Early step-down weaning of dairy calves from a high milk volume with glutamine supplementation

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ABSTRACT

Weaning dairy calves from a high milk volume (≥ 8.0 kg/d) can negatively affect the growth and welfare even if it is performed in a step-down manner. Supplementation of Gln improved gut development of preweaning calves and mitigated weaning stresses of piglets to extents achieved with antibiotics. The study objective was to examine the effect of initiating a step-down weaning scheme with a Gln supplement at an early age on calf starter intake (CSI), average daily gain (ADG), and paracellular permeability of the intestinal epithelium of calves fed a high volume of milk (9.0 kg/d). Thirty-six Holstein heifer calves were assigned to 3 treatments ($n = 12$) as follows: (1) initiating weaning at 49 d of age (LW), (2) initiating weaning at 35 d of age (EW), and (3) initiating weaning at 35 d with a Gln supplement (2.0% of dry matter intake) from 28 to 42 d of age (EWG). Calves were fed 9.0 kg/d of whole milk until weaning was initiated by abruptly decreasing the milk volume to 3.0 kg/d. Weaning was completed once calves achieved ≥ 1.0 kg/d of CSI. The paracellular permeability of the intestinal epithelium was assessed with lactulose-to-mannitol ratio (LMR) in the blood on 1 d before, and 3 and 7 d after the initiation of weaning. The blood was analyzed for haptoglobin, lipopolysaccharide-binding protein (LBP), and metabolites including AA. The CSI increased once milk volume was restricted in all treatments. The CSI of LW was greater than that of EW and EWG during the first week of weaning. The LW, EW, and EWG took 11, 19, and 16 d to achieve ≥ 1.0 kg/d of CSI and were weaned at 60, 54, and 51 d of age, respectively. The body weight (BW) of LW, EW, and EWG at the initiation of weaning were 68.2, 58.7, and 59.5 kg, respectively. Both LW and EWG achieved similar ADG, but ADG of EW was lower than LW during the first week of weaning. All calves had similar

ADG during the second week of weaning. The BW of LW, EW, and EWG at weaning were 74.8, 66.5, and 66.4 kg, representing a 2.0, 1.8, and 1.8-fold increase in birth weight, respectively. All calves had similar BW of 88.6 and 164.3 kg at 10 and 20 wk of age, respectively. Regardless of the age, serum haptoglobin and plasma LBP concentrations increased on d 3 and returned to baseline concentrations on d 7 during weaning. The EW had a lower plasma LBP concentration than LW and EWG on d 3 during weaning. The LMR was similar between treatments on d 3 but increased by 44% for EW and LW on d 7, whereas the LMR of EWG remained unchanged during weaning. The postprandial serum concentration of Gln, Met, Trp, and β -hydroxybutyrate were greater for EWG than EW during weaning. Beginning step-down weaning at 35 d with a Gln supplement can help maintain the gut barrier function and wean dairy calves with a satisfactory CSI at 7 wk of age without affecting postweaning growth.

Key words: intestinal permeability, lactulose, lipopolysaccharide-binding protein, methionine

INTRODUCTION

Early weaning is considered a promising way to decrease the cost of raising dairy heifer calves (Owen and Larson, 1982). However, weaning-associated stresses can adversely affect calf's health and wellbeing (Weary et al., 2008; Hulbert and Moisé, 2016). Therefore, determining the optimum age and most appropriate weaning method is a critical decision in calf management. The Bovine Alliance on Management and Nutrition (BAMN) recommends weaning when dairy calves consume 0.9 to 1.4 kg/d of calf starter over at least 2 consecutive days (BAMN, 2003, 2017). Nonetheless, only 22% of US dairy producers wean calves based on calf starter intake (CSI), whereas the majority of calves are simply weaned at 9 wk of age (USDA, 2016; Urie et al., 2018). Several studies have shown a potential to wean dairy calves as early as 4 wk of age, highlighting a satisfactory ruminal development that could ensure a

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smooth transition from a liquid to a solid diet (Owen and Larson, 1982; Anderson et al., 1987; Quigley et al., 1991). However, it should be noted that calves in those studies (Owen and Larson, 1982; Anderson et al., 1987; Quigley et al., 1991) consumed less milk (~4.0 kg/d) and more calf starter, potentially leading to accelerated ruminal development (Khan et al., 2007; Khan et al., 2016). Over the past 2 decades, there has been an increased interest in feeding high volumes of milk or milk replacer to dairy calves as data highlight positive relationships of preweaning nutrition with the growth and welfare of young heifer calves and their future milk production (Diaz et al., 2001; Soberon et al., 2012). A recent survey indicates that 31% of dairy calves in the United States receive 7.5 kg of milk or milk replacer per day (USDA, 2016). Calves on high planes of nutrition can encounter a significant decline in dietary nutrient supply as they increase CSI at a slow rate at weaning (Cowles et al., 2006). The reduced nutrient supply demands certain metabolic adjustments that can adversely affect the growth and wellbeing of calves, particularly when weaning is abrupt (de Passillé et al., 2011; Khan et al., 2011; Le Floch et al., 2014). Therefore, step-down weaning schemes are recommended to promote CSI to mitigate the nutritional stresses at weaning (Khan et al., 2007). Nonetheless, Eckert et al. (2015) observed a postweaning growth slump of dairy calves, initiating a step-down weaning scheme at 35 d versus 49 d, suggesting a need for an improved understanding of interactions between age and nutrition at weaning.

As shown in piglets (Smith et al., 2010) and calves (Wood et al., 2015), weaning can impair the intestinal mucosal barrier function that precludes the translocation of pathogens and toxins from the intestinal lumen to the blood (Turner, 2009). The compromised intestinal barrier function is associated with inflammation and immune activation, which is unfavorable to the growth and wellbeing of the animals (Moeser et al., 2007; Moeser et al., 2017). Moeser et al. (2007) highlights the importance of optimizing nutrition and the immune status to help animals cope with weaning stresses. In this regard, the supplementation of Gln, a NEAA, has shown promising results in mice (Rogero et al., 2011; Sellmann et al., 2015) and pigs (Lee et al., 2003; Hsu et al., 2012). Moreover, Duttlinger et al. (2020) demonstrated a potential to replace antibiotics with Gln to alleviate weaning and transportation stresses. Data on weaning piglets indicate that Gln can positively affect intestinal health and development by improving the intestinal barrier function (Potsic et al., 2002; Qin et al., 2018), mitigating intestinal atrophy (Wu et al., 1996), and optimizing the redox status of

the epithelium (Degroote et al., 2020). Because the physiology of neonatal calves is close to piglets, and van Keulen et al. (2020) showed improved intestinal morphology for a Gln supplement in preweaning calves, similar effects of Gln on gut health can be assumed for weaning calves. We hypothesized that supplementing Gln would improve paracellular permeability of the intestinal epithelium (**PPE**) of calves being weaned from a high volume of milk (≥ 8.0 kg/d) at an early age and ensure similar postweaning growth compared with calves weaned at a later age without Gln supplementation. The study objective was to examine the effect of beginning a step-down weaning scheme with or without Gln at 35 d of age on CSI, growth, and PPE of dairy calves fed a high allowance of milk (9.0 kg/d) compared with the responses of calves beginning the same weaning scheme without Gln at a later age of 49 d.

MATERIALS AND METHODS

Animals and Treatments

All experimental procedures were approved by the Animal Care and Use Committee at Iowa State University (IACUC# 18–163). The experiment was conducted at the Dairy Research and Teaching Farm at Iowa State University (Ames, IA). Thirty-six Holstein heifer calves at 28 d of age were matched for the week they were born and parity of the dam, and assigned randomly to 3 treatments involving a step-down weaning scheme ($n = 12$). The treatments were as follows: (1) initiating weaning at 49 d of age (**LW**), (2) initiating weaning at 35 d of age (**EW**), and (3) initiating weaning at 35 d with a Gln supplement (**EWG**). The sample size was determined with a power analysis (power = 0.80 and $\alpha = 0.05$) targeting the statistical power to capture 1 standard deviation change in ADG and CSI of dairy heifer calves using data from Wickramasinghe et al. (2019). Calves were bottle-fed daily 9.0 kg of pasteurized waste milk 3 times per day (3.0 kg per feeding at 0600, 1400, and 2200 h) until weaning was initiated by abruptly decreasing the milk volume to 3.0 kg/d on d 35 or 49, depending on the treatment. That 3.0 kg of milk was fed at the 2200 h feeding until calves were weaned completely. The weaning was completed once a calf consumed ≥ 1.0 kg of calf starter (as-fed basis) over 2 consecutive days (BAMN, 2003, 2017). In this paper, we use the terms “preweaning,” “during weaning,” and “postweaning” to describe the period before restricting milk, the period from the milk restriction to the completion of weaning, and the period after the completion of weaning, respectively. L-Glutamine (Ajinomoto Health and Nutrition North America, Inc.) at 2.0% of daily

DMI was fed in milk (3.0 kg at 2200 h) during the last week preweaning (28–35 d) and the first week during weaning (35–42 d). The Gln dose was determined based on milk solids and DM in CSI and was added into the pasteurized milk (~40°C) in bottles. The bottles were shaken thoroughly for about 20 s to dissolve Gln before offering to calves. The dose at 2.0% of DMI was chosen by considering the improvements in feed efficiency, health status, and intestinal barrier function shown in weaning piglets fed similar doses (Lee et al., 2003; Hsu et al., 2012; Qin et al., 2018). All calves were housed in individual pens inside the Calf Unit of Dairy Research and Teaching Farm at Iowa State University and were bedded with straw (floor area = 1.2 m × 1.8 m). In addition, they all had access to ad libitum amounts of drinking water and a texturized calf starter with whole corn grain (Vita Plus Corp.) throughout the study. A representative sample of calf starter was analyzed for DM, ether extract, ADF, CP (AOAC International, 2000), and NDF (Van Soest et al., 1991) at Cumberland Valley Analytical Services (Waynesboro, PA). Accordingly, the calf starter contained 90.2% of DM, and 24.0, 3.9, 20.2, and 12.8% of CP, crude fat, NDF, and ADF, respectively.

Measurements and Sample Collection

Body weight, heart girth, hip height (**HH**), hip width, and body length were measured weekly by the same person immediately before the evening (2200 h) feeding. Ambient temperature (°C) and relative humidity at each pen was recorded daily using temperature loggers (Lascar Electronics Inc.). The weight of milk, calf starter, and drinking water offered and leftover were recorded daily. Paracellular permeability of the intestinal epithelium was determined by measuring the ratio of lactulose and mannitol in the blood (**LMR**) according to the method described in Araujo et al. (2015). Lactulose (Alfa Aesar) and D-mannitol (Acros Organics) at 0.36 and 0.09 g/kg of BW, respectively, were dissolved in milk (3.0 kg at 2200 h feeding) and fed to calves on the last day preweaning, and 3 and 7 d after weaning was initiated by restricting the milk volume. Those days corresponded to 35, 38, and 42 d of age for EW and EWG, and 49, 52, and 56 d of age for LW, respectively. In compliance with Araujo et al. (2015), blood was drawn from the jugular vein 1.0 h after feeding the sugar markers. The serum was separated by centrifuging at $2,000 \times g$ at 4°C for 20 min and stored at -20°C until analysis of lactose and mannitol concentrations. Extra blood was collected, and serum or plasma was harvested with similar conditions for analysis of serum haptoglobin (**HPT**) and plasma LPS-binding protein (**LBP**) concentrations. Milk solid

consumption was determined assuming 87% moisture in whole milk (Winchester and Morris, 1956).

Analyses of Serum Haptoglobin and Plasma LPS-Binding Protein Concentrations

HPT. Serum HPT concentration was measured using a commercial kit (Life Diagnostics Inc., catalog# HAPT-11) in accordance with the manufacture's protocol. Briefly, serum samples were diluted (1:50) first with the diluent of the kit. The diluted samples and the standards (100 µL) were transferred in duplicate to a 96-well plate that was then incubated on an orbital shaker (150 rpm) at room temperature for 45 min. After washing 5 times with the wash solution of the kit, 100 µL of a horseradish peroxidase conjugate was added into each well and incubated on the orbital shaker (150 rpm) at room temperature for 45 min. After washing as described above, 100 µL of tetramethylbenzidine substrate was dispensed into each well and the plate was incubated on the orbital shaker (150 rpm) at room temperature for 20 min. Immediately after adding the stop solution of the kit to each well, the optical density was read at 450 nm using an Eon microplate spectrophotometer (BioTek Instruments, Inc.). The samples were reanalyzed if the coefficient of variation (**CV**) across duplicates was >20%. The intra-assay CV of 93% of the samples were <10%, and the rest had CV < 20%. The interassay CV was <14%.

LBP. Plasma LBP concentration was measured using a commercial kit (Hycult Biotech Inc., catalog# HK503) by following the manufacture's protocol. Briefly, plasma samples were diluted (1:250) first with the diluent of the kit. The diluted samples and the standards (100 µL) were transferred in duplicate into a 96-well plate that was incubated then at room temperature for 1 h. After washing each well 4 times with the wash buffer of the kit, 100 µL of the biotinylated tracer was added to each well, and then the plate was incubated at room temperature for another 1 h. The washing was repeated as detailed above, and 100 µL of diluted streptavidin-peroxidase was added to each well. The plate was incubated again at room temperature for 1 h. After repeating the same washing steps, 100 µL of tetramethylbenzidine substrate was added to each well, and the plate was incubated at room temperature in the dark for 30 min. Immediately after the stop solution of the kit was added to each well, the optical density was read at 450 nm using an Eon microplate spectrophotometer (BioTek Instruments, Inc.). The samples were reanalyzed if the CV across duplicates was >20%. The intra-assay CV of 89% of the samples were <10%, whereas the rest had CV <20%. The interassay CV was <11%.

Analyses of Lactulose, Mannitol, and Other Metabolites in Blood

Determination of serum lactulose and mannitol concentrations and other serum metabolites such as AA, glucose, BHB, and urea were performed by GC-MS at the W. M. Keck Metabolomics Research Laboratory at Iowa State University (Ames, IA). Briefly, ribitol (10 μ L from 1 mg/mL stock) was added to 100 μ L of each serum sample as an internal standard for polar compounds (e.g., lactulose and mannitol). On the other hand, 10 μ L of nonadecanoic acid (1 mg/mL) was added to the same serum sample as an internal standard for nonpolar compounds. After adding 0.9 mL of methanol (8:2 MeOH:H₂O), the serum samples for both polar and nonpolar compound analyses were incubated for 10 min on ice, followed by a 10-min sonication in an ice-cold water bath. The samples were then centrifuged for 7 min at 4°C and 13,000 $\times g$, and 500 μ L of the supernatant was transferred into GC-MS vials and dried in a speed-vac concentrator (Savant SpeedVac SPD120 Vacuum Concentrator, Thermo Fisher Scientific) overnight. Next, we added 50 μ L of methoxyamine hydrochloride (20 mg/mL of pyridine) and incubated the samples at 30°C for 1.5 h. Trimethylsilylation was then performed by incubating samples with 70 μ L of bis-trimethyl silyl trifluoroacetamide containing 1% trimethylchlorosilane for 30 min at 60°C. The GC-MS analyses were performed for 1 μ L of the sample using an Agilent 6890N GC coupled to a mass selective detector (model 5975, Agilent Technologies). The column used was 5% phenyl methyl siloxane with 30 m $\times$ 250 μ m $\times$ 0.25 μ m film thickness (Agilent 19091S-433, Agilent Technologies). The oven temperature was increased first from 50 to 245°C at a rate of 5°C/min, and then to 320°C at a rate of 20°C/min. Helium was used as a carrier gas at an initial flow rate of 1.2 mL/min. The quantifications were performed using electron ionization at 70 eV by setting the source and quadrupole temperatures at 230°C and 150°C, respectively. The mass data were collected in the range from m/z 50 to m/z 800. Agilent Enhanced ChemStation version D.02.00.275 was used for the identification of compounds. The abundance of compounds was quantified by integrating the corresponding peak areas relative to the area of the internal standards.

Statistical Analysis

Treatment effects on the responses of interest were determined using the following statistical model:

$$Y_{ijkl} = \mu + T_i + PR_j + P_k + (T \times PR)_{ij} + TMP_{ijkl} + C_l + e_{ijkl},$$

where Y_{ijkl} = the response variable of interest, μ = the overall mean of the response, T_i = the fixed effect of the i th treatment (i = LW, EW, and EWG), PR_j = the fixed effect of the j th period (j = preweaning, during weaning, and postweaning), P_k = the fixed effect of k th parity (k = primiparous and multiparous), $(T \times PR)_{ij}$ = the fixed effect of the interaction between i th treatment and j th period, TMP_{ijkl} = fixed covariate effect of the average ambient temperature of the day the response was measured, C_l = the random effect of the l th calf, and e_{ijkl} = the random error assumed to be independent and identically distributed from a normal distribution with a mean of 0 and a variance of σ^2 [$\sim N(0, I\sigma^2)$]. When growth performance was analyzed, the model included birth weight as a covariate. The preweaning and weaning periods corresponded to different windows of age for LW versus EW and EWG. On the other hand, postweaning performance corresponded to ages of 10 and 20 wk, common for all calves. Because ambient temperature explained much of the variability among sampling days that were different for calves at a given age, it was included in the model instead of week that calves were born. All analyses were performed using the MIXED procedure of SAS (version 9.4, SAS Institute Inc.) with the REPEATED option and the unstructured variance-covariance structure. The treatment means were compared using the Tukey-Kramer adjustment test. The P -value for treatment effect within a given period was obtained with the SLICE option included in the lsmeans statement. Statistical differences were declared at $P \leq 0.05$, and a tendency toward significance was considered at $0.05 < P \leq 0.10$.

RESULTS

Intake and Growth

Treatment effects on nutrient intake and growth during preweaning, weaning, and postweaning are presented in Tables 1 and 2, respectively. The CSI and BW changing with age across the periods are presented in Figure 1. Calves in all 3 treatments had similar milk intake at 8.51 kg/d ($P = 0.140$), which was the sole contributor to DMI because CSI was negligible preweaning (Table 1). There was a significant treatment $\times$ period interaction on CSI and drinking water intake ($P < 0.001$, data not shown). The CSI was similar among treatments preweaning ($P = 0.515$) but increased and decreased for LW compared with EW during weaning ($P < 0.001$) and postweaning ($P = 0.006$), respectively. In line with CSI, drinking water intake also increased and decreased for LW compared with EW during weaning ($P = 0.010$) and postweaning ($P < 0.001$), respectively. Drinking

water intake, CSI, and DMI of EW and EWG were similar in each period ($P > 0.140$) but decreased ($P < 0.010$) and increased ($P < 0.055$) for EWG compared with LW during weaning and postweaning, respectively (Table 1). Moreover, drinking water intake of EWG was less than LW ($P = 0.014$) despite the similar milk intake among calves ($P = 0.140$) preweaning. Initiation of weaning by restricting the milk allowance from 9.0 to 3.0 kg/d increased CSI in all 3 treatments ($P < 0.001$, Figure 1A). In line with the differences in CSI, LW had greater DMI than EW or EWG in the first week (0.95 vs. 0.53 and 0.63 kg/d) as well as the rest of the days (1.00 vs. 0.73 and 0.76 kg/d) during weaning ($P < 0.003$). The LW achieved ≥ 1.0 kg/d of CSI (as-fed basis) 11 d after weaning was initiated, and thus weaned completely at 60 d of age. In the week before and after the initiation of weaning, 25.2 and 14.4 g of Gln (0.41 and 0.24 g/kg of BW) were fed to EWG, respectively (data not shown). The EWG achieved ≥ 1.0 kg/d of CSI 16 d after weaning was initiated. Therefore, EWG was weaned completely at 51 d of age, which was 3 d earlier than EW that was weaned completely at 54 d of age ($P < 0.001$, Table 1).

Overall, the growth responses to treatments were consistent across the periods ($P > 0.180$ for treatment $\times$ period interaction, data not shown). The age differences

of LW versus EW and EWG seemed to dictate treatment differences around weaning. Body weight (68.2 vs. 58.7 and 59.5 kg) and body frame measurements of LW (49 d of age) were greater than EW and EWG (35 d of age) at the initiation of weaning ($P < 0.050$, Table 2). The ADG of LW tended to be greater than EW and EWG preweaning ($P = 0.061$). The LW and EWG maintained similar ADG from the last week preweaning to the first week of weaning ($P > 0.650$), whereas EW experienced a growth slump ($P = 0.007$). The ADG of EWG tended to be greater than EW ($P = 0.067$) but was similar to LW ($P = 0.168$) during the first week of weaning. Signifying again the age difference, LW had greater BW, heart girth, HH, and hip width than both EW and EWG when measured at the end of the first week of weaning ($P < 0.015$, Table 2). Similarly, LW tended to have a greater BW, representing a 1.99-fold increase in birth weight compared with the BW of EW and EWG, representing 1.77- and 1.76-fold increases in birth weight at complete weaning, respectively ($P = 0.069$, Table 2). The EW and EWG exhibited a marked recovery of growth postweaning and had BW, heart girth, HH, hip width, and body length that were similar to LW at 10 wk of age ($P > 0.210$, Figure 1B and Table 2). The BW and HH were also similar among the treatments at 20 wk of age ($P > 0.750$, Table 2).

Table 1. Milk intake, calf starter intake (CSI), DMI (milk solid + starter DM), and drinking water intake (from buckets) of calves undergoing a step-down weaning scheme beginning at 49 d of age (LW) or earlier (35 d of age) with (EWG) or without (EW) an oral supplementation of Gln from 28 to 42 d of age ($n = 12$)

Variable	Least squares means			SEM	P-value
	LW	EW	EWG		
Preweaning ¹					
Milk intake, kg/d	8.48	8.58	8.48	0.05	0.140
CSI, kg/d	0.06	0.01	0.04	0.06	0.515
DMI, kg/d	1.08	1.00	1.04	0.05	0.518
Drinking water intake, kg/d	1.52 ^a	1.12 ^{ab}	0.73 ^b	0.19	0.014
First week of weaning ²					
Milk intake, kg/d	2.80	2.79	2.80	0.09	0.984
CSI, kg/d	0.57 ^a	0.23 ^b	0.34 ^b	0.05	<0.001
DMI, kg/d	0.95 ^a	0.53 ^b	0.63 ^b	0.05	<0.001
Drinking water intake, kg/d	3.14 ^a	2.43 ^b	2.77 ^{ab}	0.19	0.035
During remainder of weaning					
CSI, kg/d	1.00	0.73	0.76	0.07	0.029
Drinking water intake, kg/d	3.59	2.52	2.20	0.18	0.003
Days to reach 1.0 kg/d CSI	11.0 ^c	18.6 ^b	16.0 ^a	0.20	<0.001
Age when weaning completed, d	60.0 ^a	53.6 ^b	51.0 ^c	0.20	<0.001
Postweaning ³					
CSI, kg/d	2.55 ^b	2.73 ^a	2.83 ^a	0.05	0.001
Drinking water intake, kg/d	6.64 ^a	7.59 ^b	7.10 ^{ab}	0.17	<0.001

^{a-c}Least squares means with different superscripts indicate statistical difference ($P < 0.050$).

¹Week immediately before initiating weaning (42–49 d for LW and 28–35 d for EW and EWG).

²During the first week of weaning (49–56 d for LW, and 35–42 d for EW and EWG).

³During 63 to 70 d of age.

Lactulose-to-Mannitol Ratio and Acute Phase Proteins in Blood

Serum lactulose and mannitol concentrations and LMR are presented in Table 3. Figure 2A presents how LMR changed across the last day preweaning and 3 and 7 d after initiating weaning by restricting milk from 9.0 to 3.0 kg/d. The mannitol concentration was similar at 1.57 µg/L across treatments ($P = 0.867$) as well as across the days ($P = 0.912$). Serum lactulose concentration was also similar among treatments ($P = 0.801$) but tended to increase during weaning versus preweaning ($P = 0.069$). Regardless of treatments, calves had similar LMR preweaning and 3 d after initiating weaning (Figure 2A). Relative to d 3 values, LMR of LW and

EW increased ($P < 0.040$, Figure 2A), whereas that of EWG remained unchanged on d 7 during weaning. Figures 2B and C present how serum HPT and plasma LBP concentrations changed during the first week of weaning relative to preweaning concentrations, respectively. All calves had similar serum HPT concentrations preweaning ($P > 0.320$, Figure 2B). The HPT concentration of LW increased 3 d after the initiation of weaning ($P = 0.030$) and tended to remain higher on d 7 relative to preweaning concentration ($P = 0.077$, Figure 2B). The serum HPT concentration of LW was greater than that of EW on d 3 ($P = 0.025$) and tended to be greater than that of EWG on d 7 during weaning ($P = 0.098$). The plasma LBP concentrations of all 3 treatments increased ($P < 0.001$) compared with pre-

Table 2. Growth performance of calves undergoing a step-down weaning scheme beginning at 49 d of age (LW) or earlier (35 d of age) with (EWG) or without (EW) an oral supplementation of Gln from 28 to 42 d of age (n = 12)

Variable	Least squares means			SEM	P-value
	LW	EW	EWG		
Birth weight, kg	37.7	38.0	38.1	0.4	0.780
Preweaning ¹					
BW, kg	68.2 ^a	58.7 ^b	59.5 ^b	3.2	0.040
Heart girth, cm	88.5 ^a	86.5 ^b	86.6 ^b	0.5	<0.001
Hip height, cm	87.4 ^a	85.3 ^b	85.5 ^b	0.5	<0.001
Hip width, cm	20.8 ^a	20.2 ^b	20.5 ^{ab}	0.2	0.027
Body length, cm	79.7 ^a	77.2 ^b	77.1 ^b	0.5	<0.001
ADG, kg/d	0.66	0.50	0.42	0.09	0.061
First week of weaning ²					
BW, kg	72.4 ^a	59.5 ^b	62.0 ^b	3.0	0.003
Heart girth, cm	90.9 ^a	87.1 ^b	87.6 ^b	0.8	0.011
Hip height, cm	91.2 ^a	87.6 ^b	88.6 ^b	0.8	0.010
Hip width, cm	22.1 ^a	20.7 ^b	21.5 ^{ab}	0.3	0.006
Body length, cm	83.5 ^a	80.8 ^b	80.1 ^b	0.9	0.052
ADG, kg/d	0.47 ^a	0.03 ^b	0.25 ^{ab}	0.11	0.005
During remainder of weaning ³					
ADG, kg/d	0.94	0.83	0.79	0.12	0.493
BW at complete weaning	74.8	66.5	66.4	3.1	0.064
BW:birth weight ⁴	1.99	1.77	1.76	0.08	0.069
Postweaning					
10 wk of age ⁵					
BW, kg	88.5	87.6	89.7	3.0	0.871
Heart girth, cm	97.6	97.5	96.4	0.7	0.314
Hip height, cm	94.3	93.7	94.4	0.6	0.669
Hip width, cm	24.6	24.4	24.7	0.2	0.673
Body length, cm	89.0	89.1	90.6	0.7	0.211
ADG, kg/d	1.11	0.92	1.18	0.11	0.188
20 wk of age					
BW, kg	165.4	166.4	161.1	10.0	0.908
Hip height, cm	109.9	109.0	109.7	1.1	0.775

^{a,b}Least squares means with different superscripts indicate significant difference ($P < 0.050$).

¹BW and body frame measurements at 49 d for LW and 35 d for EW and EWG. ADG was during 42 to 49 d for LW and 28–35 d for EW and EWG.

²BW and body frame measurements at 56 d for LW and 42 d for EW and EWG. ADG was during 49 to 56 d for LW and 35 to 42 d for EW and EWG.

³BW and body frame measurements at 60, 54, and 51 d for LW, EW, and EWG, respectively.

⁴The ratio between BW at weaning and birth weight.

⁵BW and body frame measurements at 70 d for all treatments. The ADG was during 63 to 70 d for all treatments.

Figure 1. Weekly mean ($\pm$ SEM) for calf starter DMI (A), and BW (B) of calves undergoing a step-down weaning scheme that was initiated by restricting the milk volume at 49 d of age (LW) or earlier (35 d of age) with (EWG) or without (EW) an oral supplementation of Gln from 28 to 42 d of age ($n = 12$). All calves were weaned completely once they achieved 1.0 kg/d of calf starter intake over 2 consecutive days. Trt = treatment; Wk = week.

weaning concentrations (Figure 2C), but EW tended to be associated with lower plasma LBP than LW and EWG ($P = 0.082$) 3 d after the initiation of weaning. The LBP concentrations in all 3 groups had returned to preweaning concentrations 7 d after the initiation of weaning.

Postprandial Serum Concentrations of Metabolites

Postprandial serum concentrations of AA, glucose, BHB, and urea preweaning and in the first week during weaning are presented in Table 3. Changes in the

concentrations of some of those metabolites during the first week of weaning relative to preweaning concentrations are presented in Figure 3. The concentrations of all metabolites except Ile and Gly ($P < 0.035$ for treatment $\times$ period interaction, data not shown) responded consistently to treatments before and during weaning. The serum Gln concentration of EWG was greater than EW and LW preweaning ($P < 0.010$) and during the first week of weaning ($P < 0.020$). Relative to preweaning concentrations, serum Gln concentration of EWG decreased by 32% in the first week during weaning ($P = 0.009$, Figure 3A). The concentration of total serum EAA (Ile + Leu + Lys + Met + Phe + Thr + Trp + Val) was greater in LW ($P = 0.041$) and EWG ($P = 0.007$) than EW preweaning. However, the concentration of total serum EAA was similar among treatments in the first week during weaning ($P = 0.239$, Figure 3B). The concentrations of total serum branched-chain AA, including Ile, Leu, and Val, were greater in EWG than EW preweaning ($P < 0.035$, Figure 3C). Similarly, the Phe concentration of LW was greater than EW ($P = 0.022$) but not different from EWG ($P = 0.766$) preweaning (Figure 3D). However, none of those EAA concentrations were different between treatments during weaning ($P > 0.102$, Table 3). On the other hand, serum Met of EWG was greater than EW preweaning as well as during the first week of weaning ($P < 0.020$, Figure 3E). Serum Met concentration of EWG was even greater than that of LW preweaning ($P = 0.010$, Table 3). Postprandial serum glucose concentration was not affected by treatments but tended to be greater for EWG versus EW preweaning ($P = 0.091$, Table 3). The EWG had greater serum urea concentrations than EW preweaning ($P = 0.020$), but the Gln supplementation did not change the serum urea concentration during the first week of weaning. Regardless of treatments, serum BHB increased ($P < 0.001$) once weaning was initiated by restricting the milk allowance (Figure 3F). The LW calves had greater serum BHB concentrations than EW during the first week of weaning ($P = 0.018$).

DISCUSSION

To ensure less stressful weaning and to mitigate potential growth slumps, step-down weaning schemes are recommended over abrupt weaning (Khan et al., 2007), particularly for dairy calves that are fed high volumes of milk or milk replacer (Steele et al., 2017). Eckert et al. (2015) used a step-down scheme to compare weaning from a high milk replacer allowance at 42 versus 56 d of age. They initiated the scheme by decreasing milk replacer allowance by 50% (4.0 vs. 8.0 L/d) at 35 or 49 d of age and weaned calves completely 7 d later. In the present study, we initiated step-down weaning with a

Table 3. Postprandial (1 h) serum metabolite concentrations of calves undergoing a step-down weaning scheme beginning at 49 d of age (LW) or earlier (35 d of age) with (EWG) or without (EW) an oral supplementation of Gln from 28 to 42 d of age (n = 12)

Metabolite	Prewaning ¹				During weaning ²			
	LW	EW	EWG	P-value	LW	EW	EWG	P-value
Gut permeability markers								
Mannitol, µg/L	1.526	1.462	1.685	0.861	1.589	1.453	1.573	0.914
Lactulose, µg/L	2.707	2.768	2.337	0.911	3.794	3.270	2.972	0.686
Lactulose:mannitol	1.853	1.706	1.479	0.682	2.284	2.422	1.786	0.115
EAA, µmol/L								
Met	15.5 ^b	14.2 ^b	22.3 ^a	<0.001	12.5 ^{ab}	11.2 ^b	15.0 ^a	0.046
Lys	37.5	27.5	39.2	0.355	23.1	26.3	35.9	0.145
Ile	65.7 ^{ab}	56.3 ^a	78.1 ^b	0.021	44.0	48.7	49.1	0.616
Leu	78.2 ^a	62.0 ^b	76.8 ^a	0.043	64.5	65.4	69.7	0.592
Val	123.7 ^a	100.9 ^b	125.0 ^a	0.030	96.0	97.4	107.7	0.265
Phe	14.0 ^a	10.1 ^b	13.5 ^a	0.043	10.5	9.6	12.5	0.103
Thr	34.6	33.9	36.6	0.834	28.2	30.5	31.3	0.653
Trp	7.3	4.6	6.8	0.210	6.8 ^a	3.5 ^b	4.9 ^{ab}	0.040
Total EAA	376.4 ^a	309.2 ^b	398.2 ^a	0.020	285.3	292.5	325.5	0.239
NEAA, µmol/L								
Gln	33.7 ^b	27.6 ^b	60.9 ^a	<0.001	25.6 ^b	23.03 ^b	41.2 ^a	0.009
Glu	21.2	16.6	21.8	0.194	14.4	11.7	15.5	0.219
Gly	101.7 ^a	78.5 ^b	108.1 ^a	0.025	98.9	111.5	119.6	0.070
Ala	62.9	42.6	53.8	0.089	41.7	46.9	56.5	0.138
Ser	46.7	36.7	49.9	0.120	34.6	40.3	47.3	0.076
Asp	0.6	0.4	0.5	0.150	0.3	0.3	0.3	0.619
Pro	68.2	53.7	64.1	0.261	40.6	44.2	53.7	0.139
Tyr	25.3	17.6	30.6	0.097	24.7	21.0	24.0	0.654
Other metabolites								
Glucose, mmol/L	5.797	5.422	6.500	0.226	5.331	5.907	5.611	0.514
BHB, mmol/L	0.038	0.030	0.034	0.599	0.066 ^a	0.051 ^b	0.055 ^{ab}	0.049
Urea, mmol/L	0.516 ^{ab}	0.463 ^b	0.575 ^a	0.020	0.505	0.483	0.532	0.407

^{a,b}Different superscripts in a given row indicate significantly different ($P < 0.050$) means in each period separately.

¹One day before weaning began (at 34 d for EW and EWG, and 48 d for LW).

²Two days during the first week of weaning (38 and 42 d for EW and EWG, and 52 and 56 d for LW).

more severe decrease (67%) in milk volume (9.0 to 3.0 kg/d) at the same ages. Moreover, we continued with the 3.0 kg/d of milk until calves achieved a 1.0 kg/d of CSI to wean them completely. Calves in the present study had a negligible CSI when receiving the full milk allowance, testifying the negative relationship between liquid diet allowance and CSI (Leaver and Yarrow, 1972; de Passillé and Rushen, 2012; Hill et al., 2012). Once the milk allowance was restricted, CSI increased in both EW and LW, but remained fairly low (<0.30 kg/d) for EW in the first week during weaning. The high milk allowance seemed to play a role in determining the CSI independent of age as calves weaned from a lower milk replacer allowance (5.4 L/d of milk replacer with 14.7% solid) at 35 d consumed >1.0 kg/d of calf starter during the first week after weaning in Jaeger et al. (2020). Owing to 0.60 kg/d of CSI, LW was able to maintain a constant DMI, whereas EW encountered a significant DMI decline that compromised its ADG during the first week of weaning. The lower CSI of EW most probably suggested a lack of ruminal development at 35 versus 49 d of age (Khan et al., 2011). The literature emphasizes that a successful transition from

liquid to solid diets requires the physical and metabolic development of the rumen accompanied by the development of the salivary apparatus, rumination behavior, and physiological adjustments of other tissues (Baldwin et al., 2004; Khan et al., 2011). However, the metabolic development of ruminal epithelium seems similar at 35 and 49 d of age as serum BHB were comparable between EW and LW preweaning. Although LW increased CSI faster and achieved the 1.0 kg/d target sooner than EW (11 vs. 19 d), EW could be weaned still at an earlier age than LW (54 vs. 60 d) in the present study. Because raising preweaning calves is twice as costly as raising weaned calves (Hawkins et al., 2019, 2020), weaning 6 d earlier can be economically advantageous. Nonetheless, it is noteworthy that weaning schemes should be evaluated not only based on economic gains but also related to the effects on calf health and welfare and future performances (Khan et al., 2011; Soberon et al., 2012; Hulbert and Moisés, 2016).

Both EW and LW had similar BW and HH at 10 and 20 wk of age, indicating no adverse effect of initiating weaning with the present scheme at 35 versus 49 d of age on the growth postweaning, and perhaps the future

Figure 2. Least squares means $\pm$ SEM of serum lactulose:mannitol ratio (A), serum haptoglobin (B), and plasma LPS-binding protein (LBP; C) of calves undergoing a step-down weaning scheme that was initiated by restricting the milk volume at 49 d of age (LW) or earlier (35 d of age) with (EWG) or without (EW) an oral supplementation of Gln from 28 to 42 d of age ($n = 12$). Trt = treatment.

milk production as well (Soberon et al., 2012). Even though calves in the EW group had a low CSI in the first week during weaning, they increased it sharply during the following week, resulting in a quick recovery from the growth slump. On the contrary, in Eckert et al. (2015), beginning weaning at 35 versus 49 d of age was related to a postweaning growth slump at 10 wk, albeit the early-weaned calves had achieved >1.0 kg/d

of CSI at complete weaning (42 d of age). Perhaps, extending the duration of weaning (e.g., 14 vs. 7 d) with some volume of milk, regardless of CSI, could have mitigated such a postweaning growth slump (Pluske et al., 1996). However, a comparison between the present weaning scheme and that in Eckert et al. (2015) should acknowledge several other differences between the 2 studies such as type and nutrient composition of liquid and solid diets, health status of calves, and the season during when studies were conducted.

Despite the unaffected postweaning growth, one cannot overlook the growth slump of EW at the onset of weaning as it can reflect multiple forms of nutritional stress (e.g., inflammation and infections linked to compromised gut barrier function; Campbell et al., 2013; Khan et al., 2016; Moeser et al., 2017). The literature highlights that dietary supplements of Gln improves gut barrier function, inflammation markers, and behavior, which are related to growth improvements in piglets subjected to heat stress, transportation stress, or an LPS challenge (Johnson and Lay, 2017; Qin et al., 2018). In the present study, we demonstrated those effects of Gln could also be true in weaning calves as EWG tended to improve ADG at the onset of weaning. Because protein synthesis represents 70% of water-free empty BW gain (Bartlett et al., 2006), the improvement in ADG could be a result of protein synthesis enhanced by Gln in weaning calves (Ji et al., 2019). The supplementation of Gln seems to increase the precursor supply and the stimulatory signals of protein synthesis, as EWG had greater serum EAA (particularly branched-chain AA) concentrations than EW during weaning. One way dietary Gln could increase postprandial serum EAA is by improving the capacity of the small intestine for digestion and absorption of proteins. Supporting this notion, van Keulen et al. (2020) demonstrated improved villus heights in the duodenum and jejunum of dairy calves supplemented with Gln in milk. Among EAA responding to Gln supplementation, Met showed the most consistent increase in the serum concentrations. No data describe an association of Gln and Met, even in mature cattle, for which Met is the most limiting AA. The literature on weaning piglets, however, highlights a possibility that Gln supplementation can increase serum Met by decreasing the splanchnic clearance (Hu et al., 2012; Dong et al., 2019).

Moreover, the present study results highlighted, for the first time, the potential of Gln to improve PPE in weaning calves. The PPE is a major mechanism that contributes to the intestinal barrier function (Moeser et al., 2017), and the role of Gln in improving PPE in weaning piglets has been well studied (Ji et al., 2019). Orally administered lactulose and mannitol recovered in urine is frequently used as a noninvasive method

Figure 3. Least squares means $\pm$ SEM of serum Gln (A), total EAA (B), branched-chain amino acids (BCAA; C), Phe (D), Met (E), and BHB (F) of calves undergoing a step-down weaning scheme that was initiated by restricting the milk volume at 49 d of age (LW) or earlier (35 d of age) with (EWG) or without (EW) an oral supplementation of Gln from 28 to 42 d of age ($n = 12$). Trt = treatment.

to assess PPE in humans. Elevated LMR in urine is indicative of increased PPE. Considering the challenges of obtaining representative urine samples, Fleming et al. (1996) developed a method to determine LMR in serum, which was highly correlated with LMR in urine ($r = 0.88$) in humans. Araujo et al. (2015) developed a similar method to determine LMR in the blood of calves. Therefore, we used that method to assess PPE of weaning calves in the present study. Our LMR revealed that an aggressive restriction of milk can increase PPE in calves, agreeing with increased PPE observed for feed restrictions in mature cows (Kvidera et al., 2017). Our results also highlighted a lag time (4–7 d) for the PPE response, which is in agreement with Degroote et al. (2020). Those authors assessed PPE in the small intestine of weaned piglets and did not see a PPE increment until 5 d postweaning. Degroote et al. (2020) also showed a markedly induced oxidative damage to the intestinal mucosa that was closely in line with the PPE response for weaning. Perhaps, the Gln supplementation mitigated the potential increase in PPE by alleviating oxidative damage to the intestinal mucosa of weaning calves (Ji et al., 2019). Concerning the positive link between PPE and the likelihood of luminal LPS translocation to the bloodstream, one would expect LMR to be in line with HPT and LBP concentrations in the blood (Wright et al., 1990). Our data, however, do not show such synchrony. For instance, plasma LBP concentrations seemed to respond far more quickly to the milk volume restriction than LMR did. One potential mechanism that can create a quick surge of plasma LBP is inflammation in the intestine and corresponding production of cytokines such as IL-6 and TNF- α (McCracken et al., 1999; Pié et al., 2004; Kim et al., 2011), which can stimulate the production of LBP in the liver (Wolk et al., 2007). In that sense, the low plasma LBP of EW could reflect a less severe inflammation in the intestine or an inadequate immune response to the inflammation. Jafari et al. (2006) and McLamb et al. (2013) support the latter. It is also fair to mention that many commercial kits including the one used in the present study are unable to distinguish free and bound forms of LBP, limiting the ability for drawing robust conclusions on treatment effects.

CONCLUSIONS

The present study offered a step-down scheme to wean calves fed a high milk allowance (9.0 kg/d) without affecting CSI and growth postweaning. In the present scheme, weaning was initiated at 35 d of age with an abrupt restriction of the milk allowance to 3.0 kg/d, and calves were weaned completely once they consumed ≥ 1.0 kg/d of calf starter (as-fed basis). Initiating wean-

ing at 35 d decreased ADG and was associated with a lower rate of CSI increase during weaning as opposed to initiating weaning at 49 d. Supplementation of Gln (2.0% of DMI) at 35 d tended to improve the ADG. Calves with Gln also increased CSI faster and weaned completely 3 d earlier than calves without Gln (51 vs. 54 d of age). Regardless of the age, the milk allowance restriction was associated with an increased PPE, suggesting an impairment of the gut barrier function. The supplementation of Gln mitigated such impairment. The present step-down scheme with a Gln supplement (2% of DMI) could help in weaning dairy calves from a high milk volume without compromising the growth during and postweaning.

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