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Changes in fermentation profile of the reticulorumen and hindgut, and nutrient digestion in dry cows fed concentrate-rich diets supplemented with a phytogenic feed additive

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ABSTRACT

This study evaluated the effects of duration of high-concentrate feeding on ruminal and fecal fermentation profile, as well as selected systemic health biomarkers in nonlactating cows supplemented with or without a phytogenic feed additive (PHY). In addition, ruminal degradation kinetics and total-tract nutrient digestibility were evaluated when feeding either only forage or a high-concentrate diet. Nine nonlactating, cannulated Holstein cows were used in a crossover design. Each period included 1 wk of forage feeding (wk 0), diet transition, and 4 wk on the high-concentrate diet (1, 2, 3 and wk 4; 65% dry matter basis). Cows received PHY or not (control). Compared with wk 0, from wk 1 onward, cows on high concentrate showed greater reticular, ruminal, and fecal total volatile fatty acids (VFA), with a greater level of VFA in the rumen than in the hindgut. However, ruminal fermentation was modulated differently by PHY, which showed increased total VFA in wk 1 and increased butyrate in wk 2 in the particle-associated fluid of rumen. In the hindgut, PHY increased propionate in wk 3. Cows fed a high-concentrate diet from wk 1 and onward also showed greater ruminal lactate, as well as lower ruminal and fecal pH, independent of PHY. In addition, compared with cows in wk 1 on a high-concentrate diet, cows in wk 4 had a greater total VFA in free fluid of the rumen and lower fecal pH. Compared with cows at wk 0, cows at wk 1 on high concentrate onward showed greater serum amyloid A and greater activity of glutamate dehydrogenase. In contrast, the high-concentrate diet decreased in situ ruminal degradability of grass silage but increased degradability of corn grain as well as total-tract nutrient digestibility, with total-tract neutral detergent fiber digestibility being greater for cows on the PHY treatment. Overall, from the start of high-concentrate feeding, gut fermentation increased, but differently according to location or PHY, with a stronger build-up of VFA in the rumen compared with the hindgut. In addition, a longer duration on high concentrate exacerbated gut acidification. The enhancing effects of PHY on total VFA and butyrate in particle-associated fluid of the rumen suggest beneficial effects of PHY on particle-associated bacteria, likely contributing to the increased neutral detergent fiber digestibility. The greater production of ruminal butyrate with PHY may be beneficial for the host, given the health benefits of this acid, but more research is needed to elucidate the effects on gut microbiota and the effects of increased butyrate in nonlactating dairy cows.
Key words: gut fermentation, phytogenic feed additive, short-chain fatty acids, dairy cow

INTRODUCTION

Cattle meet the vast majority of their energy requirements from VFA, mainly acetate, propionate, and butyrate, which are produced primarily in the rumen as a result of microbial fermentation (Bergman, 1990). Concentrate-rich diets are highly fermentable, greatly increasing ruminal VFA yield, especially propionate yield, often at the expense of acetate (Duncan et al., 2002). This enhances the glucose (energy) supply for the host, stimulating rapid weight gain or high milk production. However, the fermentation of concentrate-rich diets and the resulting change in fermentation profile in the rumen can be detrimental for cattle health and metabolism. For example, excessive accumulation of VFA leads to acidification of the rumen milieu and ruminal acidosis, which impairs fiber degradation, because some bacterial taxa thrive at the expense of fiber-degrading strains due to low pH (Russell, 2002). Depending on the duration and severity, the drop of

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ruminal pH can also lead to systemic inflammation (Khafipour et al., 2009). In addition, the reduction of acetate and butyrate could contribute to milk fat depression, because it lowers the availability of carbons for the synthesis of de novo fatty acids as well as cholesterol and other body lipids (Steele et al., 2011; Izumi et al., 2019). From VFA, the butyrate has attracted particular research interests recently, not only as stimulator of mammary lipid synthesis (Izumi et al., 2019), but also as an important signaling molecule, able to regulate ruminal gene expression (Baldwin et al., 2018), intestinal gluconeogenesis (De Vadder et al., 2014), and the host inflammatory response (Flint et al., 2012). Additionally, shifting to ruminal butyrate production instead of propionate during high-concentrate feeding might be advantageous, because butyrate releases fewer protons from the fermentation of hexoses than acetate or propionate (Owens and Goetsch, 1988).

Feed additives that can influence rumen microbiota may shift the VFA profile and mitigate the effects of concentrate diets on ruminal fermentation; therefore, studies have indicated the potential of phytogenic additives to modulate rumen microbial fermentation (Rodriguez-Prado et al., 2008; Tager and Krause, 2011; Bueno et al., 2020). In a previous study, Neubauer et al. (2018) demonstrated that phytogenic additives modulated the rumen microbiota increasing ruminal pH and butyrate production of cows fed concentrate-rich diets. Thus, when supplemented in diets with the same chemical composition (i.e., high in starch), it seems that phytogenic compounds enhanced butyrate producers rather than propionate or acetate producers. Yet, it is not clear whether this effect is related to pH changes per se and whether it persists across various locations within the rumen, namely particle-associated fluid (**PAF**) and free fluid (**FF**), or in the hindgut. Research has shown that within the reticulorumen, microbial profiles and activities are different (Klevenhusen et al., 2017), which is understandable because of the differences in substrates (particulate matter is more insoluble; Zebeli et al., 2008). Thus, the effect of diet on the VFA production and profile might be different as well. In addition, when concentrate-rich diets are fed to cows, large amounts of starch are fermented in the hindgut, which could increase the contribution of the concentrate-rich diet to both energy supply and health issues (i.e., hindgut dysbiosis; Neubauer et al., 2020). Thus, we evaluated the effects of duration on a high-concentrate diet on ruminal and fecal fermentation profiles, as well as on selected health biomarkers in nonlactating cows supplemented or not with a phytogenic feed additive (**PHY**). Ruminal degradation kinetics and total-tract nutrient digestibility were also evaluated when feeding only forage or the high-concentrate diet. The hypothesis was that fermentation will increase throughout the gut from the start of high-concentrate feeding, and that further duration of high-concentrate feeding will exacerbate gut acidification. We also hypothesized that PHY would shift the fermentation profile toward butyrate production, with this shift being greater in the rumen than in the hindgut.

MATERIALS AND METHODS

Animals, Experimental Design, and Animal Management

The methods and protocols followed in this experiment were approved by the institutional ethics and animal welfare committee of the University of Veterinary Medicine Vienna, Austria, and the Austrian national authority (according to §§26ff of the Animal Experiments Act, Tierversuchsgesetz 2012; protocol number: BMBWF- 68.205/0003-V/3b/2019).

Nine nonlactating, multiparous, cannulated Holstein cows (916 ± 22.9 kg of BW) fitted with ruminal cannulas (Bar Diamond) were used in a crossover design. The experiment consisted of 2 periods. During each period, cows were first fed a forage-only diet for 1 wk (wk 0) and then transitioned over 1 wk to a 65% concentrate diet (DM basis; Table 1) by increasing the concentrate by 10% daily, which they consumed for an additional 4 wk (wk 1, 2, 3, and 4). Before initiation of the study, cows had grazed on pasture for 14 wk and did not receive concentrate supplementation. During the 10-wk washout interval between the 2 experimental periods, cows grazed on pasture with no supplementation.

Cows were divided according to BW in 2 blocks of 4 and 5 cows, and they were allocated to either a control diet without supplementation (**CON**) or a diet supplemented with 0.04% (DM basis) of a PHY characterized by a blend of herbs, spices, and their extracts or pure compounds that include menthol, thymol, and eugenol (PHY, Digestarom, Biomin GmbH). Due to the difficulty of homogenizing the phytogenic additive with the TMR during the week of forage feeding, PHY cows received the mineral and vitamin mix containing the additive through the ruminal cannula, while CON cows received only the mineral and vitamin mix. In the week of diet transition, the amounts dosed were adjusted according to the increasing level of dietary concentrate. Throughout the weeks on a high-concentrate diet, the phytogenic additive was first combined with the corresponding concentrate and then integrated in the TMR.

Animals were housed in a freestall barn equipped with deep litter cubicles (2.6 × 1.25 m, straw litter),

Table 1. Ingredients, chemical composition, and particle size distribution of the diets fed to nonlactating cows during the week of forage feeding and during the 4 wk of high-concentrate feeding

Item	Forage diet	High-concentrate diet	
		CON	PHY
Ingredients, % of DM			
Grass hay	10.0	0	0
Grass silage	45.0	26.3	26.3
Corn silage	45.0	8.75	8.75
CON concentrate[1]	0	65.0	0
PHY concentrate[2]	0	0	65.0
TMR chemical composition			
DM, % as fresh	34.0	47.1	47.3
CP, %	11.5	17.8	17.4
NDF, %	55.5	32.0	31.2
ADF, %	34.2	21.7	21.3
Starch, %	17.0	28.8	28.6
Ether extract, %	1.98	2.79	2.77
NFC, %	22.9	39.3	41.6
Residual OM, %	5.9	10.5	13.0
Ash, %	6.70	6.76	6.68
Particle fraction (% retained)[3]			
Long	64.5	28.6	29.6
Medium	21.3	29.0	31.2
Short	13.6	40.1	37.4
Fine	0.51	2.13	1.66

[1]The control pelleted concentrate mixture (CON) contained wheat (30.36%), triticale (18.06%), bakery by-product (23.02%), rapeseed meal (23.94%), molasses (2.99%), mineral-vitamin premix for dairy cattle (1.53%), and limestone (1.0%).

[2]The phytogenic pelleted concentrate mixture (PHY) contained wheat (30.36%), triticale (18.06%), bakery by-product (23.02%), rapeseed meal (23.94%), molasses (2.99%), mineral-vitamin premix for dairy cattle (1.53%), and limestone (1.0%). In addition, it was formulated to provide 0.04% of a phytogenic feed additive based on menthol, thymol, and eugenol in the TMR.

[3]Particle fractions were determined by Penn State Particle Separator with a 19-mm screen (long), 8-mm screen (medium), 1.18-mm screen (short), and a pan (fine) according to Kononoff et al. (2003).

and mineral blocks were freely available. Water and feed were available for ad libitum consumption, except during the short periods for blood sample collection. The TMR was mixed once daily at 0600 h using an automated feeding system (Trioliet Triomatic T15), and was offered in individual feeding troughs. Individual feed intake was continuously recorded, as feed bunks were equipped with electronic weighing scales and computer-regulated access gates (Insentec B.V.). Dry matter of TMR was determined daily by drying samples at 100°C for 24 h. Due to the low proportion of moisture in feed ingredients used in the rations, water was added to the TMR during mixing, with a target of approximately 46% of DM content. Feed offered and refusals were also recorded daily.

Collection of Feed Samples and Chemical Analyses

Individual feed ingredient samples were collected at the beginning and end of each period, whereas samples of the diets were collected and pooled weekly. At the end of the experiment, samples were analyzed. Briefly, ash was analyzed by combustion in a muffle furnace overnight at 580°C. Crude protein was analyzed following the Kjeldahl method (VDLUFA, 2012) and ether extracts using the Soxhlet extraction system (Extraction System B-811, BÜCHI Corporation). The NDF and ADF contents were determined with sodium sulfite and reported exclusive of residual ash following the official analytical methods of VDLUFA (2012) using the Fiber Therm FT 12 (Gerhardt GmbH & Co. KG) with heat-stable α-amylase for NDF analysis. Starch content was measured (K-TSTA kit; Megazyme Ltd.). Nonfiber carbohydrates were calculated as 100 − (% CP + % NDF + % ether extract + % ash); residual OM was calculated by portioning NFC into starch and residual OM (Weiss and Tebbe, 2018). Particle size distribution of TMR was measured using the method described by Kononoff et al. (2003) with a Penn State Particle Separator equipped with 3 screens (19.0, 8.0, and 1.18 mm) and a pan.

Collection of Ruminal pH Data and Analyses

Ruminal pH was monitored using the Lethbridge Research Center Ruminal pH Measurement System (LRCpH; Dascor Inc.) and following the methodology described by Penner et al. (2006). The pH systems were calibrated to pH 4.0 and 7.0 before inserting the sensors into the ventral sac of the rumen and after removal. Ruminal pH was measured every 15 min, and the data were downloaded weekly. The appropriate location of probes was confirmed at the moment of retrieval. Calculations for ruminal pH variables were conducted similar to that described in Castillo-Lopez et al. (2013) by calculating maximum, mean, minimum, and the magnitude variation in pH, as well as the time and area below pH 5.8. In addition, the ruminal acidosis index was evaluated by calculating the time that ruminal pH was below 5.8 per kg of DMI (Khiaosa-ard et al., 2018).

Collection of FF from the Reticulum and Rumen and Analysis for VFA, Lactate, and Ammonia

Samplings of FF from the reticulum and rumen were conducted at 0, 4, 8, and 12 h after feeding. These samplings were performed weekly including the week of forage feeding and the 4 wk on the high-concentrate diet using the procedure described by Zebeli et al. (2008). Briefly, 10 mL of fluid was collected from each site using a single-use 20-mL syringe each time. Samples were immediately frozen at −20°C; at the end of the experiment, VFA were determined in samples. Sample preparation and measurements of VFA were conducted

according to the protocol reported by Qumar et al. (2016) using a gas chromatography apparatus (Shimadzu GC Plus with flame-ionization detector), which was equipped with a 30 m × 0.53 mm ID (internal diameter) × 0.53 μm capillary column (Trace TR Wax, Thermo Fisher Scientific). Lactate (D-lactate, L-lactate, and total lactate) was evaluated in the FF of the rumen for samples collected at 4 h post-feeding (D-/L-Lactate assay; Megazyme Ltd.). Ammonia was determined in FF of the rumen using the indophenol reaction (Weatherburn, 1967).

Collection of PAF of the Rumen and Analysis for VFA and Lactate

Samples of PAF were collected weekly at 4 h after feeding, similar to Castillo-Lopez et al. (2014). The number of sample collection time points and the time after feeding were defined, taking into consideration the laborious nature of the sampling technique and sample processing, as well as to avoid jeopardizing feed intake and gut fermentation due to prolonged animal stress. Briefly, samples of rumen contents were collected from 4 regions (caudal ventral sac, cranial ventral sac, and 2 samples from the feed mat in the middle and dorsal rumen) using a disposable palpation sleeve for each collection. Ruminal contents were composited in a sterilized container and strained through 4 layers of gauze. Around 2 mL of PAF was frozen in liquid nitrogen, and then stored at −80°C until later analyses for ruminal VFA and lactate.

Collection of Fecal Samples and Analysis of pH and VFA

Fecal samples were collected weekly at 0, 4, 8 and 12 h after feeding. Samples were taken rectally using a palpation sleeve, and 8-mL samples were frozen at −20°C. At the end of the trial, fecal pH was measured (Mettler-Toledo AG Analytical) by direct insertion of the pH sensor into the sample. Measurements were taken in duplicate, and values were averaged. Then, VFA in feces were measured using 1 g of sample, which was diluted in 1 mL of water and followed the laboratory protocol previously described.

Evaluation of In Situ Ruminal Degradation Kinetics and Total-Tract Nutrient Digestibility

In situ ruminal incubations of corn grain, wheat grain, and grass silage were conducted similar to Paz et al. (2014) in the week of forage feeding and in wk 4 of high-concentrate feeding. The grains and silage were ground to pass through a 4- or 6-mm screen, respectively. Samples were placed in bags with a 50-μm pore size (Ankom Technologies). Incubation of all bags (20 for each type of grain and 17 for grass silage per cow) started at 0600 h. Samples were incubated for 0, 2, 4, 8, 12, and 24 h for the grains, and for 0, 2, 4, 8, 12, 24, and 48 h for grass silage. The rapidly degradable fraction (a, %), potentially degradable fraction (b, %), rate of degradation (kd, %/h), effective rumen degradability (%), and lag time (h) were determined (Ørskov and McDonald, 1979; Krieg et al., 2017).

Analysis of total-tract nutrient digestibility was conducted similar to that described by Castillo-Lopez et al. (2014). Fecal samples were taken twice a day at 0800 and 1600 h during 3 consecutive days in the week of forage feeding and in wk 4 on high concentrate. Samples were composited by cow, within sampling week. Acid-insoluble ash was used as a digesta marker. Digesta flow was calculated based on the amount of marker fed and its concentration in fecal samples. Total-tract nutrient digestibility was then calculated (May et al., 2010).

Collection of Blood Samples and Analyses

Blood samples were collected weekly from the jugular vein before the morning meal (Stauder et al., 2020). Tubes were centrifuged at 2,000 × *g* at 4°C for 15 min (Centrifuge 5804 R, Eppendorf), the supernatant was pipetted into 2-mL tubes (Eppendorf), and stored at −80°C. At the end of the trial, blood concentration of serum amyloid A (**SAA**) was determined using an ELISA kit (Tridelta Ltd.). Activities of aspartate aminotransferase (**AST**), gamma-glutamyl transferase (**GGT**), and glutamate dehydrogenase (**GLDH**) were evaluated at the Central Clinical Pathology Unit, University of Veterinary Medicine (Vienna, Austria) with an automated autoanalyzer (Cobas 6000/c501; Roche Diagnostics GmbH).

Statistical Analyses

Statistical a priori power analysis was conducted with PROC Power of SAS (version 9.4; SAS Institute) using similar fermentation data from previous experiments (Castillo-Lopez et al., 2021), which indicated a statistical power ≥85% with $\alpha = 0.05$. Data were analyzed with the PROC Mixed procedure of SAS, with sequence, experimental period, duration of high-concentrate feeding (wk 0 to wk 4), and supplementation (CON and PHY) as fixed effects, and cow within period as a random effect. The interaction between duration of high-concentrate feeding and supplementation was also tested. Data from different times (hours, weeks) from the same cow in the same treatment

were processed as repeated measures with first-order variance-covariance structure matrices, considering that the variance-covariance decays with time. Before analysis, data were checked for outliers, which were removed based on Cook's distance. Normal distribution was verified using PROC Univariate followed by the normal and plot options. When normality was not met, square root or log-transformation was applied following evaluation with the Box-Cox transformation in the TRANSREG procedure, which determined the transformation mode. The PDIFF option was also tested, allowing multiple comparisons of means. To illustrate the profile of VFA with time post-feeding in different locations of the gut, boxplot figures were constructed with R (R Core Team, 2020) and using the ggplot2 package version 3.3.5 (Wickham, 2016). The largest standard error of the mean is reported. Statistical significance was declared when $P \leq 0.05$ and tendency discussed if $0.05 < P \leq 0.10$.

RESULTS

Dietary Transition

The forage diet had 17.0% starch and 55.5% NDF, and the high-concentrate diet had 28.7% starch and 31.6% NDF (Table 1). During dietary transition, there was a decline of ruminal pH and an increment in the time that ruminal pH was <5.8 (Figure 1). Feed intake ($P < 0.01$) also increased with diet transition (Table 2).

Ruminal and Fecal pH Variables

Compared with wk 0, from wk 1 on high concentrate onward, ruminal acidification was greater ($P < 0.01$), as revealed by diverse acidosis indices (Table 2). Lowest ($P < 0.05$) mean ruminal pH was observed in wk 1; the time below pH 5.8 and ruminal acidosis indices were greatest ($P < 0.05$) in wk 1 and 4 on high concentrate. The greatest diurnal variation in ruminal pH ($P < 0.01$) was observed in wk 4. In general, ruminal pH peaks were observed before the morning meal, which decreased in the 6 h following feeding (Supplemental Figure S1, https://doi.org/10.5281/zenodo.6522815; Castillo-Lopez et al., 2022).

Compared with wk 0, from wk 1 on high concentrate onward, fecal pH was lower ($P < 0.01$) independent of PHY. Mean fecal pH reached minimum ($P < 0.05$) in wk 4 (Supplemental Figure S2, https://doi.org/10.5281/zenodo.6522815; Castillo-Lopez et al., 2022). In wk 1 on high concentrate, CON cows had a drastic reduction in fecal pH after feeding ($P < 0.05$), but PHY cows maintained fecal pH. Compared with wk 0, from wk 1 on high concentrate onward, the pattern of diurnal variation of fecal pH shifted, with pH being lowest in early morning and increasing after feeding (Figure 2).

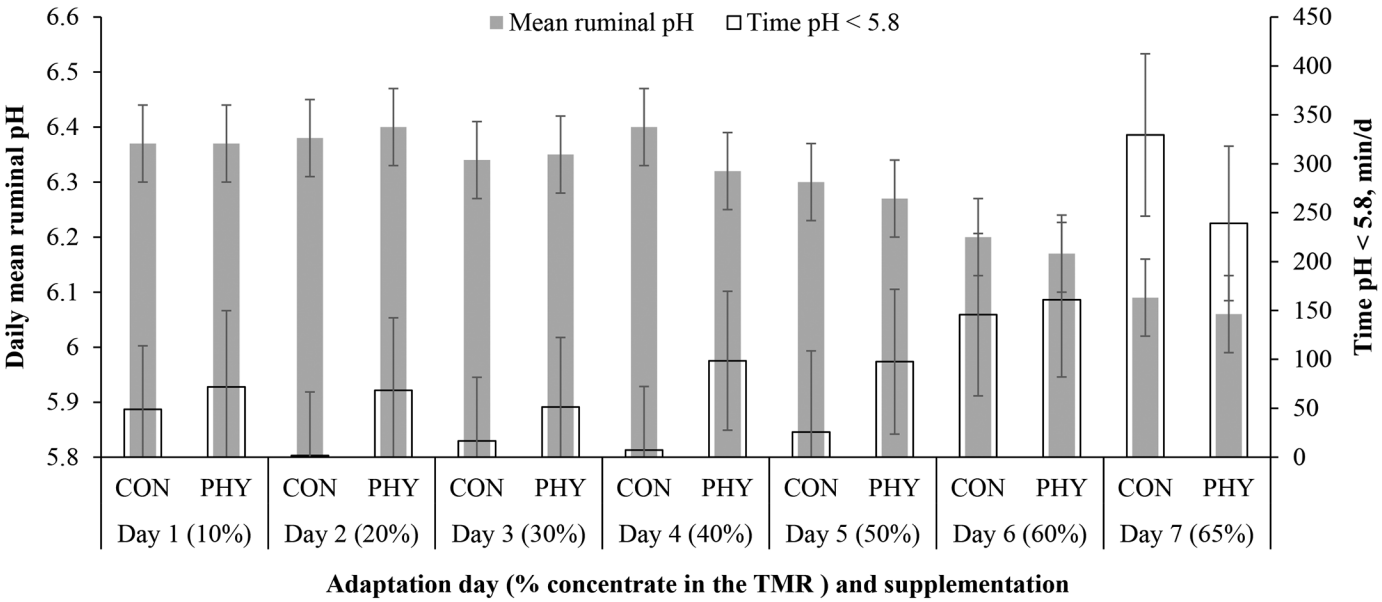

Figure 1. The change in ruminal pH variables of nonlactating cows during a 7-d adaptation period to a high-concentrate diet not supplemented (CON) or supplemented with a phytogenic feed additive (PHY). For mean ruminal pH, *P*-values were $P < 0.01$ for day, $P = 0.96$ for supplementation, and $P = 0.57$ for their interaction. For time pH <5.8, *P*-values were $P < 0.01$ for day, $P = 0.69$ for supplementation, and $P = 0.79$ for their interaction. Error bars indicate SEM.

Table 2. Effects of duration on a high-concentrate diet and supplementation with a phytogenic feed additive on dry matter intake and ruminal pH of nonlactating cows

Item	Duration on high concentrate and feed supplementation[1]										*P*-value[3]		
	Wk 0		Wk 1		Wk 2		Wk 3		Wk 4				
	CON	PHY	CON	PHY	CON	PHY	CON	PHY	CON	PHY	SEM[2]	DU	SU	I
DMI, kg	10.2	10.2	12.7	12.3	11.0	10.4	11.2	10.9	11.1	11.4	0.57	<0.01	0.69	0.41
Maximum pH	6.80	6.80	6.63	6.61	6.72	6.81	6.73	6.86	6.74	6.82	0.04	<0.01	0.29	0.16
Minimum pH	6.11	6.12	5.59	5.60	5.64	5.59	5.56	5.62	5.52	5.57	0.05	<0.01	0.90	0.45
Mean pH	6.45	6.46	6.07	6.06	6.14	6.18	6.10	6.22	6.11	6.18	0.04	<0.01	0.54	0.12
Magnitude variation	0.68	0.67	1.03	1.01	1.07	1.20	1.17	1.23	1.22	1.25	0.05	<0.01	0.58	0.21
Time for pH <5.8, min/d[4]	2.73	0.52	238	218	126	169	151	126	247	182	53.8	<0.01	0.94	0.48
Area for pH <5.8, min × pH[4]	0.35	0.04	36.6	30.3	26.3	43.6	22.0	21.7	50.2	37.8	0.49	<0.01	0.97	0.26
Acidosis index, time pH <5.8/kg of DM[4]	0.18	0.09	18.6	18.7	11.0	17.3	13.0	11.6	22.6	15.0	6.59	<0.01	0.81	0.18

[1]CON = control diet without phytogenic supplementation; PHY = supplementation with 0.04% of a phytogenic feed additive based on menthol, thymol, and eugenol. Wk 0 corresponds to measurements taken before the start of high-concentrate feeding.
[2]The largest standard error of the mean.
[3]*P*-values for the main effects of duration on high concentrate in weeks (DU), the main effects of feed supplementation (SU), and the interaction of duration on high concentrate × feed supplementation (I).
[4]Because of a lack of normal distribution, data were subjected to square root transformation before statistical analysis, and then back-transformed.

Profile of VFA

VFA in FF of the Rumen and Reticulum. Compared with wk 0, from wk 1 on high concentrate onward, total VFA was greater ($P < 0.01$) in the FF of both rumen (Table 3) and reticulum (Supplemental Table S1, https://doi.org/10.5281/zenodo.6522815; Castillo-Lopez et al., 2022), notably with time after feeding (Supplemental Figures S3 and S4, https://doi.org/10.5281/zenodo.6522815; Castillo-Lopez et al., 2022). Specifically, total VFA was greatest ($P < 0.05$) in wk 4. In addition, compared with wk 0, acetate was lower ($P < 0.01$) from wk 1 onward, and we found an interaction ($P < 0.05$) between duration on high concentrate and PHY, with acetate being lower for PHY in wk 2. Acetate decreased with time post-feeding ($P < 0.01$) from wk 1 to wk 4. In contrast, propionate ($P < 0.01$) was greater, which also increased with time post-feeding. There was an interaction between duration on high concentrate and PHY for butyrate in FF, with this acid being greater for PHY in wk 2 ($P < 0.01$) but

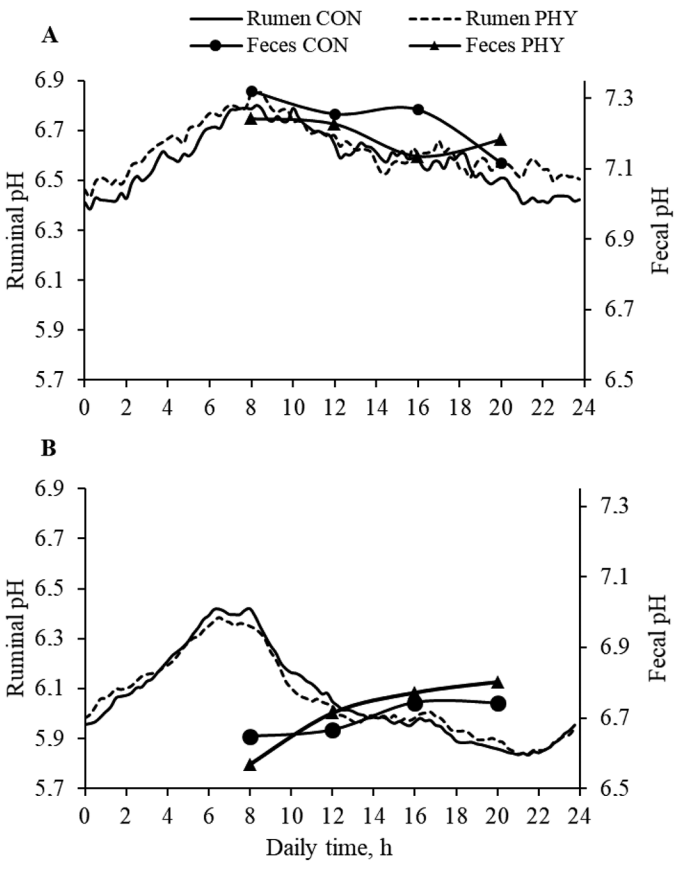

Figure 2. Diurnal variations of ruminal and fecal pH for forage feeding (A), and for high grain feeding (B) in nonlactating cows not supplemented (CON) or supplemented with a phytogenic feed additive (PHY).

lower in wk 4. Butyrate generally increased ($P < 0.01$) with time post-feeding, especially on high concentrate. Compared with wk 0, isobutyrate and isovalerate were lower in the FF of both rumen and reticulum from wk 1 onward ($P < 0.01$).

VFA in PAF of the Rumen. Similar to FF, compared with wk 0, from wk 1 onward, total VFA, propionate, butyrate, and valerate were greater ($P < 0.01$), but acetate, isobutyrate, and isovalerate were lower in PAF (Table 4). Contrary to FF, PHY increased total VFA in PAF in wk 1, as demonstrated by the interaction ($P = 0.05$) between duration on high concentrate and PHY. In addition, PHY increased ($P < 0.05$) butyrate and tended to lower acetate in wk 2.

VFA in Feces. Overall, the fermentation profile of feces was different from any of the rumen locations (Supplemental Table S2, https://doi.org/10.5281/zenodo.6522815; Castillo-Lopez et al., 2022), particularly with time post-feeding (Supplemental Figures S3 and S4), with greater acetate but lower butyrate and propionate; however, similar to rumen variables, from wk 1 onward, total VFA, propionate, butyrate, and valerate were greater ($P < 0.01$), but acetate, isobutyrate, and isovalerate were lower ($P < 0.01$), compared with wk 0. Specifically, total VFA was greatest ($P < 0.01$) in wk 1. We observed an interaction between duration on high concentrate and PHY ($P < 0.05$) on propionate, valerate, and isobutyrate, with greater values for PHY in wk 3 on high concentrate. In addition, we observed an interaction ($P < 0.05$) between duration on high concentrate and PHY on butyrate and isovalerate, with lower values for PHY in wk 4.

Lactate and Ammonia in the Rumen

Supplemental Figures S5, S6, and S7 (https://doi.org/10.5281/zenodo.6522815; Castillo-Lopez et al., 2022) illustrate ruminal D-, L-, and total lactate, respectively, in PAF and FF of the rumen. In both PAF and FF, greater ($P < 0.01$) D-, L-, and total lactate were observed from wk 1 onward, compared with wk 0. D-Lactate and total lactate were greater ($P < 0.05$) in FF compared with PAF in wk 3 and 4. In addition, from wk 1 onward, L-lactate was generally greater in FF ($P < 0.05$) compared with PAF, and PHY tended ($P = 0.06$) to increase L-lactate. Compared with wk 0, we found greater ($P < 0.05$) ammonia in FF from wk 1 onward (Supplemental Figure S8, https://doi.org/10.5281/zenodo.6522815; Castillo-Lopez et al., 2022).

In Situ Ruminal Degradation Kinetics

For corn DM (Table 5), degradability of potentially degradable fraction and effective rumen degradability

Table 3. Effects of duration on a high-concentrate diet and supplementation with a phytogenic feed additive on the profile of volatile fatty acids in the free fluid of the rumen of nonlactating cows (values are daily means)

	Duration on high concentrate and feed supplementation[1]										*P*-value[3]		
	Wk 0		Wk 1		Wk 2		Wk 3		Wk 4				
Item	CON	PHY	CON	PHY	CON	PHY	CON	PHY	CON	PHY	SEM[2]	DU	SU	I
Total VFA, m*M*	89.5	89.3	123	123	116[x]	130[y]	130	124	127	136	5.09	<0.01	0.69	0.17
% of total VFA														
Acetate (A)[4]	64.6	65.0	58.6	57.7	59.1[a]	54.6[b]	60.6	60.2	62.0	62.9	1.02	<0.01	0.38	<0.01
Propionate (P)	18.0	17.9	20.0	20.3	21.5	24.2	20.9	21.4	21.0	22.2	1.33	<0.01	0.64	0.16
Butyrate[4]	10.9	10.8	15.4	16.1	13.6[b]	14.9[a]	13.4	13.2	11.8[a]	10.3[b]	0.44	<0.01	0.98	<0.01
Valerate[4]	1.79	2.12	2.23	2.25	2.19	2.43	1.81	1.75	1.80	1.84	0.14	<0.01	0.36	0.05
Isobutyrate	1.58	1.65	1.23	1.25	1.28	1.17	1.15	1.38	1.21	1.20	0.07	<0.01	0.53	0.12
Isovalerate	2.45	2.52	1.85	1.95	1.72	1.95	1.54	1.74	1.48	1.57	0.15	<0.01	0.74	0.81
A:P	3.68	3.65	3.05	2.90	2.96	2.56	3.30	3.36	3.16	3.02	0.26	<0.01	0.88	0.35

[a,b]Within corresponding week, means with different superscripts differ between CON and PHY ($P < 0.05$).

[x,y]Within corresponding week, means with different superscript tended to differ between CON and PHY ($0.05 < P \leq 0.10$).

[1]CON = control diet without phytogenic supplementation; PHY = supplementation with 0.04% of a phytogenic feed additive based on menthol, thymol, and eugenol. Wk 0 corresponds to measurements taken before the start of high-concentrate feeding.

[2]The largest standard error of the mean. Means include the values of samples collected at 0, 4, 8, and 12 h post-feeding.

[3]*P*-values for the main effects of duration on high concentrate in weeks (DU), the main effects of feed supplementation (SU), and the interaction of duration on high concentrate × feed supplementation (I).

[4]Because of a lack of normal distribution, data were subjected to log-transformation before statistical analysis, and then back-transformed.

were greater ($P < 0.01$), but lag time ($P = 0.09$) tended to be lower for the high-concentrate diet. For wheat DM, degradability of potentially degradable fraction increased ($P < 0.01$) with PHY, and lag time tended to increase ($P = 0.07$) with PHY. For grass silage DM, the potentially degradable fraction and effective rumen degradability decreased ($P < 0.01$), but lag time was higher ($P < 0.05$) with high concentrate.

Apparent Total-Tract Nutrient Digestibility

With high-concentrate feeding (Table 6), DM digestibility was increased ($P < 0.01$), independent of PHY. In addition, intakes and digestibility of protein ($P < 0.05$), ether extract ($P < 0.05$), and starch ($P < 0.05$) were greater with high-concentrate feeding. The intake ($P < 0.05$) of NDF decreased, but the digestibility was greater ($P < 0.01$) by 8% for the high-concentrate diet compared with the forage diet. Total-tract digestibility of NDF was enhanced ($P < 0.05$) by PHY.

Acute Phase Proteins and Liver Enzymes

Duration on high concentrate affected ($P < 0.01$) the concentrations of SAA and activity and GLDH (Table 7). Specifically, SAA was greatest ($P < 0.05$) in wk 2 on high concentrate; this value was 3.2-fold greater compared with wk 0. Activity of GLDH reached maximum value ($P < 0.05$) in wk 3 and 4. However, activity of AST ($P = 0.12$) and activity of GGT ($P = 0.26$) were not affected by duration on high concentrate. Additionally, PHY supplementation did not influence the measured variables ($P \geq 0.47$).

DISCUSSION

This study aimed to evaluate the effects of duration on a high-concentrate diet on the VFA profile in different locations of the gut of nonlactating cows supplemented, or not, with PHY. The study mimicked an acidosis challenge by changing from forage only to a high-concentrate diet, which was fed for 4 wk. In agreement with our hypothesis, results revealed that from the start of high-concentrate feeding, fermentation and total VFA increased throughout the gut, but differently according to location or PHY supplementation. Specifically, the increase in total VFA was greater in the FF of rumen and reticulum than in feces. This difference may be because of greater availability of readily fermentable substrates in the rumen, which decreased ruminal pH (Zebeli et al., 2008). In this context, contrasting in situ degradation of forages, the greater in situ rumen degradability, and the tendency for lower lag time for corn grain with a high-concentrate diet may be because

Table 4. Effects of duration on a high-concentrate diet and supplementation with a phytogenic feed additive on the profile of volatile fatty acids in particle-associated fluid of the rumen of nonlactating cows

	Duration on high concentrate and feed supplementation[1]										*P*-value[3]		
	Wk 0		Wk 1		Wk 2		Wk 3		Wk 4				
Item	CON	PHY	CON	PHY	CON	PHY	CON	PHY	CON	PHY	SEM[2]	DU	SU	I
Total VFA, m*M*	107	96.4	120[b]	155[a]	138	146	137	140	142	154	8.51	<0.01	0.07	0.05
% of total VFA														
Acetate (A)[4]	67.0	67.0	61.6	60.1	58.9[x]	55.8[y]	61.5	58.9	61.0	58.8	1.92	<0.01	0.20	0.71
Propionate (P)[4]	17.6	17.1	18.7	19.5	21.7	23.8	20.6	22.1	20.7	22.4	1.70	<0.01	0.50	0.90
Butyrate	10.3	11.1	15.4	15.9	14.7	15.3	13.4	14.2	13.4	14.3	0.76	<0.01	<0.05	0.99
Valerate	1.74	1.64	1.98	2.16	2.03[b]	2.53[a]	1.99	2.11	1.98	1.96	0.13	<0.01	0.13	0.10
Isobutyrate	1.22	1.26	0.87	0.88	0.94	0.78	0.96	1.05	1.07[a]	0.85[b]	0.08	<0.01	0.44	0.08
Isovalerate	2.00	1.89	1.44	1.50	1.72	1.81	1.58	1.62	1.77	1.66	0.14	<0.01	0.95	0.88
A:P	3.84	3.91	3.41	3.10	2.81	3.61	3.33	3.15	3.05	2.91	0.31	<0.01	0.40	0.91

[a,b]Within corresponding week, means with different superscripts differed between CON and PHY ($P < 0.05$).

[x,y]Within corresponding week, means with different superscript tended to differ between CON and PHY ($0.05 < P \leq 0.10$).

[1]CON = control diet without phytogenic supplementation; PHY = supplementation with 0.04% of a phytogenic feed additive consisting of a combination of menthol, thymol, and eugenol. Wk 0 corresponds to measurements taken before the start of high-concentrate feeding.

[2]The largest standard error of the mean. Means include the values of samples collected 4 h post-feeding.

[3]*P*-values for the main effects of duration on high concentrate in weeks (DU), the main effects of feed supplementation (SU), and the interaction of duration on high concentrate × feed supplementation (I).

[4]Because of a lack of normal distribution, data were subjected to log-transformation before statistical analysis, and then back-transformed.

Table 5. Effects of feeding all forage or a high-concentrate diet and supplementation with a phytogenic feed additive[1] on in situ ruminal degradation kinetics of DM of corn grain, wheat grain, and grass silage in nonlactating cows

	Forage		High concentrate			*P*-value[4]		
Item[2]	CON	PHY	CON	PHY	SEM[3]	D	SU	I
Corn								
a, %	31.5	32.6	32.6	31.9	0.82	0.78	0.83	0.26
b, %	43.6	41.4	59.9	60.0	3.17	<0.01	0.70	0.68
kd, %/h	8.56	10.1	8.05	10.7	1.82	0.98	0.08	0.61
Lag time, h	6.30	7.47	5.81	5.47	0.73	0.09	0.56	0.30
Effective rumen degradability	57.5	58.6	68.8	70.9	1.14	<0.01	0.09	0.69
Wheat								
a, %	33.2	33.0	36.3	32.2	1.71	0.50	0.21	0.26
b, %	41.6	42.3	36.9	42.1	1.42	0.08	<0.05	0.11
kd, %/h	29.2	28.1	30.5	34.5	2.63	0.14	0.58	0.34
Lag time, h	1.80	3.10	2.07	2.81	0.55	0.99	0.07	0.61
Effective rumen degradability	67.0	67.1	66.3	66.8	0.84	0.59	0.73	0.79
Grass silage								
a, %	34.0	34.8	34.8	35.8	0.66	0.20	0.16	0.89
b, %	45.0	42.5	38.3	38.1	1.73	<0.01	0.41	0.51
kd, %/h	5.70	6.80	5.90	7.10	1.01	0.74	0.27	0.97
Lag time, h	6.36	5.84	7.92	7.87	0.75	0.02	0.68	0.74
Effective rumen degradability	57.8	59.2	55.2	54.9	0.90	<0.01	0.53	0.25

[1]CON = control diet without phytogenic supplementation; PHY = supplementation with 0.04% of a phytogenic feed additive based on menthol, thymol, and eugenol.

[2]a = rapidly degradable fraction; b = potentially degradable fraction; kd = constant rate of degradation of fraction b; effective ruminal degradability with a passage rate of 6% for grains and 4% for grass silage.

[3]The largest standard error of the mean.

[4]*P*-values for the main effects of diet (D), the main effects of supplementation (SU), and the diet × supplementation interaction (I).

of lower ruminal pH. These findings might be explained by the proliferation of amylolytic bacteria in cattle fed high amounts of concentrate (Fernando et al., 2010). In contrast, the enhanced total-tract nutrient digestibility with high-concentrate diets may reflect greater availability of nutrients, due to smaller feed particle size, which increased surface area and facilitated microbial attachment as previously observed (McAllister et al., 1993); however, the accumulation of VFA with time post-feeding was different across the gut, with the hindgut showing a smoother build-up of acids throughout the day, especially during high-concentrate feeding, which agrees with the subtler diurnal changes in hindgut pH compared with the rumen. The latter findings may be due to a more uniform flow of nutrients to the intestines during the day, as opposed to a sudden arrival of feed in the rumen during each meal. These data support the different patterns of diurnal variation of pH across the gut. Interestingly, during most of the high-concentrate feeding, fecal pH was lowest in the early morning, which suggests that for evaluation of hindgut acidification, measurements should include data from early morning to capture the nadir of pH, a pattern that opposes ruminal pH variation, where the nadir is reached after the first meal. The reduction of fecal pH with high-concentrate feeding may be because of an increase in digesta passage rate, which likely increases the flow of starch to the hindgut; however, results suggest that PHY may modulate hindgut pH, particularly at the start of high-concentrate feeding, possibly by enhancing the uptake of protons across the intestinal mucosa and their exchange with buffers (Hopfer and Liedtke, 1987).

Our results also revealed that PHY increased total VFA in PAF during wk 1 on high concentrate, suggesting stimulation of microbial activity of particle-associated ruminal bacteria, which represent the largest proportion of ruminal bacteria (Sung et al., 2013). The lack of an effect of PHY on total VFA in FF during high-concentrate feeding suggests that produced acids were rapidly absorbed (Bergman, 1990), possibly enhancing metabolizable energy supply. In addition, the enhanced total VFA in PAF with PHY may reflect greater feed degradation and agrees with the greater total-tract NDF digestibility in PHY-supplemented cows. However, the lack of an effect of PHY on total VFA in the hindgut might be due to the presence of a different microbial community (Dankwa et al., 2021), which did not respond as the foregut microbiota in terms of VFA production; therefore, findings show differential effects of diet and PHY on total VFA across the gut.

The total VFA and fermentation dynamics observed within the rumen are also in agreement with the content of lactate. Specifically, during the acidosis challenge, lactate was greater for PHY compared with CON cows in FF. It is possible that the increased fermentation in

Table 6. Effects of feeding all forage or a high-concentrate diet and supplementation with a phytogenic feed additive[1] on apparent total-tract nutrient digestibility in nonlactating cows

	Forage		High concentrate			*P*-value[3]		
Item	CON	PHY	CON	PHY	SEM[2]	D	SU	I
DM								
Intake, kg/d	10.3	10.1	11.1	11.4	0.59	<0.05	0.87	0.65
Digestibility, %	68.3	68.0	81.8	84.1	1.62	<0.01	0.61	0.48
CP[4]								
Intake, kg/d	1.19	1.14	1.98	2.00	0.09	<0.01	0.93	0.50
Digestibility, %	65.3	62.4	81.7	81.8	2.06	<0.01	0.47	0.44
Ether extract[4]								
Intake, kg/d	0.20	0.19	0.30	0.32	0.02	<0.01	0.71	0.36
Digestibility, %	65.0	63.1	79.5	83.2	2.42	<0.01	0.76	0.34
NDF[4]								
Intake, kg/d	5.71	5.70	3.55	3.59	0.26	<0.01	0.97	0.85
Digestibility, %	62.7	67.5	69.8	74.7	1.95	<0.01	<0.05	0.65
Starch[4]								
Intake, kg/d	1.65	1.70	3.18	3.26	0.14	<0.01	0.67	0.78
Digestibility, %	99.2	98.0	99.6	99.6	0.12	<0.01	0.43	0.12

[1]CON = control diet without phytogenic supplementation; PHY = supplementation with 0.04% of a phytogenic feed additive based on menthol, thymol, and eugenol.

[2]The largest standard error of the mean.

[3]*P*-values for the main effect of diet (D), the main effects of supplementation (SU), and diet × supplementation interaction (I).

[4]Because of a lack of normal distribution, data were subjected to log-transformation before statistical analysis, and then back-transformed.

PAF by PHY resulted in greater production not only of VFA, but also lactate with subsequent release in the FF; however, in contrast to the absorption of VFA, lactate is not absorbed and, thus, accumulates. The greater total lactate in the FF in PHY cows during high-concentrate feeding may also reflect the dynamics of microbial digestion of readily available carbohydrates, whereby primary microbial colonizers digest feed and release soluble nutrients such as glucose and other sugars (Mackenzie, 1967; McAllister et al., 1994; Wang and McAllister, 2002). Then, the released nutrients are fermented with subsequent reduction of pyruvate to lactate (Mackenzie, 1967), but without negative effects on ruminal pH or fiber degradation, as shown by improved total-tract NDF digestibility. Additionally, the greater lactate in the FF with PHY could be due to the enhanced degradation of carbohydrates, allowing proliferation of lactate producers, with the levels of lactate from this study being comparable to reported values (Nagaraja and Titgemeyer, 2007; Khafipour et al., 2009).

Our results further show that the duration of the ruminal acidosis challenge can influence concentration of total VFA and the regulation of gut pH. Specifically, the increase in total VFA in the FF of the rumen in wk 4 on the high-concentrate diet agrees with the increased duration of pH being <5.8 and ruminal acidosis index in that week. These observations support the notion that further duration on a concentrate-dense ration may impair absorption of VFA (Wilson et al., 2012), compromise ruminal pH balance (Wilson et al., 2012), and increase the severity and risk for SARA (Dohme et al., 2008). Our findings also suggest that increased time on high concentrate may lead to erratic ruminal pH, exacerbating the negative effects of high concentrate intake, as demonstrated by the greater magnitude of pH variation in wk 4 of the acidosis challenge. In contrast, total VFA in the hindgut was greater by at least 10 m*M* in the week immediately after diet transition compared with the rest of the weeks, which suggests limited absorption or utilization of VFA shortly after adaptation to high concentrate; however, similar to the rumen, hindgut acidification may be exacerbated with increased time on high concentrate, as illustrated by lowest fecal pH in wk 4, and reflect impaired regulation of pH and buffering with duration on high concentrate. This condition can affect animal health and production, because of the role of the hindgut in nutrient utilization and microbial fermentation of feed (Gressley et al., 2011). In this regard, our observations for changes in gut pH when cows transitioned to high concentrate agree with the levels of systemic health biomarkers. That is, the concomitant increase of the acute phase protein SAA and ruminal acidosis index probably reflected a response by the host for detoxification and effective clearance of produced lipopolysaccharides, which resulted from the negative effect of low pH on gut bacteria. Findings indicate that cows develop signs of systemic inflammation, although the level of SAA was low and comparable with values within the normal range (Cannizzo et al., 2012), and suggest a low degree of inflammation. Furthermore, the increment in

Table 7. Effects of duration on a high-concentrate diet and supplementation with a phytogenic feed additive on systemic and liver health biomarkers in nonlactating cows

	Duration on high concentrate and feed supplementation[2]													
	Wk 0		Wk 1		Wk 2		Wk 3		Wk 4			*P*-value[4]		
Item[1]	CON	PHY	CON	PHY	CON	PHY	CON	PHY	CON	PHY	SEM[3]	DU	SU	I
SAA, μg/mL	3.33	3.05	6.77	4.66	9.63	11.1	5.44	10.6	6.09	10.1	1.32	<0.01	0.47	0.29
AST, U/L	70.9	68.9	73.3	74.2	82.2	92.6	88.7	90.5	84.6	87.5	14.3	0.12	0.82	0.88
GLDH, U/L	3.65	3.14	6.59	5.82	6.71	8.45	8.90	8.30	8.01	7.82	2.54	<0.01	0.75	0.58
GGT, U/L	20.6	22.6	22.3	23.3	25.0	26.0	25.6	26.3	17.2	25.3	3.64	0.26	0.59	0.58

[1]Because of a lack of normal distribution, data for these variables were subjected to log-transformation before statistical analysis, and then back-transformed. SAA = serum amyloid A; AST = aspartate aminotransferase; GLDH = glutamate dehydrogenase; GGT = gamma-glutamyl transferase.

[2]CON = control diet without phytogenic supplementation; PHY = supplementation with 0.04% of a phytogenic feed additive based on a combination of menthol, thymol, and eugenol. Wk 0 corresponds to measurements taken before the start of high-concentrate feeding.

[3]The largest standard error of the mean.

[4]*P*-values for the main effects of duration on high concentrate in weeks (DU), the main effects of feed supplementation (SU), and the interaction of duration on high concentrate × feed supplementation (I).

the enzymatic activity of GLDH during the acidosis challenge reflects only mild hepatic damage or impaired liver function, because values were within the normal range (Bobe et al., 2004).

In further agreement with our hypothesis, PHY supplementation influenced production of individual VFA, but differed according to location. Specifically, PHY increased the production of butyrate in PAF of the rumen. This observation suggests stimulation of the butyryl CoA-acetyl CoA transferase pathway (Duncan et al., 2002), which has been reported in some ruminal bacteria such as *Butyrivibrio* sp. (Diez-Gonzalez et al., 1999), a major butyrate producer (Russell, 2002). In this metabolic route, after production of pyruvate through the Embden-Meyerhof-Parnas pathway, butyryl CoA is exchanged with acetate to yield acetyl-CoA and butyrate. Then, acetate is utilized for the generation of acetoacetyl-CoA with subsequent synthesis of butyrate and regeneration of acetate; therefore, findings support previous reports regarding effects of phytogenic compounds (Rodriguez-Prado et al., 2008; Neubauer et al., 2018), which could have positive effects on animal physiology and production performance because of the role of this VFA (Allen, 2020). For example, butyrate is important for maintaining ruminal health and function, because it is extensively used as an energy source by the ruminal epithelium (Miguel et al., 2019). In addition, butyrate plays an active role as a signaling molecule in several metabolic processes (Baldwin et al., 2018). In particular, butyrate is known to act as a histone deacetylase inhibitor or as an activator of the G protein-coupled receptor 43 in the gut, processes that have been shown to have anti-inflammatory functions (Johnstone, 2002; Flint et al., 2012) or improve glucose homeostasis (Lin et al., 2012; De Vadder et al., 2014).

The lower butyrate in FF of rumen and reticulum in PHY cows in wk 4 on high concentrate might reflect enhanced utilization of this acid once released into the FF as a response to increased production from previous weeks. Nevertheless, the consequences of this fermentation shift remain to be completely elucidated; however, the increase in butyrate due to PHY did not occur in the hindgut, even though some intestinal bacteria such as *Roseburia* sp. and *Faecalibacterium* sp. have been reported to use the butyryl CoA-acetyl CoA transferase pathway (Duncan et al., 2002). The latter findings suggest that PHY could only stimulate bacteria using the butyryl CoA-acetyl CoA transferase pathway in the rumen. In fact, in the hindgut, butyrate was lower for PHY during wk 3 and 4 on high concentrate, but with a constant supply after feeding. This observation may reflect improved use of this acid during the day, which could be beneficial, given the positive role of butyrate on intestinal health (Vital et al., 2017).

This study demonstrated that diet and PHY can modulate other VFA differently across the gut. For example, the lower percentage of propionate and the lack of sudden accumulation after feeding in the hindgut, compared with the rumen, may be because starch is extensively fermented in the rumen, so that digesta reaching the hindgut contains low levels of starch (Brake and Swanson, 2018). In addition, an increase in the proportion of propionate was found in the hindgut in PHY-supplemented cows in wk 3 of high-concentrate feeding. This is a beneficial outcome, given the role of propionate as a glucose precursor and suggests stimulation of propionate-producing bacteria by PHY in the hindgut. Moreover, our findings show that the percentage of acetate in the hindgut was greater compared with the rumen, but with less variation post-feeding, and emphasize a more uniform fermentation of fiber throughout the day. Furthermore, in this study, isobutyrate and isovalerate decreased with diet transition, which may reflect enhanced use of these acids and increased bacterial protein synthesis resulting from increased supply of degradable protein and available carbohydrates; however, the differential effect of PHY on branched VFA across the gut may be due to differential effects of PHY on specific bacterial taxa participating in generation and utilization of these acids across the gut.

CONCLUSIONS

The ruminal acidosis challenge stimulated fermentation throughout the gut of nonlactating cows, but differently according to location or supplementation, with greater total VFA in the rumen than hindgut. The steadier fermentation in the hindgut, compared with the rumen, may reflect a more uniform flow of nutrients during the day, contrasting with the sudden arrival of substrates in the rumen during meals. The PHY increased total VFA in PAF when feeding high concentrate, possibly reflecting increased nutrient digestion and increasing the supply of energy for the host, but without affecting ruminal pH during the acidosis challenge. The PHY elicited changes on ruminal fermentation, increasing butyrate in PAF, and suggested enhancement of the butyryl CoA-acetyl CoA transferase pathway in bacteria. The enhanced butyrate may be beneficial for the host, but further research is needed to fully elucidate effects on the gut microbiota, as well as the effects of increased butyrate in nonlactating cows.

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