth and Kolver, 2001) or pH (Calsamiglia et al., 2008) influenced the C2/C3 more. In many studies with CCF, changes in the ruminal fermentation pattern

as pH decreases have been largely ascribed to shifts in bacterial species (Yang et al., 2002; Colombatto et al., 2003; Carro et al., 2009). The effect of F/B on ruminal fermentation parameters found in this study could be explained by a pH-mediated shift in the bacterial species present in the fermenters. Furthermore, the concentration of purine bases in EB (Figure 2) differed ($P < 0.05$) in fermenters with different F/B for 3 out of 4 diets, supporting the hypothesis that changes in the F/B can promote the presence of different bacterial species (Obispo and Dehority, 1999), which likely occurs through a pH-mediated effect.

Table 2. Average values of pH, VFA concentration, and molar proportions in single-flow continuous-culture fermenters (CCF) receiving the experimental diets at 2 different feed-to-buffer ratios (F/B)¹

Item	Diet with low F/B ²				Diet with high F/B ³				SEM	P-value		
	GF	GC	AF	AC	GF	GC	AF	AC		F/B	Diet	F/B $\times$ diet
Average pH ⁴	6.50 ^a	6.22 ^b	6.53 ^a	6.31 ^b	6.08 ^w	5.94 ^x	5.79 ^y	5.70 ^z	0.021	<0.001	0.001	0.003
Total VFA, ⁵ mmol/L	112 ^a	115 ^a	125 ^b	120 ^{ab}	152 ^x	173 ^y	168 ^{xy}	166 ^{xy}	1.9	<0.001	0.002	0.03
VFA, mol/100 mol												
Acetate	63.9 ^{ab}	62.1 ^b	65.1 ^a	63.0 ^b	57.1 ^x	49.7 ^y	63.5 ^w	50.6 ^y	0.47	<0.001	<0.001	0.001
Propionate	21.0 ^b	22.4 ^a	16.6 ^c	20.9 ^b	22.3 ^x	27.8 ^w	21.8 ^x	28.7 ^w	0.26	<0.001	<0.001	0.002
Butyrate	11.8 ^b	11.1 ^b	12.8 ^a	11.0 ^b	16.8 ^{wx}	18.5 ^w	9.93 ^y	15.2 ^x	0.41	<0.001	0.04	0.001
Isoacid ⁶	1.75 ^b	2.15 ^{ab}	2.60 ^a	2.81 ^a	1.34	1.34	1.82	1.95	0.073	<0.001	0.001	0.69
Valerate	1.65 ^c	2.26 ^b	2.93 ^a	2.65 ^a	2.42 ^y	2.64 ^y	2.94 ^x	3.54 ^w	0.061	<0.001	<0.001	0.01
C2/C3 ratio ⁷	3.06 ^b	2.85 ^b	3.94 ^a	3.06 ^b	2.57 ^x	1.80 ^y	2.92 ^w	1.77 ^y	0.043	<0.001	<0.001	0.03

a-c; w-z. In a row, within each F/B, means without a common superscript letter differ ($P < 0.05$); observations, n = 4.

¹Grass hay-high forage (GF) = 70% of grass hay and 30% of concentrate; grass hay-high concentrate (GC) = 30% of grass hay and 70% of concentrate; alfalfa hay-high forage (AF) = 70% of alfalfa hay and 30% of concentrate; alfalfa hay-high concentrate (AC) = 30% of alfalfa hay and 70% of concentrate (DM basis).

²Low feed-to-buffer ratio = 23.5 g of DM/L.

³High feed-to-buffer ratio = 35 g of DM/L.

⁴Average pH at 0 to 12 h after the morning feeding measured every 2 h.

⁵The VFA concentration was measured in effluents collected every morning before feeding from d 8 to 11, representing 24-h average values.

⁶Isobutyrate plus isovalerate.

⁷Acetate-to-propionate ratio.

Figure 2. Purine bases (PB) concentration ($\mu\text{mol/g}$ of DM) in effluent bacteria isolated from single-flow continuous-culture fermenters (CCF) receiving the experimental diets at low (23.5 g of DM/L; open bars) and high (35 g of DM/L; dotted bars) feed-to-buffer ratio. Grass hay-high forage (GF) diet = 70% of grass hay and 30% of concentrate; grass hay-high concentrate (GC) diet = 30% of grass hay and 70% of concentrate; alfalfa hay-high forage (AF) diet = 70% of alfalfa hay and 30% of concentrate; alfalfa hay-high concentrate (AC) diet = 30% of alfalfa hay and 70% of concentrate (DM basis). Asterisks indicate a significant difference between the 2 feed-to-buffer ratios used within the same diet (* indicates $P < 0.05$; ** indicates $P < 0.01$). Number of observations per mean = 4.

In order to illustrate to what extent the bacterial communities in CCF can change according to the F/B used, both the similarity and the diversity of bacterial composition in fermenters were assessed by T-RFLP. Because samples were submitted to digestion using 4 different enzymes and because the obtained profiles were combined, the results likely provide a more comprehensive profile of the diverse bacterial communities present in each sample. The use of more than one restriction enzyme has been shown to facilitate the resolution of bacterial populations (Liu et al., 1997; Marsh 1999) because different bacterial populations can share the same terminal restriction fragment length for a particular primer-enzyme combination but not for others (Marsh et al. 2000). Figure 3 shows the dendrogram based on Bray-Curtis distances and the Ward method analysis, the pH values in the fermenters, and the Shannon index for every sample. The dendrogram shows 2 well-defined clusters in which samples are almost completely separated according to the F/B used in the fermenters. Moreover, differences ($P < 0.05$) in fermenters belonging to these 2 clusters were found with regard to both the average pH (5.81 vs. 6.38) and bacterial diversity (3.09 vs. 3.42), suggesting a positive relationship between fermenter pH and bacterial diversity. Although a lower Shannon index does not necessarily mean a lower richness of bacterial species, the average number of peaks in the electropherograms obtained in this study for the 4 enzymes was numerically lower in the cluster corresponding to the fermenters with high F/B than in the one grouping most fermenters with low F/B (152 vs. 172, $P = 0.06$; data not shown), which

certainly would indicate a loss of bacterial species. The T-RFLP data analysis also revealed that the SIMPER for samples of high-F/B fermenters (42.7%) was higher ($P = 0.006$) than that observed in samples of low-F/B fermenters (36.2%), indicating a greater homogeneity in terms of the bacterial communities as the F/B increases in CCF. Therefore, the set of data shown in Figure 3 may indicate that the lower average pH reached in fermenters with high F/B (35 g of DM/L) could lead to a decrease in or disappearance of certain bacterial species compared with what occurs in fermenters with low F/B (23.5 g of DM/L) and that this decrease in the number of species may involve a greater similarity in bacterial communities across diets in high-F/B fermenters. In addition, the reduced fiber digestibility in the high-F/B fermenters, discussed later, supports the hypothesis that the lower diversity found in fermenters with high F/B in comparison with those with low F/B could be partially due to the decrease in cellulolytic bacterial populations. On the other hand, the dendrogram shows no clustering based on the diet supplied to the fermenters, suggesting that diet composition does not have an obvious effect on the structure of the bacterial community in CCF. The lack of effect of diet on the microbial community composition in this in vitro study could be partially explained by the following factors: 1) the fermenters receiving the same treatment were inoculated with rumen fluid belonging to different goats, and 2) the standardized in vitro conditions among fermenters did not mimic the potential diet $\times$ animal interactions, which might alter the saliva yield, buffering capacity, and rumen dilution rate, among

Figure 3. Dendrogram illustrating the relationship among bacterial communities. Results of terminal RFLP (T-RFLP) analysis of fermenter contents were subject to Bray-Curtis distance analysis to elucidate relationships. Fermenters ($n = 8$) were fed 1 of 4 diets at a low or high feed-to-buffer ratio (F/B; 23.5 vs. 35 g of DM/L, respectively). Grass hay-high forage (GF) diet = 70% of grass hay and 30% of concentrate; grass hay-high concentrate (GC) diet = 30% of grass hay and 70% of concentrate; alfalfa hay-high forage (AF) diet = 70% of alfalfa hay and 30% of concentrate; alfalfa hay-high concentrate (AC) diet = 30% of alfalfa hay and 70% of concentrate on a DM basis. Incubations were repeated (e.g., high GF1 and high GF2) to provide a total of 16 observations. Scaled bar above dendrogram indicates Bray-Curtis distances. Mean incubation pH and bacterial diversity measured by the Shannon index are indicated. Similarity percentages (SIMPER) were 36.3 and 42.7 for low and high F/B fermenters, respectively ($P = 0.006$). ^{a,b} Average pH and Shannon index values in the cluster without a common superscript letter differ ($P < 0.05$).

other factors, and thus, decrease the potential to detect the effects of the diet used. The experimental design in this work involved the donor animal and the corresponding inoculated fermenter receiving the same diet. This ensured that the microbial ecosystem present in the inoculum was a result of the fermentation of the substrate incubated in the fermenter. This design has been used in in vivo-in vitro comparisons (Mansfield et al., 1995; Molina-Alcaide et al., 2009) and was supported by Bach et al. (2005), who highlighted the importance of the environment pre-existing in the rumen for accurate evaluations of different substrates in vitro. It is well accepted that each animal harbors a specific bacterial community (Winkler et al., 2007) and, thus, that the inherent differences among individuals as well as their distinctive responses to changes in diet make interpretation at this point more difficult. However, in spite of having the influence of animals and diets confounded, the experimental design allowed us to detect the effect of the F/B on the bacterial structure in CCF; in almost all cases, bacterial communities from the 2 fermenters supplied with the same diet and inoculated

with rumen fluid from the same goat fell into 2 different clusters, and this certainly seems to be ascribed solely to the effect of F/B.

The digestibility of all of the studied nutrients decreased ($P < 0.001$) in fermenters as F/B increased from 23.5 to 35 g of DM/L (Table 3), likely due to the associated fall in pH (Crawford et al., 1980). Moreover, the effect of F/B on the true OM and the apparent NDF digestibility changed according to the type of diet (F/B $\times$ diet, $P \leq 0.003$), which is consistent with the same interaction found in the total VFA concentration, molar proportions of most individual VFA, and the average pH. All of these F/B $\times$ diet interactions suggest that the amount of feed supplied to the fermenters for a given dilution rate could affect not only the fermentation parameters themselves but also response patterns to diets. Calsamiglia et al. (2008) have recently shown that the pH in CCF was the major determinant driving the changes in the true OM digestibility and was the sole source of variation in the apparent NDF digestibility when the pH values dropped below 6.0. The decrease ($9.1\% \pm 2.31$) in the true OM digestibility

Table 3. Nutrient digestibility in single-flow continuous-culture fermenters (CCF) receiving the experimental diets at 2 different feed-to-buffer ratios (F/B)¹

Item	Diet with low F/B ²				Diet with high F/B ³				SEM	<i>P</i> -value		
	GF	GC	AF	AC	GF	GC	AF	AC		F/B	Diet	F/B × diet
True digestibility, %												
DM	55.6 ^a	73.2 ^c	64.3 ^b	73.6 ^c	51.4 ^x	64.6 ^z	55.8 ^{xy}	61.5 ^{yz}	0.54	<0.001	<0.001	0.11
OM	51.8 ^a	63.2 ^c	57.3 ^b	63.8 ^c	49.7 ^y	56.7 ^z	52.6 ^{yz}	55.6 ^z	0.37	<0.001	<0.001	0.002
NDF	49.6 ^a	57.2 ^b	51.3 ^a	57.9 ^b	46.3	48.3	44.0	44.7	0.42	<0.001	<0.001	0.003
ADF	51.2 ^a	55.7 ^b	48.3 ^a	55.1 ^b	44.5 ^{yz}	49.9 ^z	43.0 ^y	46.1 ^{yz}	0.43	<0.001	<0.001	0.45
Apparent digestibility, %												
NFC ⁴	41.5 ^a	55.1 ^b	53.3 ^b	58.4 ^b	38.1 ^y	53.7 ^z	48.0 ^z	54.0 ^z	0.81	0.001	<0.001	0.47

^{a-c}; ^{x-z} In a row, within each F/B, means without a common superscript letter differ ($P < 0.05$); observations, $n = 4$. Superscripts a–c indicate differences among mean values for diets with low F/B and superscripts w–z indicate differences among mean values of diets with high F/B.

¹Grass hay-high forage (GF) = 70% of grass hay and 30% of concentrate; grass hay-high concentrate (GC) = 30% of grass hay and 70% of concentrate; alfalfa hay-high forage (AF) = 70% of alfalfa hay and 30% of concentrate; alfalfa hay-high concentrate (AC) = 30% of alfalfa hay and 70% of concentrate (DM basis).

²Low feed-to-buffer ratio = 23.5 g of DM/L.

³High feed-to-buffer ratio = 35 g of DM/L.

⁴Calculated as: NFC = OM – (NDF – CP – ether extract).

with increased F/B seemed to be more the result of a reduction ($15.3\% \pm 3.65$) in NDF digestibility than in the apparent NFC digestibility ($7.0\% \pm 1.94$). The larger decrease in fiber digestibility in comparison with that of NFC as pH decreases is in agreement with observations from other studies with CCF (Shriver et al., 1986; de Veth and Kolver, 2001; Yang et al., 2002). The decrease in the digestibility of fiber compared with that of the NFC fraction may be due to the lower tolerance of cellulolytic microorganisms than that of amylolytic and saccharolytic microorganisms to low pH (Russell and Dombrowski, 1980). The results found by Russell and Dombrowski (1980) indicate that one of the major reasons for fibrolytic bacteria elimination in the rumens with low pH values is the increase in the energy requirements for bacteria maintenance, as in this scenario; bacteria need to maintain the proton-motive force across the membrane. This involves a reduced rate of replication, which may result in a wash-out of this microbial community from the fermenter. The disappearance of the fibrolytic bacteria as the pH decreases in vitro (Colombatto et al., 2003) could partially explain the lower bacterial diversity found in most of the fermenters with high F/B.

The effects of variations in F/B and diet on N metabolism in fermenters are shown in Table 4. Total, microbial, and undegraded dietary N output increased ($P < 0.001$) as the F/B increased due to the higher N input in fermenters with high vs. low F/B. However, the ammonia-N output was not affected ($P = 0.11$) by the F/B, which is likely because of the lower ($P < 0.001$) dietary CP degradation found in fermenters with high vs. low F/B. Lower CP degradation in dual-flow CCF as pH decreases has been reported elsewhere (Calsamiglia

et al., 2002; Cerrato-Sánchez et al., 2008) and has been related to the association between the reduction in the digestibility of fiber and protein (Wallace and Cotta, 1989). Consequently, it seems that the lower pH values found in fermenters with high F/B may have promoted a lower dietary CP degradation, mainly through a drop in fiber digestibility, as the proteolytic enzymes produced by rumen microorganisms are generally active across a wide range of pH values (Wallace and Cotta, 1989). Both the PB flow and the estimated microbial N flow differed ($P < 0.001$) among diets and increased ($P < 0.001$) with increases in F/B. The effect of diet on PB flow in fermenters with low F/B was comparable to that found for the urinary purine derivatives excretion in goats fed the same diets (Cantalapiedra-Hijar et al., 2009), where the GF diet promoted a lower urinary excretion of purine derivatives compared with that of the other 3 diets due to a lower intake of N. Because the urinary purine derivative excretion in ruminants is highly correlated with the flow of PB to the duodenum (Pérez et al., 1996; Martín-Orúe et al., 1998), these results could indicate a better simulation of rumen fermentation in CCF with low F/B compared with those with high F/B.

CONCLUSIONS

The results suggest that the F/B ratio used in single-flow CCF affects the community structure and diversity of the bacteria found in the fermenters. The shift in bacterial species seems to be responsible for most of the discrepancies in fermentation parameters found between fermenters with low and high F/B ratios. Therefore, the amount of feed supplied to single-flow

Table 4. Nitrogen metabolism in single-flow continuous-culture fermenters (CCF) receiving the experimental diets at 2 different feed-to-buffer ratios (F/B)¹

Item	Diet with low F/B ²				Diet with high F/B ³				SEM	P-value		
	GF	GC	AF	AC	GF	GC	AF	AC		F/B	Diet	F/B × diet
N input, mg/d	605	805	932	945	907	1,207	1,398	1,418				
N output, mg/d												
Total	589 ^a	782 ^b	924 ^c	948 ^d	889 ^w	1,170 ^x	1,345 ^y	1,369 ^y	5.7	<0.001	<0.001	0.003
Ammonia	18.9 ^a	64.8 ^b	275 ^d	196 ^c	8.7 ^w	39.1 ^w	350 ^y	236 ^x	5.3	0.11	<0.001	0.009
Nonammonia	570 ^a	718 ^c	649 ^b	752 ^c	881 ^w	1,131 ^y	995 ^x	1,133 ^y	8.0	<0.001	<0.001	0.16
PB ⁴ flow, µmol/d	294 ^a	478 ^b	458 ^b	481 ^b	415 ^x	703 ^z	607 ^y	580 ^y	4.5	<0.001	<0.001	0.22
Microbial N flow, mg/d	336 ^a	493 ^b	452 ^b	506 ^b	463 ^w	658 ^y	570 ^x	608 ^{xy}	6.8	<0.001	<0.001	0.44
Dietary N flow, mg/d	234	224	197	245	418	472	425	524	8.6	<0.001	0.08	0.29
Dietary CP degradation, %	58.7 ^a	69.4 ^b	78.0 ^b	74.3 ^b	52.0 ^w	57.8 ^{wx}	65.8 ^x	59.6 ^{wx}	0.80	<0.001	<0.001	0.37
EMPS ⁵ , g of microbial N/kg of OM truly digested	23.4 ^a	28.5 ^b	29.4 ^b	29.3 ^b	22.3 ^w	28.6 ^x	27.0 ^x	27.2 ^x	0.44	0.07	<0.001	0.54

^{a-c}; ^{w-z} In a row, within each F/B, means without a common superscript letter differ ($P < 0.05$); observations, $n = 4$.

¹Grass hay-high forage (GF) = 70% of grass hay and 30% of concentrate; grass hay-high concentrate (GC) = 30% of grass hay and 70% of concentrate; alfalfa hay-high forage (AF) = 70% of alfalfa hay and 30% of concentrate; alfalfa hay-high concentrate (AC) = 30% of alfalfa hay and 70% of concentrate (DM basis).

²Low feed-to-buffer ratio = 23.5 g of DM/L.

³High feed-to-buffer ratio = 35 g of DM/L.

⁴Purine bases.

⁵Efficiency of microbial protein synthesis.

CCF in which pH is not under control with regard to the infused buffer should be carefully chosen for the purpose of simulating ruminal fermentation.

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