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Shifts in Rumen Fermentation and Microbiota Are Associated with Dissolved Ruminal Hydrogen Concentrations in Lactating Dairy Cows Fed Different Types of Carbohydrates[1–3]

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Abstract

Background: Different carbohydrates ingested greatly influence rumen fermentation and microbiota and gaseous methane emissions. Dissolved hydrogen concentration is related to rumen fermentation and methane production.

Objectives: We tested the hypothesis that carbohydrates ingested greatly alter the rumen environment in dairy cows, and that dissolved hydrogen concentration is associated with these changes in rumen fermentation and microbiota.

Methods: Twenty-eight lactating Chinese Holstein dairy cows [aged 4–5 y, body weight 480 ± 37 kg (mean ± SD)] were used in a randomized complete block design to investigate effects of 4 diets differing in forage content (45% compared with 35%) and source (rice straw compared with a mixture of rice straw and corn silage) on feed intake, rumen fermentation, and microbial populations.

Results: Feed intake (10.7–12.6 kg/d) and fiber degradation (0.584–0.692) greatly differed ($P \leq 0.05$) between cows fed the 4 diets, leading to large differences ($P \leq 0.05$) in gaseous methane yield (27.2–37.3 g/kg organic matter digested), dissolved hydrogen (0.258–1.64 μmol/L), rumen fermentation products, and microbiota. Ruminal dissolved hydrogen was negatively correlated ($r < -0.40$; $P < 0.05$) with molar proportion of acetate, numbers of fungi, abundance of *Fibrobacter succinogenes*, and methane yield, but positively correlated ($r > 0.40$; $P < 0.05$) with molar proportions of propionate and *n*-butyrate, numbers of methanogens, and abundance of *Selenomonas ruminantium* and *Prevotella* spp. Ruminal dissolved hydrogen was positively correlated ($r = 0.93$; $P < 0.001$) with Gibbs free energy changes of reactions producing greater acetate and hydrogen, but not correlated with those reactions producing more propionate without hydrogen.

Conclusions: Changes in fermentation pathways from acetate toward propionate production and in microbiota from fibrolytic toward amylolytic species were closely associated with ruminal dissolved hydrogen in lactating dairy cows. An unresolved paradox was that greater dissolved hydrogen was associated with greater numbers of methanogens but with lower gaseous methane emissions. *J Nutr* doi: 10.3945/jn.116.232462.

Keywords: rumen fermentation pathway, hydrogen, methane, dietary carbohydrate, starch, fiber, dairy cow, rumen microbe, Gibbs free energy

Introduction

Plant fiber and starch are the most important dietary carbohydrates for dairy cows and provide energy for rumen microorganisms and host animals. Carbohydrate fermentation in the rumen leads to the formation of volatile FAs (VFAs)[9], carbon dioxide, hydrogen, and microbial biomass. Acetate and propionate are the main precursors of milk FAs and glucose, respectively. The hydrogen and carbon dioxide can be used by methanogens to produce methane, which is an important greenhouse gas. Starch fermentation in the rumen leads to more propionate production and less gaseous methane emissions than fiber fermentation (1), because shifting acetate to propionate production creates an alternative electron sink, directing electrons away from hydrogen production (2, 3). Hungate (4) proposed that hydrogen is an important intermediate that is linked with VFA production pathway and methanogenesis.

The process of rumen carbohydrate degradation is carried out by a wide range of microbial groups working together that can be broadly divided into 4 main groups: bacteria, protozoa, anaerobic fungi, and archaea (5). Some of the carbohydrate degraders are hydrogen-producing microorganisms, including protozoa, fungi, and fibrolytic bacteria such as *Ruminococcus*

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albus and *R. flavefaciens* (6). Other microorganisms can produce very little hydrogen, instead producing reduced VFAs such as propionate. These include *Selenomonas ruminantium* and *Prevotella* spp., which are abundant microbes in the rumen and possess xylanolytic and oligosaccharolytic activities (7). *Prevotella* spp. in particular may change the balance between the hydrogen and VFAs they produce, depending upon the carbohydrate ingested (8, 9); thus, they may be important regulators of the availability of ruminal hydrogen. Although a shift in rumen microbiota has been observed in cows during adaptation from forage to starchy diets (8), the association between hydrogen in the rumen and particular microorganisms is not yet fully understood.

Ruminal hydrogen exists in 2 forms: gaseous hydrogen and dissolved hydrogen. Dissolved hydrogen, biologically available for hydrogen-consuming microbiota (10), can be supersaturated in the liquid phase and cannot be estimated from rumen headspace or exhaled gaseous hydrogen with the use of Henry's law (11, 12). Few studies, to our knowledge, have been performed to investigate the biological effects of hydrogen by measuring ruminal dissolved hydrogen. In this study, we hypothesized that cows ingesting different types of carbohydrate would exhibit a range in rumen fermentation variables, such that changes in ruminal dissolved hydrogen would be closely associated with differences in rumen fermentation and selected microbial groups. To test this hypothesis, this study used correlation analysis to verify natural associations of dissolved hydrogen concentration with ruminal fermentation end products, selected microbial groups, and exhaled gases in cows ingesting different types of carbohydrate.

Methods

The animal procedures were approved by the Animal Care Committee, Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, China.

Cows and diets. A randomized complete block design with factorial arrangement of treatments was used to investigate the effects of 4 diets containing 2 levels of forage content (FC) and 2 types of forage source (FS) on the rumen ecosystem in lactating dairy cows. Twenty-eight multiparous Chinese Holstein dairy cows (aged 4–5 y; mean ± SD body weight, 480 ± 37 kg; milk yield, 15 ± 3.5 kg/d; days in milk, 150 ± 7; and parity, 1.75 ± 0.96) in midlactation were allocated to 7 blocks according to parity and milk production. Each block contained 4 cows, and each cow within a block was randomly assigned to 1 of the 4 dietary treatments. Thus, each dietary treatment had 7 replications (cows). The 2 FC treatments were 45% [high forage content (HF)] and 35% [low forage content (LF)] forage on a dry matter (DM) basis, whereas the two FS treatments were rice straw (RS) and a mixture of rice straw and corn silage (RC). The major difference between the RS and RC diets was that some forage in the RS diet was replaced with corn silage, whereas the major difference between the HF and LF diets was that some forage in the HF diet was replaced with corn distillers' dried grains to form the LF diet. Thus, the 4 diet groups were as follows: high forage content with 15% rice straw and 30% corn silage (HF-RC); high forage content with 45% rice straw (HF-RS); low forage content with 10% rice straw and 25% corn silage (LF-RC); and low forage content with 35% rice straw (LF-RS). All diets were formulated to have a similar crude protein content, and neutral detergent fiber (NDF)–to-starch ratios were 1.68, 2.32, 1.48, and 1.91 for the HF-RC, HF-RS, LF-RC, and LF-RS diets, respectively (**Supplemental Table 1**).

The cows were housed in a tie-stall barn, fed individually at 0630 and 1630 with the same amount of feed at each meal, and milked at 0530 and 1600. All cows had free access to fresh water. The period of diet adaptation lasted 21 d. The DM intake of total mixed ration was estimated during the initial 10 d of the diet adaptation period by offering the diet for ad libitum intake with 5% refusals. The amount of feed allocated daily thereafter was set at 100% of the DM intake during the first 10 d to minimize diet selection. The refusals, when present, were collected and analyzed to determine the actual diet intake.

Nutrient digestibility and milk production. Nutrient digestibility was determined over a 5-d period from day 22 to day 26. Total feces were collected, weighed, and mixed daily, and a subsample (1%) was stored at −20°C until the end of collection period. A composite sample was prepared for each cow, and mixed and oven dried for the analysis of DM, organic matter (OM), starch, and NDF.

Cows were milked twice daily at 0530 and 1630, and milk production was recorded at each milking by weight. The collection of milk samples for each cow was performed during the period of digestibility measurements. Milk samples (1% of total weight) were collected during each milking, treated with the addition of potassium dichromate (1 g/L) as a preservative, and stored at −20°C before analysis.

Rumen sampling. The collection of rumen contents was performed 6 h after morning feeding on days 28 and 29. Rumen contents (300 mL) were collected by oral stomach tubing according to the method described by Wang et al. (13), with the initial 150 mL discarded to avoid saliva contamination. The sample was retained for the measurement of dissolved hydrogen and dissolved methane concentrations, fermentation end products, and rumen microbiota. One subsample (100 mL) was immediately frozen at −80°C for DNA extraction and subsequent microbial quantification. Two other subsamples (35 mL each) were immediately transferred into 50-mL plastic syringes to measure dissolved hydrogen and dissolved methane concentration. The remaining sample was used for the measurements of pH, ammonia, and VFAs.

Gaseous hydrogen and methane emissions. Gaseous hydrogen and methane emissions were measured for each cow for 48 h in a respiration chamber with the use of a slightly modified protocol from Beauchemin and McGinn (14) and Wang et al. (15). Briefly, the 28 cows were sequenced individually through the chamber so that 1 from each diet group was included in each block of 4 cows, and emissions from each cow were measured for 2 d. Within the chamber, the cow was restrained with free access to a feed bin and drinking water. Airflow was maintained under negative pressure (flow rate, 190 m^3/h), controlled by the pump. The mean ambient temperature in the chamber was 17°C, and ranged from 15°C to 19°C. Approximately 30 mL of outlet gas samples were collected every 30 min, whereas 3 inlet gas samples (30 mL) were collected at 0600, 1200 and 1700, and their mean value was used to represent the gas concentrations of the inflowing air. Gas samples were saved in vacuum tubes, and gaseous hydrogen and gaseous methane concentrations were measured with the use of GC (Agilent 7890A).

Sample analysis. All samples of feeds, refusals, and feces were dried and ground to pass through a 1-mm sieve. The DM (method 945.15),

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[3] Supplemental Tables 1–3 and Supplemental Figures 1–4 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

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[9] Abbreviations used: DM, dry matter; ECM, energy-corrected milk; FC, forage content; FS, forage source; HF, high forage content; HF-RC, high forage content with 15% rice straw and 30% corn silage; HF-RS, high forage content with 45% rice straw; LF, low forage content; LF-RC, low forage content with 10% rice straw and 25% corn silage; LF-RS, low forage content with 35% rice straw; NDF, neutral detergent fiber; OM, organic matter; RC, rice straw and corn silage; R_{NH2}, net hydrogen production relative to the amount of total volatile FA produced; RS, rice straw; VFA, volatile FA.

OM (method 942.05), crude protein (method 954.01), and ether (method 920.39) extracts were determined according to published methodologies (16). Gross energy was determined with the use of an isothermal automatic calorimeter (5E-AC8018; Changsha Kaiyuan Instruments Co.). The NDF and acid detergent fiber were expressed inclusive of residual ash (17), and NDF was assayed with the addition of a heat-stable amylase, but without sodium sulfite. The starch content was determined after pre-extraction with ethanol (80%), and glucose liberated from starch by enzyme hydrolysis was measured with the use of amyloglucosidase (18). Dissolved gases in rumen fluid were measured with the use of the procedures described by Wang et al. (13).

Milk samples were pooled by cow and day, and 50 mL of milk was used for analysis of fat, protein, lactose, and total solids by infrared analysis with a spectrophotometer (MilkoScanTM FT; Foss Electric). Energy-corrected milk (ECM; 3.5% fat and 3.2% of protein) production was calculated according to Tyrrell and Reid (19), with the following equation:

$$\mathrm{ECM(kg/d)} = \left(102.77P_{\mathrm{milk}} + 4072P_{\mathrm{fat}} + 2265P_{\mathrm{protein}}\right)/314 \quad (1)$$

where P_{milk} is milk production (kg/d), P_{fat} is fat production (kg/d), and P_{protein} is protein production (kg/d).

The pH of strained rumen fluid was measured immediately after sampling with the use of a portable pH meter (Starter 300; Ohaus Instruments Co.). The frozen rumen fluid samples were thawed and centrifuged at 15,000 × *g* for 10 min at 4°C before the VFA profiles in the supernatants were measured with the use of GC (Agilent 7890A), according to the method described by Wang et al. (11). The estimated net hydrogen production relative to the amount of total volatile FA produced (R_{NH2}) was calculated according to the stoichiometric equation developed by Wang et al. (13).

qPCR analyses. Rumen samples were freeze-dried before physical disruption with the use of a bead beater. Genomic DNA was extracted with the use of the QIAamp DNA Stool Mini kit (Qiagen) following the manufacturer's instructions. The quantity of DNA was measured on the basis of absorbance at 260 and 280 nm with the use of a NanoDrop ND 100 (NanoDrop Technologies). The absolute quantification of total bacteria, protozoa, fungi, methanogens, and selected bacterial species was measured by qPCR with the use of primers validated in our laboratory as listed in **Supplemental Table 2** (20). The qPCR was performed according to the procedures described by Jiao et al. (20). Briefly, the plasmid DNA containing exact 16S ribosomal RNA gene inserts was used to generate a standard curve for each group and individual species. The qPCR was performed with a total volume of 10 μL, with the use of SYBR Premix Ex Taq (Perfect Real Time) on an ABI 7900HT Fast Real Time PCR system (Applied Biosystems). Final absolute amounts of target group or species were estimated by relating the cycle threshold (C_T) value to the standard curves and expressed as $\log_{10}$ copies/DM rumen contents.

The abundance of 6 common rumen bacteria species and 1 group (i.e., *Prevotella* spp.) were measured relative to the total bacterial DNA with the use of qPCR and species-specific 16S ribosomal RNA gene-targeted primers (Supplemental Table 2). The cycling conditions were the same as described above. Efficiencies of PCR amplification were determined by serial 10-fold dilutions of DNA standards. The abundance of each bacterial species or group was determined with the use of the ΔC_T method (7). The ratio of the amount of one-target species with respect to total bacteria (reference) was given by the following equation:

$$\mathrm{Ratio} = \left(\mathrm{E}_{\mathrm{target}}\right)^{\Delta C_{\mathrm{Ttarget(control-sample)}}} / \left(\mathrm{E}_{\mathrm{reference}}\right)^{\Delta C_{\mathrm{Treference(control-sample)}}} \quad (2)$$

where E is the efficiency of PCR amplification and ΔC_T is the number of cycles between the sample and the control (plasmid DNA).

Estimation of Gibbs free energy changes. Five pathways of glucose fermentation and methanogenesis under the conditions prevailing in the rumen were described by Janssen (3):

$$\mathrm{C_6H_{12}O_6} \rightarrow 0.66\,\mathrm{C_2H_3O_2^-} + 1.33\,\mathrm{C_3H_5O_2^-} + 0.66\,\mathrm{CO_2} + 0.66\,\mathrm{H_2O} + 2\,\mathrm{H^+} \quad (3)$$

$$\mathrm{C_6H_{12}O_6} \rightarrow \mathrm{C_2H_3O_2^-} + \mathrm{C_3H_5O_2^-} + \mathrm{CO_2} + 2\,\mathrm{H^+} + \mathrm{H_2} \quad (4)$$

$$\mathrm{C_6H_{12}O_6} \rightarrow \mathrm{C_4H_7O_2^-} + 2\,\mathrm{CO_2} + \mathrm{H^+} + 2\,\mathrm{H_2} \quad (5)$$

$$\mathrm{C_6H_{12}O_6} + 0.66\,\mathrm{H_2O} \rightarrow 0.66\,\mathrm{C_2H_3O_2^-} + 0.66\,\mathrm{C_4H_7O_2^-} + 2\,\mathrm{CO_2} + 1.33\,\mathrm{H^+} + 2.66\,\mathrm{H_2} \quad (6)$$

$$\mathrm{C_6H_{12}O_6} + 2\,\mathrm{H_2O} \rightarrow 2\,\mathrm{C_2H_3O_2^-} + 2\,\mathrm{CO_2} + 2\,\mathrm{H^+} + 4\,\mathrm{H_2} \quad (7)$$

$$\mathrm{CO_2} + 4\,\mathrm{H_2} \rightarrow \mathrm{CH_4} + 2\,\mathrm{H_2O} \quad (8)$$

Gibbs free energy changes for these 6 reactions, allowing fermentation thermodynamics per reaction to be compared, were calculated under rumen conditions, with measured values of pH, dissolved hydrogen, acetate, propionate, and *n*-butyrate; a temperature of 39°C; a dissolved CO_2 of 59 mM; and a glucose concentration of 0.6 mM (3).

Statistical analysis. Data were subjected to a linear mixed model with the use of SPSS 12.0 software. The analysis model was expressed as follows:

$$Y_{ijkl} = \mu + C_j + F_k + C_j \times F_k + B_i + D_l + e_{ijkl} \quad (9)$$

where Y_{ijkl} is the response, μ is the general mean, C_j is the fixed effect of FC, F_k is the fixed effect of FS, B_i is the random effect of the block, D_l is the random effect of the sampling day, and e_{ijkl} is the random error term. Differences of $P \leq 0.05$ were considered to be significant, and $0.05 < P \leq 0.1$ was accepted as a trend. When significant differences were found, a multiple comparison was conducted to elucidate differences between 2 particular treatments, and *P* values were adjusted with the use of the Bonferroni method.

The best linear or log linear regression between dissolved hydrogen and various markers was derived with the use of ordinary least squares, and performed with the use of SPSS 12.0 software. The Pearson correlation coefficient and statistical significance were obtained by regression analysis with the use of SPSS 12.0 software.

Results

Cows fed the HF-RS diet had a lower OM intake than cows fed the other 3 diets (−14.4%; $P < 0.05$) (**Table 1**). Although cows fed the remaining 3 diets had similar OM intake, they ingested different ($P < 0.05$) quantities of NDF (4.53–5.14 kg/d), starch (2.68–3.41 kg/d), and gross energy (250–265 MJ/d). Cows fed RC had greater apparent total tract digestibility of NDF than did cows fed RS (by 9.3%; $P = 0.01$). The cows fed the LF-RC diet had greater ($P < 0.05$) ruminal dissolved hydrogen (by 535% μmol/L) and dissolved methane (by 200% mmol/L) concentrations than cows fed the HF-RS diet. Ruminal dissolved hydrogen concentration was positively ($r = 0.52$; $P = 0.005$) correlated with rumen dissolved methane concentration (**Supplemental Figure 1**). Ruminal dissolved hydrogen concentration was positively ($r = 0.51$; $P = 0.005$) correlated with dietary starch intake, and negatively ($r = -0.59$; $P < 0.001$) correlated with the ingested NDF-to-starch ratio (**Supplemental Figure 2**).

Cows fed the HF-RS diet had a greater ($P < 0.05$) molar proportion of acetate (7.5% higher) and R_{NH2} (6.0% higher) and a lower ($P < 0.05$) total VFA concentration (−18.7%) and molar proportion of *n*-butyrate (−19.7%) than did cows fed the other 3 diets (Table 1). Ruminal dissolved hydrogen was

TABLE 1 Feed intake and digestibility, rumen dissolved gases, and fermentation end products in lactating dairy cows fed 4 diets containing 2 levels of forage and 2 forage sources after 21 d of adaptation[1]

	Diet[2]					P[3]		
	HF-RC	HF-RS	LF-RC	LF-RS	SEM	FC	FS	FC × FS
Intake								
OM, kg/d	12.5[a]	10.7[b]	12.6[a]	12.3[a]	0.08	<0.001	<0.001	<0.001
NDF, kg/d	5.14[a]	4.51[c]	4.70[b]	4.53[c]	0.057	<0.001	<0.001	<0.001
Starch, kg/d	3.41[b]	2.35[d]	3.44[a]	2.68[c]	0.007	<0.001	<0.001	<0.001
GE, MJ/d	259[b]	215[d]	265[a]	250[c]	1.6	<0.001	<0.001	<0.001
Nitrogen, kg/d	0.285[a]	0.264[b]	0.283[a]	0.283[a]	0.0016	<0.001	<0.001	<0.001
Total tract apparent digestibility								
OM	0.727	0.724	0.706	0.732	0.0198	NS	NS	NS
NDF	0.692[a]	0.621[b]	0.637[a,b]	0.584[b]	0.0231	0.05	0.01	NS
Starch	0.977	0.962	0.982	0.977	0.0048	0.07	0.07	NS
Dissolved gases								
dCH_4, mmol/L	0.674[a,b]	0.268[c]	0.804[a]	0.631[b]	0.0810	NS	<0.001	0.002
dH_2, μmol/L	1.02[b]	0.258[c]	1.64[a]	1.01[b]	0.284	<0.001	<0.001	NS
Rumen fermentation								
Ammonia, mmol/L	4.22[a]	4.41[a]	1.65[b]	2.78[b]	0.629	0.001	NS	NS
pH	6.71	6.76	6.77	6.81	0.074	NS	NS	NS
Total VFA, mmol/L	71.3[a,b]	57.8[b]	69.2[a,b]	72.8[a]	4.19	NS	NS	0.04
Acetate:propionate ratio	3.35[b]	3.96[a]	3.31[b]	3.43[b]	0.130	0.02	0.005	0.05
Molar proportion of individual VFAs, mol/100 mol								
Acetate	64.4[b]	69.6[a]	64.3[b]	65.4[b]	0.86	0.02	<0.001	0.01
n-Butyrate	13.2[a]	10.2[b]	13.0[a]	12.1[a]	0.41	0.03	<0.001	0.01
Propionate	19.0	17.8	19.5	19.3	0.52	NS	0.07	NS
Valerate	1.50[a]	0.765[b]	1.46[a]	1.34[a]	0.085	0.002	<0.001	<0.001
Isobutyrate	0.931	0.846	0.961	0.932	0.0450	NS	NS	NS
Isovalerate	1.00[a]	0.770[a,b]	0.710[b]	0.896[a,b]	0.0740	NS	NS	0.005
Estimated net H_2 production relative to the amount of total VFA produced, mol/mol	1.34[b]	1.42[a]	1.35[b]	1.35[b]	1.42[a]	0.07	0.02	0.04

[1] Values are means and pooled SEMs, $n = 7$. Labeled means within a row without a common superscript letter differ, $P \leq 0.05$. dCH_4, dissolved methane; dH_2, dissolved hydrogen; FC, forage content; FS, forage source; GE, gross energy; HF-RC, high forage content with 15% rice straw and 30% corn silage; HF-RS, high forage content with 45% rice straw; LF-RC, low forage content with 10% rice straw and 25% corn silage; LF-RS, low forage content with 35% rice straw; NDF, neutral detergent fiber; OM, organic matter; VFA, volatile FA.
[2] Diet treatments had 4 types of carbohydrates, with NDF-to-starch ratios being 1.68, 2.32, 1.48, and 1.91 for the HF-RC, HF-RS, LF-RC, and LF-RS diets, respectively.
[3] NS: $P > 0.1$.

positively correlated with total VFA ($r = 0.35$; $P = 0.06$), molar proportions of propionate ($r = 0.45$; $P = 0.02$), and *n*-butyrate ($r = 0.66$; $P < 0.001$), and negatively correlated with the molar proportion of acetate ($r = -0.65$; $P < 0.001$), acetate-to-propionate ratio ($r = -0.54$; $P = 0.003$), and R_{NH2} ($r = -0.50$; $P = 0.006$) (**Supplemental Figure 3**). Fermentation pathways producing more acetate and hydrogen per mole of glucose became rapidly less favorable (Gibbs free energy changes become more positive) with increasing dissolved hydrogen (**Figure 1**).

Consumption of the HF-RS diet led to a greater increment in the rate of gaseous methane emission after meals than did the other 3 diets (**Supplemental Figure 4**). Greater gaseous hydrogen emissions (expressed as g/kg DM intake and g/kg OM digested) were observed in cows fed the HF-RS diet ($P < 0.05$) than in cows fed the other diets (**Table 2**). Gaseous methane emissions were greater ($P < 0.05$) in cows fed the HF-RS diet than in those fed the LF-RC diet (41.2%, 37.1%, 108%, and 91% increases in terms of g/kg DM intake, g/kg OM digested, g/kg milk, and g/kg ECM, respectively). Ruminal dissolved hydrogen concentration was negatively correlated with gaseous methane emissions in terms of g/kg OM digested ($r = -0.47$; $P = 0.01$) and g/kg ECM ($r = -0.53$; $P = 0.003$) (**Figure 2**).

Cows fed the HF-RS diet had smaller numbers of protozoa (−74%; $P < 0.05$) and greater numbers of fungi (265% higher; $P < 0.05$) than did cows fed the other 3 diets, whereas cows fed the LF-RC diet had greater ($P < 0.05$) numbers of methanogens (947% higher), relative abundance of *S. ruminantium* (107% higher), and *Prevotella* spp. (57.6% higher) than did cows fed the HF-RS diet (**Supplemental Table 3**). Cows fed RC had a greater relative abundance of *Prevotella* spp. (46% higher, $P < 0.05$), and lower relative abundances of *Fibrobacter succinogenes* (−65% $P < 0.001$), *R. flavefaciens* (−50%; $P = 0.003$), and *Ruminobacter amylophilus* (−75%; $P < 0.001$) than did cows fed RS. Ruminal dissolved hydrogen was positively correlated with the number of methanogens ($r = 0.60$; $P = 0.001$), relative abundance of *S. ruminantium* ($r = 0.41$; $P = 0.03$), and *Prevotella* spp. ($r = 0.53$; $P = 0.004$), and negatively correlated with the number of fungi ($r = -0.44$; $P < 0.015$) and the relative abundance of *F. succinogenes* ($r = -0.55$; $P = 0.002$) (**Figure 3**).

Discussion

Partially replacing RS with corn silage or forage with corn distillers' dried grains increased the starch content and decreased

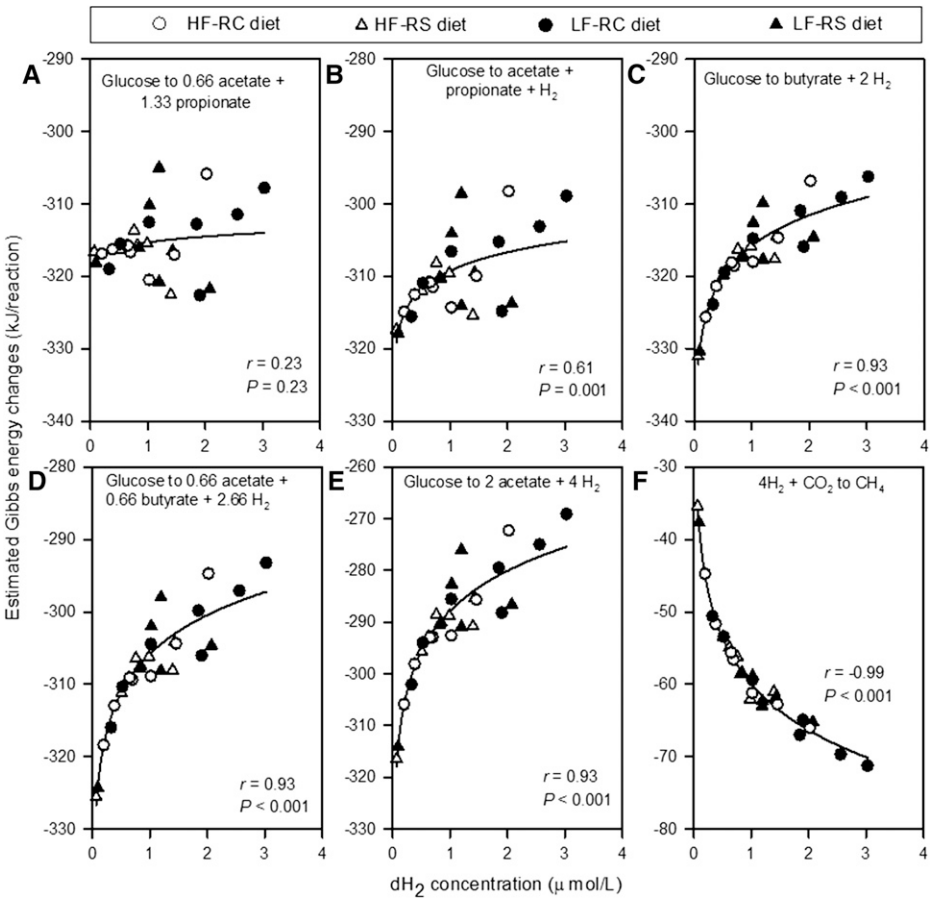

FIGURE 1 Relations between ruminal dissolved hydrogen and estimated Gibbs free energy changes of ruminal glucose fermentation via 5 different pathways (A–E) and methanogenesis (F) in lactating dairy cows fed 4 diets containing 2 levels of forage and 2 forage sources after 21 d of adaptation, $n = 7$. The lines in each panel are the best log linear regression line. Each point represents 1 cow, with a total of 28 data points. dH_2, dissolved hydrogen; HF-RC, high forage content with 15% rice straw and 30% corn silage; HF-RS, high forage content with 45% rice straw; LF-RC, LF content with 10% rice straw and 25% corn silage; LF-RS, low forage content with 35% rice straw.

the NDF content in the diets. The low OM intake observed for the fibrous HF-RS diet was due to the relatively high dietary NDF content, which had negative effects on feed intake because of increased rumen fill (21, 22). Despite relatively large differences in starch intake between diets, differences in starch digestibility were small and likely of limited biological significance, because starch digestibility was very high (>96%), which is consistent with other results in dairy cows (23). Greater NDF

TABLE 2 Gaseous methane and hydrogen emissions from lactating dairy cows fed 4 diets containing 2 levels of forage and 2 forage sources after 21 d of adaptation[1]

	Diet[2]					P[3]		
	HF-RC	HF-RS	LF-RC	LF-RS	SEM	FC	FS	FC × FS
Gaseous CH_4 emissions								
g/d	269[a,b]	289[a]	244[b]	250[b]	9.2	0.001	NS	NS
g/kg DM intake	20.7[b]	25.7[a]	18.2[c]	18.9[c]	0.96	<0.001	<0.001	0.004
g/kg NDF intake	52.2[b]	67.1[a]	49.9[b]	53.3[b]	1.86	<0.001	<0.001	0.004
g/kg OM digested	27.2[b]	37.3[a]	27.2[b]	28.4[b]	1.12	<0.001	<0.001	0.003
g/kg milk	22.6[b]	31.0[a]	14.9[c]	20.2[b,c]	1.77	0.001	<0.001	0.75
g/kg ECM	18.9[b,c]	27.9[a]	14.6[c]	19.6[b]	1.23	0.001	<0.001	0.60
Gaseous H_2 emissions								
g/d	0.469[b]	0.627[a]	0.454[b]	0.380[b]	0.0350	0.001	NS	0.003
g/kg DM intake	0.036[b]	0.056[a]	0.034[b]	0.029[b]	0.0025	<0.001	0.02	<0.001
g/kg NDF intake	0.091[b]	0.147[a]	0.092[b]	0.081[b]	0.0080	<0.001	0.01	<0.001
g/kg OM digested	0.051[b]	0.076[a]	0.048[b]	0.044[b]	0.0035	<0.001	0.006	<0.001

[1] Values are means and pooled SEMs, $n = 7$. Labeled means within a row without a common superscript letter differ, $P \leq 0.05$. DM, dry matter; ECM, energy-corrected milk; FC, forage content; FS, forage source; HF-RC, high forage content with 15% rice straw and 30% corn silage; HF-RS, high forage content with 45% rice straw; LF-RC, low forage content with 10% rice straw and 25% corn silage; LF-RS, low forage content with 35% rice straw; NDF, neutral detergent fiber; OM, organic matter.
[2] Dietary treatments had 4 types of carbohydrates, with NDF-to-starch ratios being 1.68, 2.32, 1.48, and 1.91 for the HF-RC, HF-RS, LF-RC, and LF-RS diets, respectively.
[3] NS: $P > 0.1$.

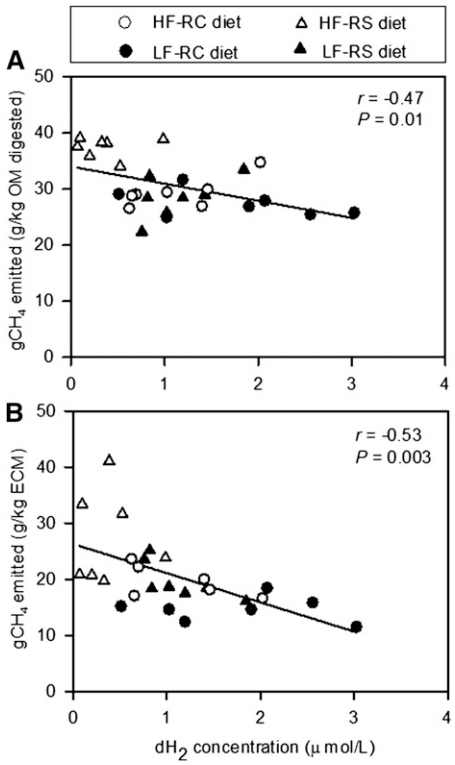

FIGURE 2 Relations between ruminal dissolved hydrogen and gaseous methane emissions expressed as yields/kg OM digested (A) or yields/kg ECM (B) from lactating dairy cows fed 4 diets containing 2 levels of forage and 2 forage sources after 21 d of adaptation, $n = 7$. The lines in each panel are the best linear regression line. Each point represents 1 cow, with a total of 28 data points. dH_2, dissolved hydrogen; ECM, energy-corrected milk; gCH_4, gaseous methane; HF-RC, high forage content with 15% rice straw and 30% corn silage; HF-RS, high forage content with 45% rice straw; LF-RC, low forage content with 10% rice straw and 25% corn silage; LF-RS, low forage content with 35% rice straw; OM, organic matter.

digestibility of the RC diet compared with that of the RS diet was consistent with expectations that the NDF of corn silage would be more digestible than that of RS. It was obvious that the 4 diets evaluated had different compositions and digestibility of carbohydrates, which would explain the numerous interactions of FS and content. These different diets also led to a large variation in dissolved hydrogen concentrations, which was important for investigating its correlations with other ruminal variables.

Hydrogen mainly is generated during fermentation of dietary fiber and starch to VFAs (3); as expected, dissolved hydrogen concentration was greatly affected by dietary FC and source. Starch is hydrolyzed to glucose more rapidly and efficiently than fiber in the rumen (24). Ruminants ingesting concentrate-forage mixed diets had greater ruminal dissolved hydrogen concentrations than those fed all-forage diets (25). Large amounts of hydrogen released from ruminal starch fermentation may overload the utilization capacity of hydrogen-consuming microorganisms, leading to elevated dissolved hydrogen in the rumen. The observed positive correlation between ruminal dissolved hydrogen and starch intake further confirms that starch intake, rather than fiber intake, is a critical factor associated with increased ruminal dissolved hydrogen. Furthermore, the strong positive correlation between dissolved hydrogen and VFA concentrations observed in this study is in agreement with results from a closed in vitro fermentation (11), in which there was no VFA absorption and outflow.

Cellulose is fermented to propionate to a lesser extent than it is to acetate and hydrogen, whereas readily degraded starch is fermented less to acetate and hydrogen and more to propionate (26). Our study indicated that molar proportions of major VFAs were greatly affected by dietary FC and source and that their interactions with individual VFA concentrations were closely associated with dissolved hydrogen concentration. Dissolved hydrogen concentration was negatively correlated with molar proportion of acetate, and positively correlated with molar proportions of propionate and *n*-butyrate. Such shifts from pathways producing acetate to propionate with increased dissolved hydrogen were further supported by the apparent Gibbs free energy changes for different pathways of glucose fermentation under the conditions prevailing in the rumen. The thermodynamics of propionate production were less affected by changes in the rumen environment associated with increasing dissolved hydrogen than were the thermodynamics of hydrogen formation. Therefore, propionate production becomes relatively more favorable as dissolved hydrogen increases. This can be explained by the increasingly unfavorable thermodynamics of hydrogen formation from electrons derived from fermentation as dissolved hydrogen increases, whereas the pathway of propionate production is not affected by dissolved hydrogen concentration. The variation of dissolved hydrogen in individual cows (44-fold; Figure 1) and diets (6-fold) was larger than that of major individual VFAs in cows (<3.7-fold; Figure 1) and diets (1.5-fold). This means that variation in VFA concentrations have a relatively minor influence on fermentation thermodynamics, so pathways not producing hydrogen are less affected by variations in end product concentrations.

Most cellulolytic microorganisms are acetate and hydrogen producers and are greatly affected by FC and source and their interaction. Cellulolytic microorganisms were negatively correlated with dissolved hydrogen concentration. For example, cows fed the fibrous HF-RS diet had greater numbers of fungi and relative abundances of *F. succinogenes*, but lower dissolved hydrogen concentrations. When more dietary starch was included, fibrolytic microorganisms were replaced by amylolytic bacteria. Fernando et al. (8) reported that populations of *S. ruminantium* and *Prevotella* spp. were greatly increased during the transition from forage to concentrate diets. Cows fed the starchy LF-RC diet had a greater relative abundance of *S. ruminantium* and *Prevotella* spp. These bacteria use electrons from fermentation to reduce organic intermediates from carbohydrates or other substrates to propionate instead of disposing of these electrons by hydrogen production (9, 27, 28). Changes in the rumen microbiota were closely associated with ruminal dissolved hydrogen concentration. Lower ruminal dissolved hydrogen was associated with more acetate and hydrogen producers, whereas higher ruminal dissolved hydrogen was associated with more propionate producers that yield less hydrogen production relative to the total VFA. Therefore, the efficiency of hydrogen production decreases as dissolved hydrogen increases, which is further confirmed by the strong negative correlation between dissolved hydrogen and R_{NH2}.

The different mixtures of carbohydrate ingested also affected gaseous methane and gaseous hydrogen emissions. Increasing dietary starch contents decreased gaseous methane emissions in cows (29–31), and decreased the amount of gaseous hydrogen emitted per unit of DM intake in steers (31). The gaseous methane and gaseous hydrogen emissions were affected greatly by dietary FC and source and their interaction, and were greater in cows fed the fibrous HF-RS diet. This may be a consequence of decreased carbohydrate degradation in the rumen and shifts

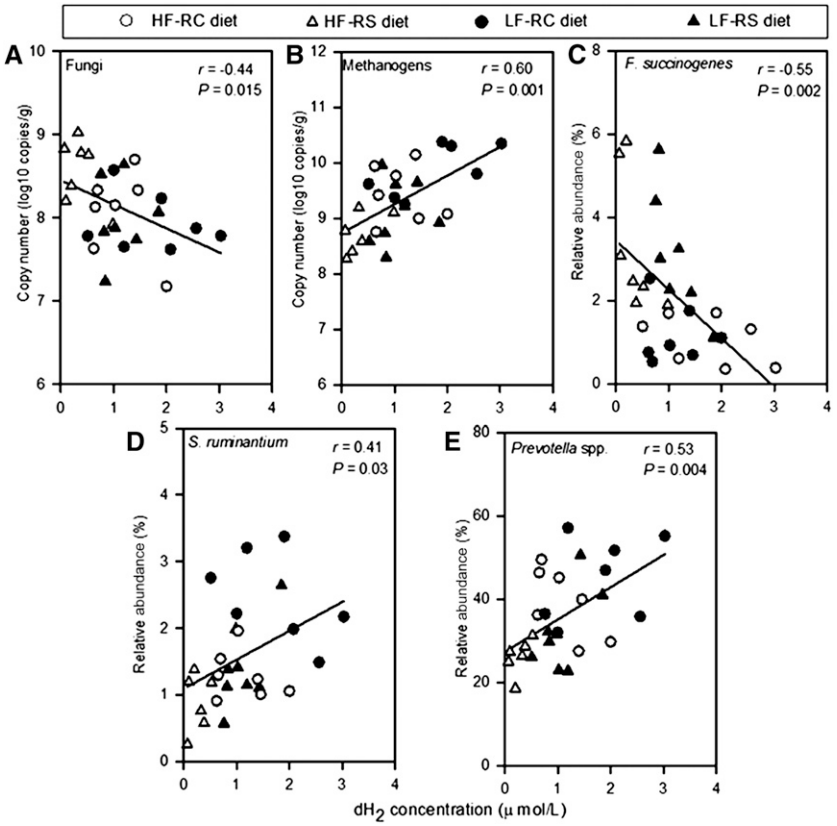

FIGURE 3 Relations between ruminal dissolved hydrogen and 5 different microbial groups (A–E) in lactating dairy cows fed 4 diets containing 2 levels of forage and 2 forage sources after 21 d of adaptation, $n = 7$. The lines in each panel are the best linear regression line. Each point represents 1 cow, with a total of 28 data points. Relative abundance was expressed as the ratio of the amount of one target species with respect to total bacteria, and microbial groups with correlation coefficients of $r > 0.40$ or < -0.40 are shown. dH_2, dissolved hydrogen; *F.*, *Fibrobacter*; HF-RC, high forage content with 15% rice straw and 30% corn silage; HF-RS, high forage content with 45% rice straw; LF-RC, low forage content with 10% rice straw and 25% corn silage; LF-RS, low forage content with 35% rice straw; *S.*, *Selenomonas*.

in the rumen fermentation pathway of less hydrogen production, such as those we describe above. Elevated starch intake would favor propionate and *n*-butyrate production at the expense of acetate (30).

Growth and metabolism of methanogens, observed as the number of methanogens and dissolved methane, were greatly affected by dietary FC and source and their interaction, and were positively correlated with dissolved hydrogen concentrations. However, a greater number of methanogens and favorable conditions for their activity did not explain the lower gaseous methane emissions of cows fed diets that resulted in higher dissolved hydrogen. This paradox needs to be understood by studying the saturation factor of ruminal hydrogen and methane and the dynamics of methanogen numbers and activity in detail over an entire feeding cycle. First, both dissolved hydrogen and dissolved methane are supersaturated (saturation factor > unity) in the rumen. We suspect that the extent of supersaturation might widely vary between the 4 treatments, because the saturation factor was reported to have a positive correlation with dissolved gas concentrations in vitro (11) and in vivo (12). Supersaturation decreased the facility of gas mass transfer from the liquid to gas phase, or exhaled gas, but may not alter the total amount of gases emitted from rumen. Second, ruminal dissolved hydrogen concentrations could be greatly affected by feeding events; thus, measuring dissolved hydrogen concentrations at one particular time after feeding may not reflect differences in dissolved hydrogen over a 24-h period (32). Third, the replacement of forage with concentrate might increase the passage of the solid phase from the rumen (33). Faster rumen passage can cause a lower extent of rumen fermentation (34), and a shorter residence time for methanogens might decrease methanogensis in the rumen (35). Furthermore, fewer methanogens that grow more rapidly and turn over more quickly in a high passage–rate rumen could produce more methane per day, keeping the dissolved hydrogen low (35).

In conclusion, lactating dairy cows adapted to the fibrous HF-RS diet with a high dietary NDF–to-starch ratio had greatly decreased OM intake, ruminal dissolved hydrogen and dissolved methane, molar proportion of *n*-butyrate, amylolytic species, and methanogens and an increased molar proportion of acetate, gaseous hydrogen, and gaseous methane emissions and fibrolytic species compared with cows adapted to the starchy LF-RC diet. Ruminal dissolved hydrogen variation was closely associated with shifts of rumen microbiota and fermentation pathways. Lower ruminal dissolved hydrogen was associated with greater numbers of fibrolytic microorganisms that enhance fiber degradation and hydrogen generation through acetate production, whereas greater ruminal dissolved hydrogen was associated with increased amylolytic microorganisms that are capable of disposing of electrons derived from fermentation through propionate production. Methane emissions were influenced by complicated interactions between factors, including supersaturation of dissolved gases, rumen passage rate, feed digestibility, fermentation pathway, and population of methanogens, and these complex interactions will need to be understood better before we can understand how these combine to control gaseous methane emissions.

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