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Replacing concentrates with a high-quality hay in the starter feed of dairy calves: II. Effects on the development of chewing and gut fermentation, and selected systemic health variables

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ABSTRACT

Early development of the rumen, rumination, and fermentation is highly important in dairy calves. Yet, common rearing practices with feeding of concentrate-rich starters may jeopardize them because of lacking physically effective fiber (peNDF). The main objective of this study was to establish the influence of the composition of the calf starter feed (only forage with 2 different qualities or concentrate-rich starter diet) on chewing behavior, rumen development, rumen and hindgut fermentation, and selected systemic health and stress variables of dairy calves. The experiment was carried out with 40 newborn Holstein-Friesian calves, randomly assigned to 4 different solid feed treatments: MQH = 100% medium-quality hay (9.4 MJ metabolizable energy, 149 g of crude protein, and 522 g of neutral detergent fiber/kg of dry matter); HQH = 100% high-quality hay (11.2 MJ of metabolizable energy, 210 g of crude protein, 455 g of neutral detergent fiber/kg of dry matter); MQH+C = 30% MQH + 70% starter concentrate; HQH+C = 30% HQH + 70% starter concentrate). All calves were up to 14 wk in the trial and received acidified whole milk ad libitum in the first 4 wk of life, thereafter in reduced quantity until weaning on 12 wk of age. Water and the solid feed treatments were available ad libitum throughout the trial. Chewing activity was measured in wk 4, 6, 10, and 12 using RumiWatch halters. Until wk 3, rumen fluid, feces and blood were sampled weekly, thereafter every 2 wk. Rumen mucosal thickness (RMT) was measured on the same days with rumen fluid samples. Data showed that calves fed the HQH diet consumed more peNDF and this was associated with longer rumination time (591 min/d) and more ruminating boli (709 boli/d) than calves fed concentrate-rich diets (MQH+C: 430 min/d, 518 boli/d; HQH+C: 430 min/d, 541 boli/d), whereas the MQH group was intermediate (539 min/d, 644 boli/d). Ruminal and fecal pH were higher in calves fed only hay (especially MQH) compared with calves with concentrate supplementation. In both hay-fed groups, ruminal and fecal short-chain fatty acids were shifted toward acetate, whereas only the HQH diet increased the butyrate proportion in the ruminal short-chain fatty acids profile. Ruminal ammonia concentration was at a high level only in the first 3 wk and decreased thereafter. Feeding HQH tended to increase ruminal ammonia, likely because of its high crude protein content and ruminal degradability as well as lower assimilation from rumen microbes. The RMT similarly, though nonlinearly, increased in all groups over the course of the experiment. When using RMT as an indicator of rumen development in dairy calves in the practice, our data suggest an RMT of 1.7 mm and >2 mm at wk 5 and 10 of life, respectively. Feeding did not affect the blood levels of aspartate aminotransferase, gamma glutamyl transferase, glutamate dehydrogenase, and cortisol. In conclusion, feeding high-quality hay, instead of concentrate-rich starter feeds, resulted in improved rumination and ruminal fermentation profile, without affecting ruminal pH and systemic and stress health variables.

Key words: hay quality, calf health, blood parameter, chewing activity, rumen fermentation

INTRODUCTION

Calf nutrition is gaining importance in today's dairy cattle production. One reason is that nutrition and development of young calves has important long-term influences on lifetime productivity of dairy cattle (Meale et al., 2017). In addition, the average life span of cows is getting shorter and, therefore, rearing is of crucial importance to maintain herd production stock by

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reaching the insemination weight early (Boulton et al., 2017). Therefore, early development of forestomaches, rumination behavior, and establishment of ruminal fermentation have become crucial aspects in dairy calf rearing. Feeding techniques with solid feeds can influence these physiological processes in dairy calves (Warner et al., 1956; Tamate et al., 1962; Khan et al., 2016). Feeding of calf starter concentrates, rich in grains and pulses, soon after birth enhances the energy and protein supply of calves and leads to higher growth rates. Calf starters are highly palatable and digestible, because of their generally low fiber content, resulting in enhanced production of short-chain fatty acids (**SCFA**) such as acetate, propionate, and butyrate, and the presence of high amount of readily fermentable carbohydrate of concentrates can increase microbial protein yield by using ammonia nitrogen in rumen in adult cattle (Aldrich et al., 1993). Among SCFA, butyrate has gained research attention as the main stimulator of rumen mucosa papillae growth and is generally considered as beneficial for the rumen development (Sander et al., 1959; Baldwin and McLeod, 2000; Castells et al., 2012).

However, feeding concentrate-rich starter feeds is increasingly associated with negative effects on calf health, such as impaired rumination and salivation, which affect digestibility processes in the rumen, and most importantly, the overall health of gastrointestinal tract (Khan et al., 2016). Accordingly, an increased risk of ruminal acidosis, hyperkeratosis (Zitnan et al., 1998), and agglutination of the rumen papillae (Bull et al., 1965; Castells et al., 2012) has been reported when starter diets containing high amounts of concentrates are fed (Khan et al., 2016; Gelsinger et al., 2020). High intakes of concentrates have been shown to compromise the rumen epithelial barrier function, which might enable the leaking of hazardous substances into the blood (Li et al., 2012; Klevenhusen et al., 2013), thereby increasing the risk for systemic health disorders. Blood metabolites such as aspartate aminotransferase (**AST**), gamma glutamyl transferase (**GGT**), glutamate dehydrogenase (**GLDH**), and cortisol are common markers to monitor systemic calf health and their stress level (Zanker et al., 2001; Yu et al., 2019). In addition, rumination behavior has been proposed as a good indicator for monitoring the rumen development and feed intake in rearing dairy calves (Eslamizad et al., 2018).

Feeding forages such as hay can promote rumination; however, insufficient hay quality might compromise feed intake in calves. We hypothesized that palatable, high-quality hay, rich in water-soluble carbohydrates (**WSC**) and CP (both approximately 200 g/kg of DM), could be an alternative to concentrate-rich starter diets in the feeding of dairy calves. Supplementing such aromatic, early-harvested, and indoor-dried hay at the expense of starchy concentrates might increase the structural effectiveness of the starter diet, enhancing chewing activity and likely rumen pH, and stimulating SCFA production and improving energy supply, as observed in dairy cows (Kleefisch et al., 2017). In a recent study, we observed that feeding young dairy calves such high-quality hay resulted in the same energy and nutrient intake and growth performance as in calves fed concentrate-rich starter feeds and enhanced ketogenesis and cholesterolgenesis on the time around weaning (Terler et al., 2022). The extent by which hay feeding and hay quality can influence the development of rumination behavior, rumen and hindgut fermentation, as well as the rumen morphology of calves, has not been tested. Therefore, the aim of this research was to evaluate the effects of starter diets differing in hay quality and concentrate inclusion on chewing activity, ruminal and fecal fermentation parameters, rumen mucosal thickness (**RMT**), and selected health and stress-related blood variables in dairy calves. Our main hypothesis was that the replacement of starchy concentrates (in the form of calf starter) with high-quality hay will improve chewing activity (eating and ruminating) and thus promote the ruminal and systemic health. In addition, increased fiber ingestion through the provision of hay will support rumen and hindgut fermentation and stimulate the rumen wall development. Data on nutrient intake, growth performance, and blood parameters related to nutrition status are reported in our companion paper (Terler et al., 2022).

MATERIALS AND METHODS

Animals, Diets, and Experimental Setup

The experimental protocols were approved by the national authority according to §26 of the Law for Animal Experiments, Tierversuchsgesetz 2012-TVG (GZ: BMBWF-66.019/0016-V/3b/2019).

The results presented here are part of larger study (Terler et al., 2022) and detailed information about the calves, feeding, feeds, and experimental setup is given in that article. In brief, the feeding experiment was carried out at the Agricultural Research and Education Centre Raumberg-Gumpenstein (Irdning-Donnersbachtal, Austria) and involved a total of 40 newborn Holstein-Friesian calves (20 males and 20 females). Calves were randomly allocated to 4 different dietary treatments, yielding 4 well-balanced groups (n = 10 per group) based on the birth weight (42.9 ± 6.2 kg) and sex. The experimental groups were (1) 100% medium-quality hay without concentrate (**MQH**), (2) 100%

high-quality hay without concentrate (**HQH**), (3) 30% medium-quality hay plus 70% concentrate mixture (**MQH+C**), and (4) 30% high-quality hay plus 70% concentrate mixture (**HQH+C**). The concentrate mixture consisted of wheat (36%), barley (35%), soybean meal (17%), linseed meal (10%), and a mineral premix (2%). Nutritional quality differed considerably between MHQ and HQH hays, as shown in contrasting CP (149 vs. 210 g/kg of DM), WSC (124 vs. 205 g/kg of DM), and NDF (522 vs. 455 g/kg of DM) contents (Table 1). The MQH was a second-cut hay harvested from permanent grassland at AREC Raumberg-Gumpenstein at ear emergence and dried indoor. The permanent grassland consisted of approximately 75% grasses (mainly *Alopecurus pratensis*, *Dactylis glomerata*, *Festuca pratensis*, *Trisetum flavescens*), 15% clover (mainly *Trifolium repens*), and 10% herbs. The HQH was a mixture of first and second-cut hay harvested at the beginning of ear emergence and dried indoor in the Austrian federal state Vorarlberg (Rhine valley) and consisted mainly of *Lolium perenne*. Both hays were chopped to approximately 4.0 cm particle length before feeding. Hay and concentrate samples were taken weekly for DM determination and every 4 consecutive weeks a pooled sample was chemically analyzed (Terler et al., 2022). The chemical composition of the experimental feeds and the milk is shown in Terler et al. (2022) and in Table 1. From the day of birth on fresh feed and water were provided ad libitum in a separate bucket once a day at 0700 h, after recording the remaining amount from the previous day. Concentrate and hay were well mixed before feeding. The experimental diets and water were offered to the calves ad libitum during the entire experimental period. Immediately after birth, calves were fed warm colostrum from their dams ad libitum with an ensured minimum intake of 2.5 L via a bottle. From d 2 of life on, all calves from the 4 experimental groups were offered acidified milk according to the same milk feeding regimen. Accordingly, all calves had access to milk in a milk feeding bucket with teat the whole day, and fresh acidified milk was provided twice per day at 0600 and 1700 h. During the first 4 wk of life, acidified milk was offered ad libitum. In wk 5 and 6, milk allowance was limited to 8 kg of acidified milk/d and from wk 7 on, daily milk supply was reduced by 1 kg each week. Calves were weaned at the end of wk 12, so that in the last 2 experimental weeks (i.e., wk 13 and 14), calves were fed the experimental solid feeds and water only (Terler et al., 2022). Feed samples were sieved for particle size distribution using the Penn State Particle Separator (model C24682N, Nasco) with 3 screens (>19 mm, 8–19 mm, 1.18–8 mm, and <1.18 mm) as described by Kononoff et al. (2003). The physically effective NDF of the ration was calculated by multiplying the analyzed NDF content with the proportion of particles >8 mm (**peNDF$_{>8mm}$**; Kononoff et al., 2003).

Sampling Procedures

Rumen Fluid and Fermentation Variables. Rumen fluid was collected on d 7, 14, 21, 35, 49, 63, 77, 91, and 98 of life, each time at 0900 h. For sampling, a tube protector made of iron and wrapped with plastic was placed in the mouth of the calf and then the plastic tube (inside diameter: 7 mm, length: 1,500 mm) was gently pushed into the rumen. A manually operated vacuum pump was used to obtain the rumen fluid sample. After discarding the first 10 mL to avoid saliva contamination, approximately 30 mL rumen fluid were collected, filtered through gauze compresses (Wilhelm Weisweiler GmbH & Co. KG) and the pH was immediately measured, using a calibrated, portable digital pH meter (pH7+DHS, XS Instruments). Aliquot samples were stored in −20°C for further analyses.

Concentrations of SCFA (acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate, caproate, heptanoate) were determined by GC. Therefore, ruminal fluid samples were thawed overnight at 4°C and centrifuged at 3,220 × *g* for 20 min at 4°C, and the supernatant was used for further analysis. Subsequently, 200 μL of distilled water, 200 μL of the internal standard 4-methylvaleric acid (Sigma-Aldrich) and 200 μL of 1.8 *M* HCl were added to 600 μL of supernatant. Samples were vortexed, and then centrifuged at 20,000 × *g* for 20 min at 4°C. The clear supernatant was transferred into glass vials for GC. The analysis was conducted using a GC apparatus (Shimadzu GC Plus with FID detector) equipped with a 30 m × 0.53 mm i.d. × 0.53 μm capillary column (Trace TR Wax, Thermo Fisher Scientific). Injector and detector had temperatures of 200 and 220°C, respectively. Helium was used as carrier gas with a flow rate of 1 mL/min.

The ammonia concentration in the rumen fluid samples was determined using the indophenol reaction (Weatherburn, 1967). For this, samples were thawed at room temperature and then centrifuged at 15,115 × *g* for 10 min at room temperature (20°C). Subsequently, the clear supernatant was diluted with distilled water to obtain a concentration within the standard calibration curve. Both ammonia and phenol were oxidized by sodium hydroxide in the presence of sodium nitroprusside and dichloroisocyanuric acid to form a blue complex. After 90 min of reaction, the absorbance was measured at 655 nm using UV-1800 Spectrophotometer (Shimadzu Handels GmbH). The blue color intensity linearly correlated with ammonia concentration.

Feces and Fermentation Variables. Feces samples were collected on d 1, 2, 3, 4, 5, 7, 14, 21, 35, 49, 63, 77, and 91, each time at 1000 h. Afterward, the pH value was measured as described above for rumen fluid and the samples were stored at −80°C for further analysis. The SCFA profile in fecal samples was determined by GC using the same methods as described above for rumen fluid with minor modifications. Briefly, samples were thawed overnight at 4°C and mixed thoroughly. Then, 1 g of fecal sample was diluted in 1 mL of distilled water. Subsequently, 300 μL of the internal standard 4-methylvaleric acid (Sigma-Aldrich) and 200 μL of 25% phosphoric acid were added. Samples were vortexed vigorously and centrifuged at 20,000 × *g* for 20 min at 4°C. After transferring the supernatant into a fresh tube, it was centrifuged until being clear. The measurement of SCFA was conducted with GC as described above for rumen fluid.

Chewing Activity. The measurement of chewing activity was carried out with noseband-sensor halters (RumiWatch System, ITIN + HOCH GmbH), validated previously (Eslamizad et al., 2018). The halter was applied to each calf 4 times (wk 4, 6, 10, and 12) and each time chewing behavior was measured from over 3 consecutive days, as described by (Eslamizad et al., 2018). Data were converted and stored with the software RumiWatch Converter (RumiWatch System, ITIN + HOCH GmbH) and comprised the duration of eating, ruminating and total chewing (eating + ruminating times) in minutes per day, as well as the number of ruminating boli per day and chews per bolus. Additionally, the chewing indices for each chewing category were calculated using data of chewing and feed intake of the same day.

Rumen Mucosal Thickness. Rumen mucosal thickness was measured on d 7, 14, 21, 35, 49, 63, 77, 91, and 98 at 1100 h. An ultrasound scanner (Mindray DP-3300 Veterinary Ultrasound Machine, National Ultrasound) was used to measure the thickness in the 11th intercostal space at the height of tuber coxae using a method described in details by Neubauer et al. (2018). In brief, 3 images per animal with 3 different measurement points each were taken at each day of measurement. Calves were restricted with a rope before measurement. From 9 different recordings, the average RMT was calculated and the final daily value was determined.

Blood Sampling and Analyses. Blood samples were collected on d 1, 3, 7, 21, 49, 77 and 91. On d 1, blood was collected within 6 h after birth and subse-

Table 1. The analyzed chemical composition of milk, hays, and concentrates used in the experiment (mean ± SD)

Item	Milk	MQH[1]	HQH[2]	Starter concentrate[3]
Ingredients of experimental feeds (% of fresh matter)				
HQH		—	100	—
MQH		100	—	—
HQH + concentrate		—	30	70
MQH + concentrate		30	—	70
Chemical composition (g/kg of DM unless stated)				
Number of samples (n)	80	20	20	20
DM, g/kg of fresh matter	130 ± 2	899 ± 24	887 ± 30	891 ± 13
CP	260 ± 3	149 ± 29	210 ± 11	193 ± 9
Ether extract	322 ± 6	18 ± 3	24 ± 3	18 ± 2
Ash	58 ± 1	76 ± 7	86 ± 3	39 ± 11
NDF	—	522 ± 24	455 ± 15	204 ± 12
ADF	—	329 ± 15	247 ± 11	66 ± 5
ADL	—	49 ± 7	23 ± 3	13 ± 2
NFC[4]	360 ± 6	235 ± 34	225 ± 16	547 ± 16
Water-soluble carbohydrates	—	124 ± 34	205 ± 10	—
Ethanol-soluble carbohydrates	—	99 ± 27	167 ± 4	—
Fructans	—	25 ± 10	38 ± 10	—
ME, MJ/kg of DM	19.2 ± 0.1	9.4 ± 0.4	11.2 ± 0.2	13.5 ± 0.2
Particle size distribution (% of DM)				
>19 mm	—	68 ± 13.3	94 ± 1.4	—
8.0–19.0 mm	—	4.9 ± 1.3	1.3 ± 0.4	—
<8.0 mm	—	27.2 ± 12.0	5.2 ± 1.0	100
$peNDF_{>8mm}$ (% of DM)	—	38.0 ± 6.3	43.1 ± 0.5	—

[1]MQH = medium-quality hay.

[2]HQH = high-quality hay.

[3]The starter concentrate consisted of 36% ground wheat, 35% ground barley, 17% soybean meal, 10% linseed meal, and 2% mineral premix [180 g of Ca, 40 g of P, 60 g of Mg, 80 g of Na, 1,500 mg of Cu, 7,000 mg of Zn, 4,000 mg of Mn, 40 mg of Se, 60 mg of Co, 160 mg of I, 700,000 IU of vitamin A, 100,000 IU of vitamin D_3, 2,500 IU of vitamin E (values per kg of feed)].

[4]NFC = (1,000 – ash – CP – ether extract – NDF).

[5]$peNDF_{>8mm}$ = physically effective NDF >8 mm determined according to Kononoff et al. (2003).

quent samples were taken at 0600 before morning feeding. Samples were taken from the jugular vein using 0.9 × 38 mm multiple sampling cannulas, 9-mL serum vacutainer tube, and 6-mL vacutainer tube containing sodium fluoride and potassium oxalate (Vacuette, Greiner Bio One International GmbH). The samples were allowed to clot for 1.5 h at room temperature and were centrifuged at 3,000 × *g* for 20 min at room temperature (Centrifuge 5702, Eppendorf AG). Afterward serum and plasma were stored in 2 mL tubes (SafeSeal reaction vessel, Sarstedt AG & Co KG) at −80°C until analysis. Concentrations of the liver enzymes AST, GLDH, and GGT in serum were analyzed using standard enzymatic colorimetric assays with a fully automated analyzer for clinical chemistry (Cobas 6000/c501, Roche Diagnostics GmbH) at the laboratory of the Central Clinical Pathology Unit, University of Veterinary Medicine, Vienna, Austria. Serum concentrations of cortisol were measured with a xMark Microplate Absorbance Spectrophotometer (Bio-Rad Laboratories GesmbH) using the respective kit (Cortisol ELISA, TopMed Trade).

Statistical Analyses

The power analysis and main statistical data processing methods are shown in details in Terler et al. (2022). In brief, the ANOVA was performed using the PROC MIXED of SAS 9.4 (SAS Inst. Inc.) with the fixed effects hay quality, concentrate inclusion, sampling time, and sex, as well as the corresponding interactions. Calf within the experimental group was considered as random effect. Furthermore, measurements taken on the same calf at different days were considered repeated measures in the ANOVA using variance components covariance structures according to Bayesian information criteria. Degrees of freedom were approximated with the Kenward-Roger method. Multiple comparison analysis was performed with PDIFF and Tukey adjustment. Normality distributions of residuals was tested using PROC UNIVARIATE of SAS. Significant differences were assumed, if *P*-values were below 0.05 and are indicated by different superscripts.

RESULTS

Intake of peNDF$_{>8mm}$

The data of peNDF$_{>8mm}$ intake are shown in Figure 1. The intake of peNDF$_{>8\ mm}$ increased over time, especially after wk 7 of life ($P < 0.01$). Until wk 7, the peNDF$_{>8mm}$ intake of calves was rather low (<200 g/d). There were overall effects of hay quality, concentrate inclusion, and their corresponding interaction on peNDF$_{>8mm}$ intake ($P < 0.01$). Significantly greater peNDF$_{>8mm}$ intakes were observed in calves fed only hay compared with animals fed concentrate starting from wk 7. However, starting from wk 12, the HQH calves had the highest peNDF$_{>8mm}$ intake ($P < 0.05$) of all experimental groups (Figure 1).

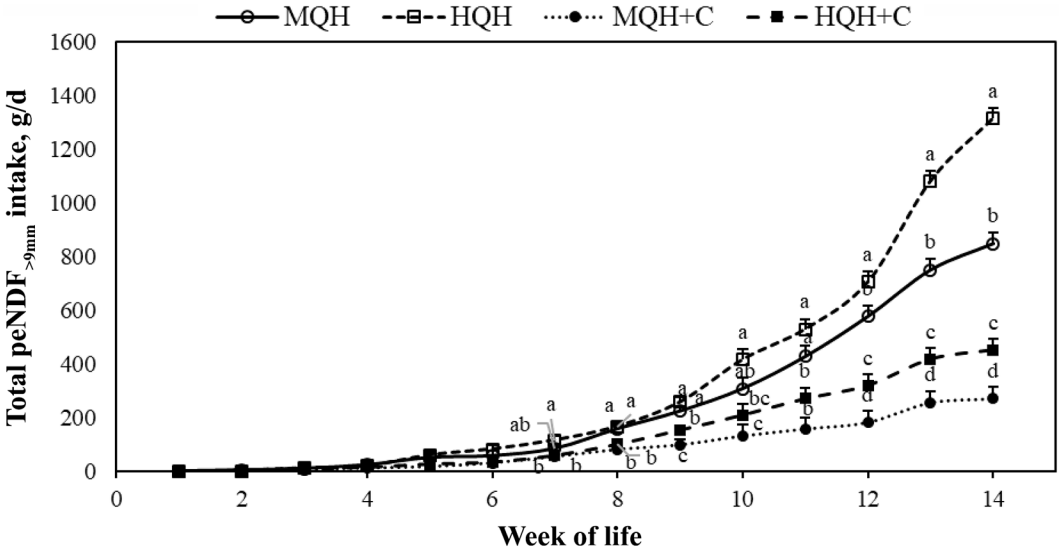

Figure 1. Variation in daily intake of physically effective fiber (peNDF$_{>8mm}$) of rearing calves fed hay of different qualities with or without supplementation of concentrates (MQH = 100% medium-quality hay; HQH = 100% high-quality hay; MQH+C = 30% medium-quality hay plus 70% concentrates; HQH+C = 30% high-quality hay plus 70% concentrates). Letters (a–d) indicate significant differences between diets in respective weeks. Error bars represent SE.

Chewing Activity

The chewing data are shown in Table 2. Both rumination and eating time, and consequently the total chewing time increased over time, whereas their indices decreased mostly with time ($P < 0.01$). There were no overall effects of hay quality and interaction between hay and concentrate. Overall effects of concentrate inclusion were seen on ruminating (min/g peNDF intake) and eating (min/d and min/g peNDF intake); however, there were interactions between dietary factors with time of measurement. Accordingly, the rumination time and the number of rumination-boli per day was different among dietary treatments only in wk 12. There, calves fed the HQH diet spent more ($P < 0.05$) time ruminating (591 min/d) and had more ruminating boli (709 boli/d) than calves fed the concentrate-rich diets (MQH+C: 430 min/d, 518 boli/d; HQH+C: 430 min/d, 541 boli/d), whereas the MQH group was intermediate (539 min/d, 644 boli/d). As well, eating time was different during wk 4, 10, and 12, with both hay groups spending more time eating in wk 10. In wk 12, the groups offered MQH and MQH+C spent more time on eating than the respective groups receiving HQH. An exemplary overview of the circadian profile of ruminating and eating time on wk 12 taken from one typical calf per group is shown in Supplemental Figure S1 (https://data.mendeley.com/datasets/2brv3zs8hx/1). Accordingly, calves spent longer time eating mainly during the day, especially in the morning after offering the fresh ration. Ruminating, on the other hand, occurred throughout the day, whereby the calves fed only hay had a continuous distribution of ruminating activity, whereas single peaks were observed in the concentrate-rich groups.

Regarding rumination indices, there were differences in the rumination time per unit of DMI only in wk 4 and in rumination time per unit of NDF intake as well as $peNDF_{>8mm}$ intake in wk 4 and 6; either with MQH calves ruminating the most per kilogram of DMI or MQH+C calves ruminating lowest per kilogram of NDF intake in wk 4 (Table 2).

Ruminal Fermentation

Data of ruminal fermentation profile of calves from d 7 to 98 (wk 1 to 14) of life are presented in Figure 2. As shown in Figure 2a, ruminal pH was affected by time ($P < 0.01$), and there were overall effects of hay quality ($P = 0.038$), concentrate inclusion ($P < 0.01$), with no interactive effect between both factors. Time effects indicated that ruminal pH was at a comparably low level (mean 6.28) until d 35 and increased afterward (the maximum overall pH of 6.79 by d 98 of all calves). Multiple comparison analysis showed that feeding only hay and MQH+C diets maintained higher ruminal pH than HQH+C on d 14 and 21 of life. The MQH-fed calves had higher ruminal pH than other groups on d 63 and higher pH than MQH+C group on d 77. Within groups, a significant increase in ruminal pH was observed from d 49 to 63 for MQH, from d 49 to 77 for HQH, as well as from d 63 to 77 for MQH+C and from d 35 to 98 for HQH+C groups. Sex of the calf had no effect on ruminal pH ($P = 0.996$).

Results of ruminal ammonia concentration indicated a day effect ($P < 0.01$; Figure 2b) with high concentrations in the first 3 wk (22 mg/100 mL) and a curvilinear decrease thereafter, reaching the nadir around d 63 (7 mg/100 mL). As well, there was an overall effect of hay quality (HQH higher; $P < 0.01$) with higher concentrations due to high-quality hay feeding. Effects of concentrate inclusion ($P = 0.11$) and the 2-way interaction between the 2 dietary factors ($P = 0.83$) were not significant.

Data of ruminal SCFA concentration indicated also a time effect ($P < 0.01$) with concentrations increasing steadily from d 7 to 63, where they reached a plateau averaging 73 μmol/g (Figure 2c). None of the dietary factors affected the concentration of total SCFA. However, there was an interaction of diet with time, so that multiple comparisons indicated lower ruminal SCFA concentration in MQH group than in both concentrate-fed groups. Furthermore, ruminal SCFA concentration of calves fed HQH+C was lower than in the HQH group on d 77 and lower than in all other groups on d 98. The proportion of individual SCFA in are shown in Supplemental Figure S2a (https://data.mendeley.com/datasets/2brv3zs8hx/1). Proportions of acetate, propionate, and butyrate of total ruminal SCFA were affected by time ($P < 0.01$), and there were overall effects of concentrate inclusion and an interaction between diet and time with no interactive effect between hay and concentrate (only acetate: ($P = 0.03$). Acetate concentration was highest in calves fed MQH from d 35 on and in calves fed MQH and HQH from d 77 to 98. In contrast, proportion of propionate in ruminal fluid was higher in both groups with concentrate inclusion than in calves fed only hay. From d 35 on, feeding HQH resulted in the highest butyrate proportions in ruminal SCFA.

Fecal Fermentation

Results on fecal fermentation are presented in Figure 3. As shown in Figure 3a, fecal pH increased from d 7 to 14 (time effect $P < 0.01$), and decreased with concen-

trate intake ($P < 0.01$), with a tendency for interaction between diet and time ($P = 0.07$). Hay quality had no effect on this variable ($P = 0.16$). Multiple comparison of data showed that from d 21 of life on (except for d 49), MQH-fed calves had higher fecal pH ($P < 0.05$) than other groups, except for MQH+C group on d 21, 35, and 63 and HQH group on d 63. Fecal pH was on a similar level in MQH+C, HQH, and HQH+C groups, except for d 91, in which it was higher in HQH-fed calves than in calves offered MQH+C. On average over the entire experiment, the MQH-fed calves had a significantly higher fecal pH of 6.29, than both concentrate groups (MQH+C: 6.05; HQH+C: 6.03), and the HQH group (6.13) showing intermediate values. Fecal pH of male and female calves did not differ ($P = 0.28$).

As shown in Figure 3b, total fecal SCFA concentration was affected by time ($P < 0.01$), but it was unaffected by hay quality and concentrate inclusion. Multiple comparison analysis showed that feeding MQH lowered fecal SCFA concentration from d 35 on

Table 2. Chewing behavior variables of calves fed different diets in different weeks of life

		Diet[1]					*P*-value[2]			
Item	Week	MQH	HQH	MQH+C	HQH+C	SEM	HQ	CON	HQ × CON	HQ × CON × Wk
Ruminating										
min/d	4	118	28	10	69	46	0.897	0.111	0.534	0.004
	6	215	197	200	206	45				
	10	427	412	331	375	43				
	12	539^{ab}	591^{a}	430^{b}	430^{b}	43				
min/g of DMI	4	1.56^{a}	0.44^{b}	0.28^{b}	0.89^{ab}	0.28	0.583	0.390	0.118	0.103
	6	1.42	0.94	1.20	1.28	0.27				
	10	0.54	0.48	0.30	0.37	0.26				
	12	0.33	0.38	0.23	0.24	0.25				
min/g of NDF intake	4	2.94^{a}	0.99^{b}	0.75^{b}	3.14^{a}	0.63	0.900	0.432	0.142	0.012
	6	2.76^{ab}	2.04^{b}	3.57^{ab}	3.75^{a}	0.61				
	10	1.02	1.05	0.98	1.24	0.59				
	12	0.61	0.85	0.80	0.80	0.58				
min/g of peNDF intake	4	4.04^{b}	1.05^{b}	3.44^{b}	11.05^{a}	1.70	0.883	<0.001	0.381	<0.001
	6	3.79^{b}	2.15^{b}	1.635^{a}	13.19^{a}	1.65				
	10	1.40	1.11	4.49	4.37	1.60				
	12	0.83	0.90	3.68	2.81	1.57				
Ruminating boli (n/d)	4	138	27	15	92	54	0.750	0.152	0.457	0.007
	6	255	233	253	256	53				
	10	498	496	387	458	50				
	12	644^{ab}	709^{a}	518^{b}	541^{b}	50				
Ruminating chews/bolus	4	30	26	23	35	4	0.528	0.582	0.617	0.014
	6	40^{ab}	36^{b}	49^{a}	45^{a}	4				
	10	54	51	53	49	4				
	12	55	55	56	50	3				
Eating										
min/d	4	118^{a}	48^{b}	37^{b}	66^{b}	19	0.121	0.017	0.350	<0.001
	6	130	111	109	97	18				
	10	183^{a}	177^{a}	122^{b}	121^{b}	18				
	12	219^{a}	168^{b}	182^{ab}	128^{c}	18				
min/g of DMI	4	1.91^{b}	3.75^{a}	1.52^{ac}	0.88^{c}	0.39	0.682	0.064	0.291	<0.001
	6	1.15	0.86	0.74	0.68	0.37				
	10	0.19	0.20	0.11	0.14	0.36				
	12	0.09	0.10	0.09	0.07	0.35				
min/g of NDF intake	4	3.65^{b}	8.22^{a}	3.97^{b}	2.92^{b}	0.90	0.538	0.343	0.257	<0.001
	6	2.21	1.89	2.22	1.95	0.87				
	10	0.37	0.45	0.36	0.48	0.84				
	12	0.17	0.22	0.33	0.22	0.83				
min/g of peNDF intake	4	5.02^{b}	8.68^{b}	18.22^{a}	10.27^{b}	1.08	0.496	0.030	0.297	0.061
	6	3.04^{b}	1.98^{b}	10.15^{a}	6.89^{ab}	1.04				
	10	0.52	0.48	1.65	1.68	0.52				
	12	0.26	0.23	1.53	0.80	0.46				

[a–c]Means bearing different superscripts within a row differ at $P < 0.05$.

[1]MQH = 100% medium-quality hay; HQH = 100% high-quality hay; MQH+C = 30% medium-quality hay plus 70% concentrates; HQH+C = 30% high-quality hay plus 70% concentrates.

[2]The effect of hay quality (HQ), concentrate inclusion (CON), their two-way interaction (HQ × CON) and the interaction of hay quality, concentrate inclusion and week (HQ × CON × Wk). Week was significant for all variables measured (P < 0.01).

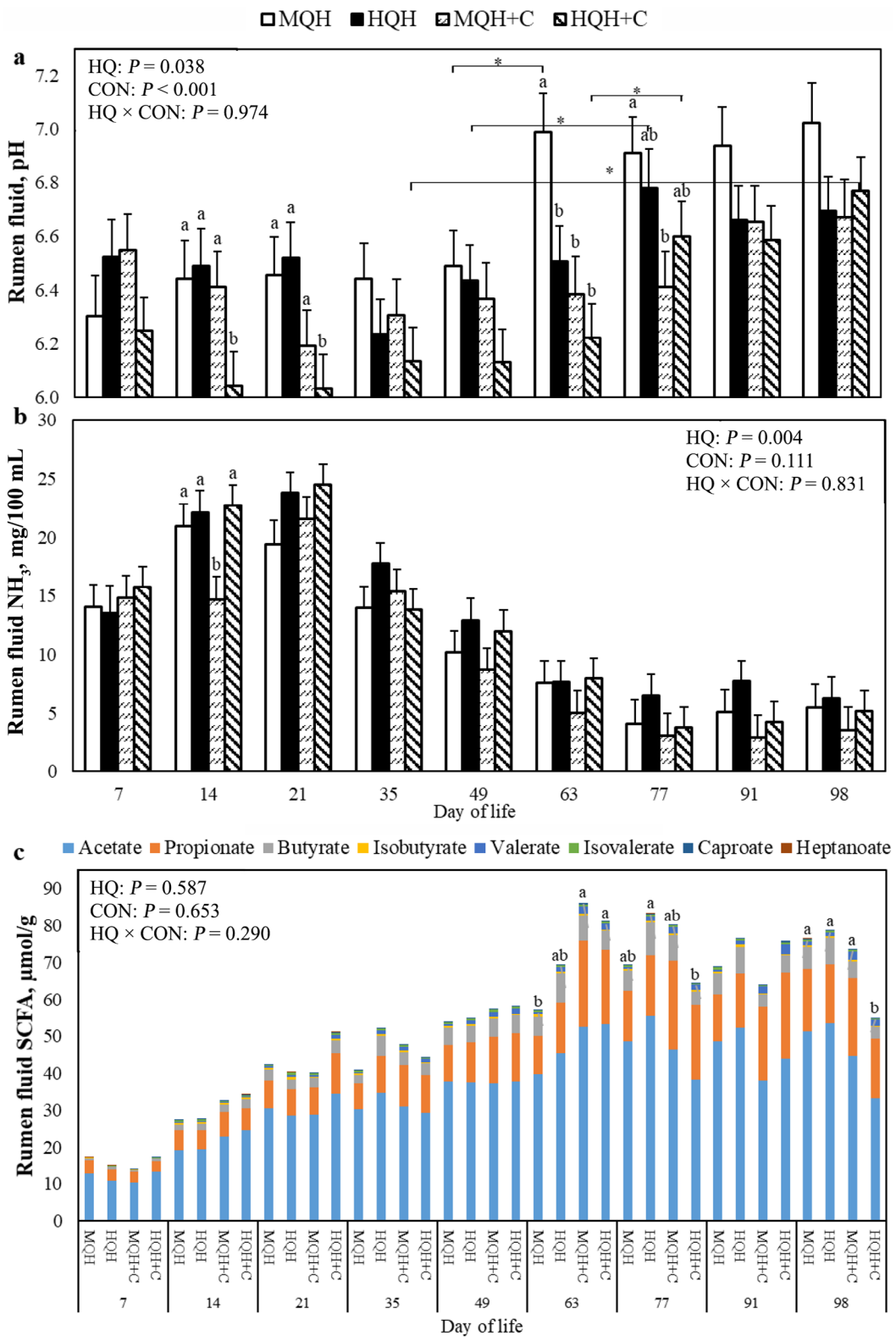

Figure 2. Effect of starter diet's hay quality (HQ), concentrate inclusion (CON) and their interaction on ruminal pH (a), ammonia (NH_3) content (b), and short-chain fatty acids (SCFA) concentration (c) in dairy calves measured on several days of life. Starter diets were medium-quality hay only (MQH), high-quality hay only (HQH), 30% medium-quality hay + 70% concentrate (MQH+C) and 30% high-quality hay + 70% concentrate (HQH+C). Effect of day was significant ($P < 0.05$) for all variables. Letters (a, b) indicate differences among dietary treatments within each day of life. Asterisks indicate differences between diets in respective days ($P < 0.05$). Error bars represent SE.

(except d 49). The HQH-fed calves and the groups with concentrate inclusion had comparable fecal SCFA concentrations over the entire experiment. Of the measured fecal SCFA concentrations revealed a fermentation capacity in the hindgut already on d 1 in the first fecal sample (meconium), with SCFA levels averaging

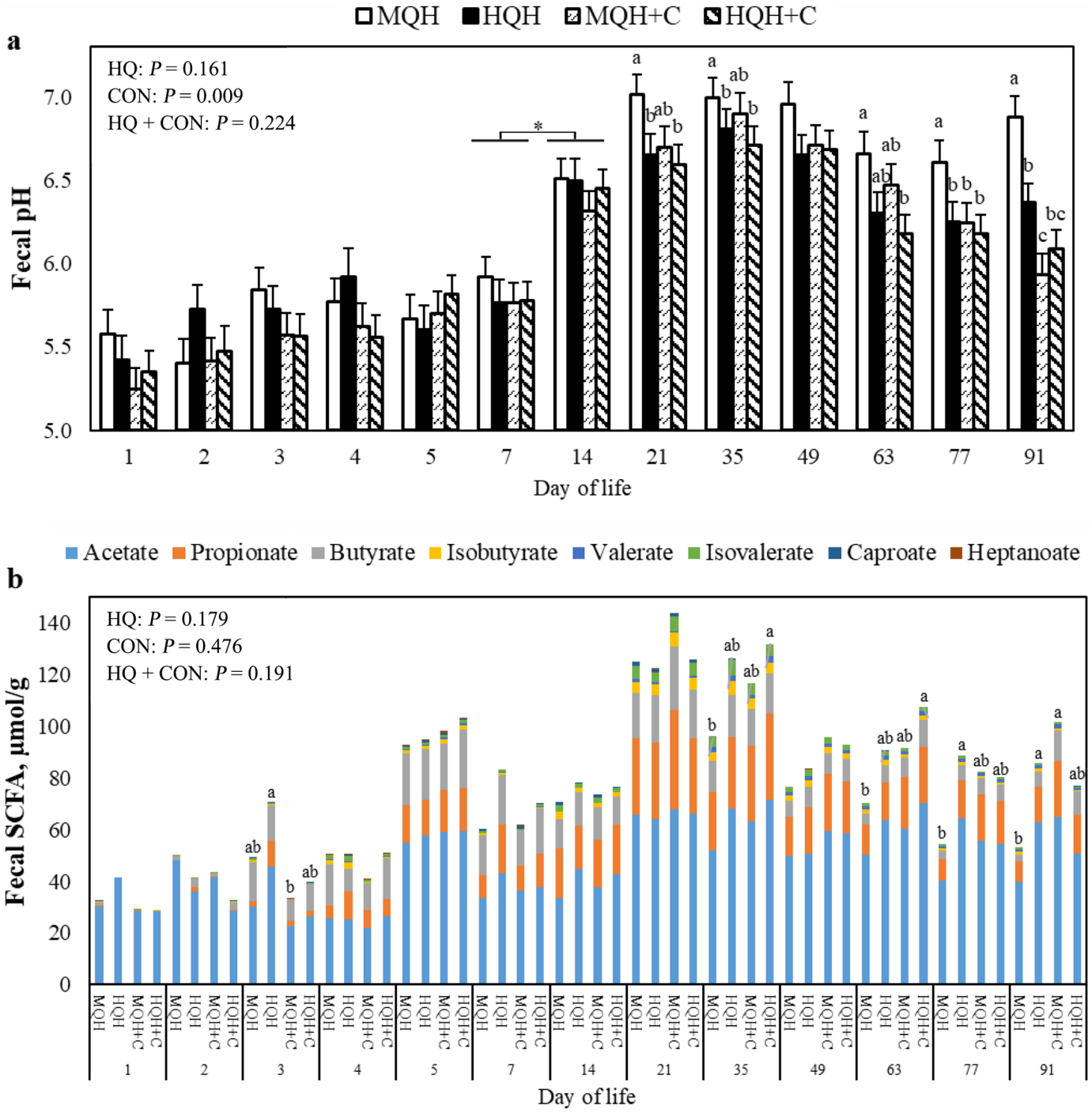

Figure 3. Effect of starter diet's hay quality (HQ), concentrate inclusion (CON) and their interaction on fecal pH (a) and short-chain fatty acids (SCFA) concentration (b) in dairy calves measured on several days of life. Starter diets were medium-quality hay only (MQH), high-quality hay only (HQH), 30% medium-quality hay + 70% concentrate (MQH+C) and 30% high-quality hay + 70% concentrate (HQH+C). Effect of day was significant ($P < 0.05$) for all variables. Letters (a–c) indicate differences among dietary treatments within each day of life. Asterisks indicate differences between diets in respective days ($P < 0.05$). Error bars represent SE.

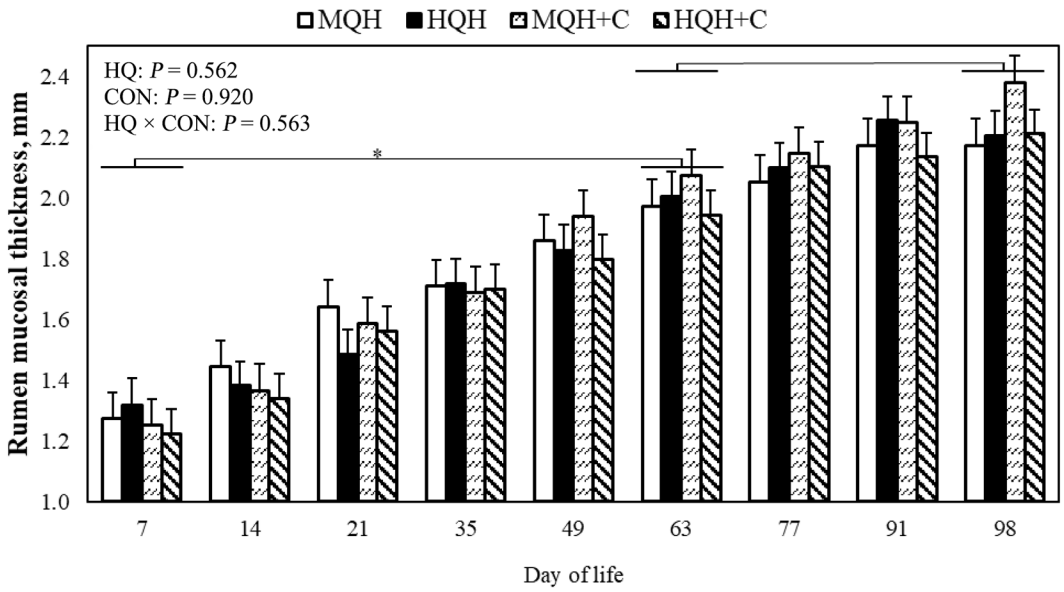

Figure 4. Effect of starter diet's hay quality (HQ), concentrate inclusion (CON) and their interaction on rumen mucosal thickness (RMT) in dairy calves measured in several days of life. Starter diets were medium-quality hay only (MQH), high-quality hay only (HQH), 30% medium-quality hay + 70% concentrate (MQH+C) and 30% high-quality hay + 70% concentrate (HQH+C). Effect of day was significant ($P < 0.05$) for all variables. Asterisks indicate differences between diets in respective days ($P < 0.05$). Error bars represent SE.

33 μmol/g (Figure 3b), and consisting mainly of acetate (>95%; Supplemental Figure S2b, https://data.mendeley.com/datasets/2brv3zs8hx/1). Beginning on d 3, small amounts of propionate and butyrate were also found in fecal samples. The highest concentration of fecal SCFA were observed on d 21 (129 μmol/g) and d 35 (118 μmol/g), after which the absolute concentration values slightly decreased. Proportion of butyrate in fecal SCFA was very high on d 3 (up to 25%) and decreased until the end of the trial (Supplemental Figure S2b). Multiple comparison showed only differences of butyrate in the first 4 d of life and d 91 (higher in groups with concentrate inclusion) between the dietary groups. In contrast, proportion of propionate in fecal SCFA of all calves was low at the beginning and increased toward the end of the trial. On d 77 and 91 the groups with concentrate inclusion had higher concentration of propionate, whereas the hay-only feeding groups had higher acetate concentrations. The remaining SCFA were low in absolute concentration and relative proportion throughout the experiment.

Rumen Mucosal Thickness

Results on RMT are in Figure 4. Overall, the RMT increased significantly over time ($P < 0.01$), but was not affected by any of the dietary factors and their interaction with average values across all treatments from 1.27 mm (d 7) to 2.24 mm (d 98). Exclusively, a trend ($P = 0.098$) existed on d 98 between MQH+C (2.38 mm) and MQH (2.17 mm) calves. From d 7 to d 63, a significant increase of RMT was observed in all feeding groups and thereafter the increase was no longer significant until the end of the experiment. The male and female calves did not differ in their RMT ($P = 0.22$).

Blood Metabolites

Results of AST, GGT, GLDH, and cortisol are shown in Figure 5. A time effect was observed for all blood parameters ($P < 0.01$). Accordingly, AST and GGT showed the maximum concentration on d 3 (95 U/L, 1,395 U/L, respectively) with AST decreasing steadily up to 36 U/L on d 21, but increasing again up to 78 U/L on d 91. In contrast, serum GGT concentration remained low after d 7. The GLDH concentration increased by the end of experiment reaching the highest level of 31 U/L on d 91. Plasma cortisol was high shortly after birth on d 1 (102 μg/mL on average), decreased to 11 μg/mL on d 21 (Figure 5d) and remained low till the end of the trial independent of the diet. Albeit the statistical analyses revealed a diet × time interaction with significant differences between dietary groups for some of the blood parameters, only differences from d 7 onward are considered relevant, due to the lack of solid feed intake until d 21. Blood levels of AST differed between sexes ($P < 0.01$) with female calves

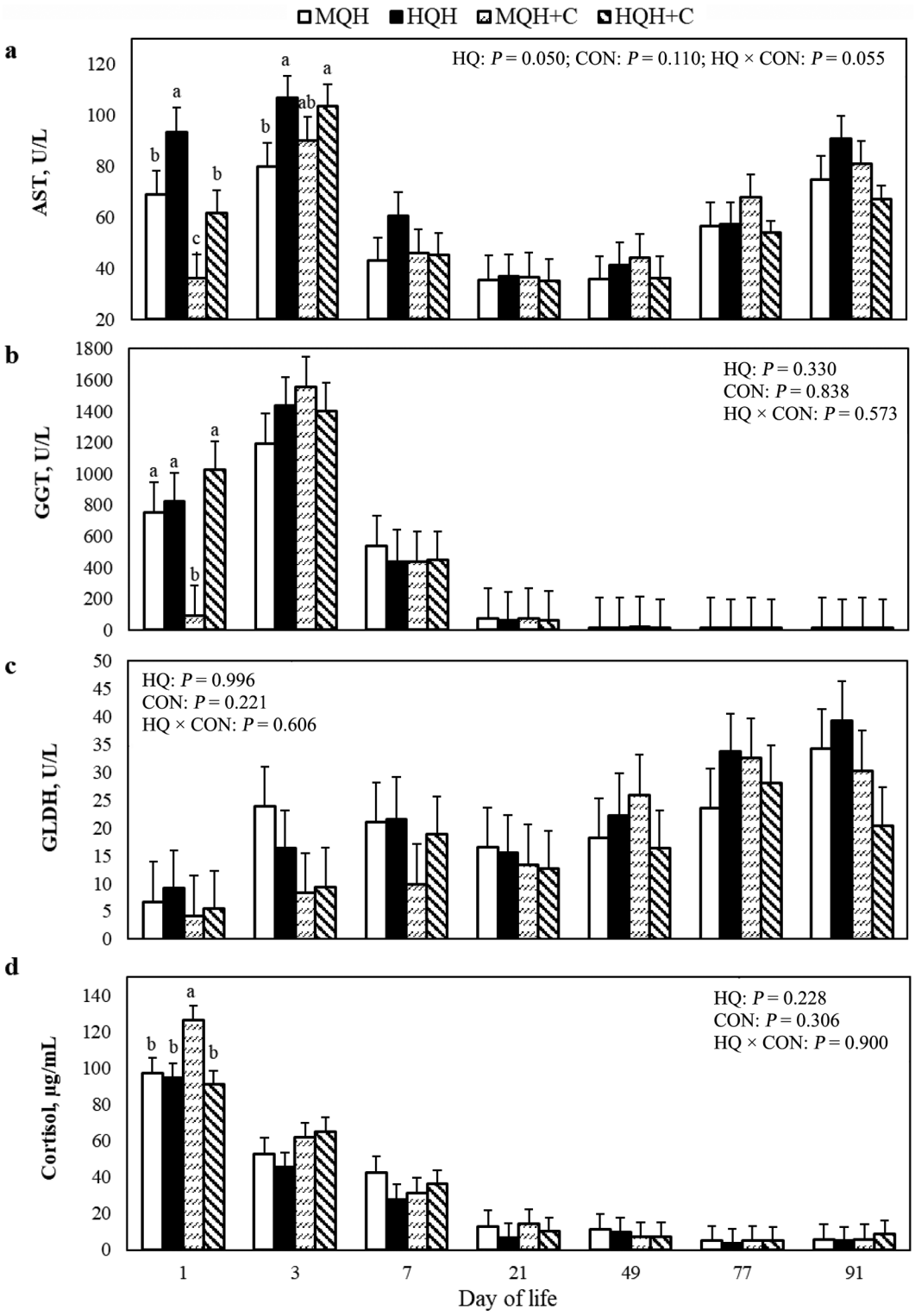

Figure 5. Effect of starter diet's hay quality (HQ), concentrate inclusion (CON) and their interaction on blood metabolites aspartate aminotransferase (AST; a), gamma glutamyl transferase (GGT; b), glutamate dehydrogenase (GLDH; c) and cortisol (d) in dairy calves measured on several days of life. Starter diets were medium-quality hay only (MQH), high-quality hay only (HQH), 30% medium-quality hay + 70% concentrate (MQH+C) and 30% high-quality hay + 70% concentrate (HQH+C). Effect of day was significant ($P < 0.05$) for all variables. Letters (a–c) indicate differences among dietary treatments within each day of life. Error bars represent SE.

having generally higher values (on average 65 U/L vs. 55 U/L).

DISCUSSION

This research primarily aimed to evaluate the effects of starter diets differing in hay quality and level of concentrate inclusion on chewing activity and the establishment of fermentation in the rumen and hindgut, as well as on selected systemic health and stress parameters in dairy calves. Chewing is an essential physiological process of young calves because it enhances salivation and rumen buffering (van Ackeren et al., 2009). In this context, an important finding of the present study was that calves fed HQH diet showed longer rumination time in wk 12 than calves fed diets with concentrates, whereas not differing from calves fed MQH diet. Thus, in addition to being rich in WSC (205 vs. 124 g/kg of DM) and hence in ME (11.2 vs. 9.4 MJ/kg of DM) as well as in CP (210 vs. 149 g/kg of DM) and other nutrients, our data showed that HQH possesses sufficient structural effectiveness to stimulate rumination in calves when offered ad libitum without concentrates. This is both due to relatively high peNDF$_{>8mm}$ content of HQH (43.1%) and greater peNDF$_{>8mm}$ intake (705 g/d) on wk 12, being considerably higher in comparison to the starter diets containing concentrates (i.e., 320 and 183 g of peNDF$_{>8mm}$/d for HQH+C and MQH+C, respectively), whereas MQH calves consumed an intermediate level (577 g/d). Physically effective fiber is mostly known for its role to stimulate chewing and therefore preventing rumen acidosis in adult cattle (Zebeli et al., 2012). Although data about peNDF role on chewing activity and rumen health are lacking in calves, our study suggests a similar role for peNDF$_{>8mm}$ in stimulating rumination in young dairy calves.

In our experiment, calves had an average rumination time of 3.5 h/d on wk 6, which more than doubled to 7.2 to 9.9 h/d by wk 12. This indicates an overall high rumination activity of young calves, although the solid feed intake was still low during this time (Terler et al., 2022). Research by Terré et al. (2013) suggested that supply of forage increased ruminating time and decreased abnormal behaviors such as tongue rolling, licking surfaces, or eating wood shavings in young calves. In addition to important physiological effects, prolonged rumination might also positively affect the welfare of young calves by increasing the time they are occupied with chewing activities. In fact, rumination time is also being used as indicator for calf healthiness. Borderas et al. (2008) described a decrease in ruminating time and hay eating time and an increase in lying time when body temperature is elevated.

Another interesting finding of the study was an improved ruminal pH by feeding hay, regardless of the quality, as early as d 14 and 21 of life, whereas the positive effect on fecal pH during this time was only evident for MQH feeding. During this early phase, calves consumed similar, though ad libitum milk amounts, and the solid feed intake was low but similar among groups (Terler et al., 2022), thus suggesting similar amounts of fermentation acids such as SCFA and lactate in the rumen. The accumulation of protons from these fermentation acids results on decreased ruminal pH, especially when combined with insufficient neutralization, likely due to low ensalivation (Castillo-Lopez et al., 2021a). A low ensalivation is expected in young calves because of not yet established rumination. In this study, the rumination time was very low and shorter than the eating time, when measured on wk 4. Indeed, on wk 4 we observed that calves spent 1.9, 3.7, 1.5, and 0.9 min eating time/g of solid DMI in groups MQH, HQH, MQH+C, and HQH+C, respectively. Calves consumed on average 10 to 30 g of DM solid feed on wk 2 and 3, respectively (Terler et al., 2022), suggesting 10 to 80 min longer time spent eating for MQH, HQH, and MQH+C group than HQH+C calves. We speculate that this increased time eating enhanced saliva secretion and this might have affected the ruminal pH of the young calves. The higher fecal pH of calves fed MQH can be explained with decreased postruminal flow of fermentable substrates, mainly lactose and starch in these calves. The fecal pH strongly depends on the amount of fermentable substrates escaping the rumen, especially lactose and starch (Wheeler and Noller, 1977; Ireland-Perry and Stallings, 1993). We generally observed lower pH in feces than in the rumen, especially during d 7, which can be explained by greater hindgut fermentation of the milk, as supported by higher total SCFA concentration in the feces than in the rumen fluid during this phase. These data emphasize the theory that hindgut fermentation plays a significant role in SCFA supply of the pre-ruminant calves till d 49 of life regardless of solid feed supply. Also differences in the neutralization of SCFA in the rumen versus hindgut and shorter retention time in the intestine (Ferreira et al., 1980) for the absorption of SCFA might have played role, though.

The higher ruminal pH on d 63 in MQH-fed calves is likely associated with the lower intake of easily-fermentable carbohydrates during this time in these calves (Terler et al., 2022). On the other hand, the fact that ruminal and fecal pH in calves fed HQH did not differ from concentrate groups rich in starch indicates that WSC of the HQH provided similar substrate amounts for fermentation as the starch of the concentrate-rich diets (Klevenhusen and Zebeli, 2021), whereas the in-

creased rumination observed on wk 12 was not enough to affect acid neutralization and modulate ruminal pH. In the groups with concentrate supplementation, feed sorting played an essential role, too. As shown by Terler et al. (2022), calves sorted in favor of hay in both groups until wk 11. Yet, from wk 11 to wk 14, MQH+C group selected for concentrate, whereas concentrate intake made up less than 70% of total DMI in the calves fed HQH+C until the end of the experiment (Terler et al., 2022). This fact might be the reason for the higher pH on d 77 and 98 in calves fed HQH+C.

The onset of microbial colonization and fermentation is a crucial physiological aspect of a newborn calf, both from the perspective of its early independent nutrient supply and its gastrointestinal health. Contrarily to the sterile theory of the fetal gut, recent studies have shown colonization of the fetal gut and that the bacterial composition of the meconium reflects the in utero microbial environment (Wilczyńska et al., 2019; Owens et al., 2021). The occurrence of rather high level of SCFA (30 μmol/g), consisting of almost exclusively acetate, almost no propionate and only 2 to 3% butyrate in the meconium of all 40 calves is an interesting finding of this study. Accordingly, our data suggest bacterial colonization of the calf's fetal gut and the onset of an acetogenic fermentation in the calf's digestive tract already during the late stage of pregnancy. Although bacterial colonization has been reported (Owens et al., 2021), to our knowledge, this is the first report of the presence of such high acetate formation in the meconium of calves. Although more research it is necessary, we hypothesize that acetate but not butyrate and propionate may serve as a first initial stimulus for the maturation of the gastrointestinal system in newborn calves. On d 2 of life we found about 3 to 10% butyrate and only 1 to 2% propionate in total fecal SCFA. During the whole first week of life, fecal butyrate concentration was higher than propionate, suggesting an increased butyrogenesis in the gut of young calves during the first week, likely to stimulate the intestinal growth and maturation (Górka et al., 2011; Steele et al., 2016). Starting from d 7, both ruminal and fecal fermentation profile resembled that of adult cattle fecal fermentation (Castillo-Lopez et al., 2020, 2021b).

Dietary effects on SCFA indicated that MQH feeding yielded less total SCFA in the rumen beginning on d 63 and in feces from d 35 on. This can be explained with the fact of lower fermentable substrate and higher NDF content of the MQH diet and agrees with previous findings in calves (Terré et al., 2013; Doolatabad et al., 2020). In the present study, calves fed the HQH diet showed higher SCFA concentration similar with concentrate-rich indicating that HQH provided similar fermentable substrates with concentrate-rich diets as became visible in similar OM digestibility of these diets and similar growth performance among calves fed HQH and concentrate-rich diets as reported in Terler et al. (2022). Both hay diets resulted in higher proportions of acetate, regardless of hay quality, and less propionate and butyrate (only feces) than concentrate-fed calves. Less propionate and butyrate due to concentrate feeding is expected because of enhancing role of starch on propionogenesis and butyrogenesis (Castillo-Lopez et al., 2021a). The proportion of butyrate in the ruminal SCFA was higher in the HQH than in the other groups, which is in agreement with Klevenhusen et al. (2017) in adult cattle.

The higher NH_3 concentration in the first 3 wk can be explained by the high milk protein intake and ruminal degradation, coupled with insufficient capacity of ruminal microbes to use it. In the further course of the experiment, feeding HQH tended to result in highest ruminal NH_3 concentration. This can be explained by the high CP content with high ruminal degradability and lower energy content of HQH, resulting in a higher ruminal NH_3 availability and decreased N utilization in the HQH group. Even in the groups with concentrate inclusion, calves fed HQH+C had a slightly higher ruminal NH_3 concentration than calves offered MQH+C. This may be due to feed sorting to hay in HQH+C and to concentrate in MQH+C group. It is known that the proportion of soluble nitrogen in total N is higher in hay than in protein-rich concentrates, such as soybean meal or linseed meal (Sniffen et al., 1992) and the energy availability often does not match the N availability in HQH (Kleefisch et al., 2017).

Dietary factors did not affect the RMT in our experiment, and RMT increased in all groups from 1.27 mm (d 7) to 2.24 mm (d 98), though with different intensity among weeks, with the highest growth intensity observed until d 63. To our knowledge, this is the first study that reports RMT in growing Holstein calves. In multiparous dairy cows, Neubauer et al. (2018) reported an RMT of 4.68 to 6.00 mm with increasing effects of lactation number and high grain feeding. Comparing the RMT of young calves with cows, our data suggest that until d 98 the RMT achieves about 30 to 40% of the adult cattle.

Most blood metabolites differed between groups on the first day of life, which is likely related to different parturition and birth circumstances and minor variation in blood sampling times after birth. Because intake of solid feed did not occur, effect of dietary treatments has to be excluded. Additionally, the supply with colostrum after birth can be excluded as reason for different contents of blood metabolites on d 1, as 2.5 L colostrum

intake was ensured within 6 h after birth. The differences in AST and GGT on d 1 might be due to the various times of blood samples taken after the birth of the respective calves in our experiment. Another reason could be the differences in the absorption of colostral GGT and AST (Hadorn and Blum, 1997; Yu et al., 2019). Furthermore, Zanker et al. (2001) described an increase in AST content in the first few days because of an increase in endogenous production and not because of colostrum intake. GLDH concentrations are generally high in colostrum and Blum and Hammon (2000) described increased activity of this enzyme in calf plasma after colostrum intake, but in our experiment, we did not detect elevated levels in blood during the first few days.

The development of the levels of blood metabolites was similar in all feeding groups suggesting that solid feeding did not affect both liver health and stress level. We expected differences in the stress level, assuming especially an increased stress around weaning and with feeding of hay only, because the energy difference from milk to solid feed is supposedly greater than with supplemented concentrates, but based on our results we could not detect any negative effects. Cortisol has been reported to increase when calves were weaned and additionally switched to group housing (Kim et al., 2019), which was not practiced in the preset study due to individual housing throughout the experiment. Similarly, Quigley et al. (2019) did not detect any increase in blood cortisol at weaning. Overall, feeding did not affect liver health and cortisol level of calves in this study, where indeed all calves remained healthy during the entire experiment.

CONCLUSIONS

Taken together, our data showed that feeding hay enhanced ruminal pH of dairy calves already during the wk 2 and 3 of life, most likely by enhancing eating time per unit of solid feed intake. During the time around weaning, feeding of HQH alone, without concentrate supplementation, resulted in increased peNDF$_{>8mm}$ intake. This event resulted in increased rumination time, suggesting sufficient structural effectiveness of HQH, in addition to being rich in energy and protein. Furthermore, feeding of HQH enhanced butyrate concentration in the rumen without affecting health and stress parameters as well as ruminal thickness as an indicator of rumen development. The occurrence of rather high levels of SCFA in meconium, consisting of almost exclusively acetate, suggest acetate fermentation serving as a first initial stimulus for the maturation of the gastrointestinal system in newborn calves. Overall, feeding high-quality hay instead of concentrate-rich starter feed resulted in improved rumination and ruminal fermentation profile, without affecting ruminal pH and systemic and stress health variables.

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