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The effect of maternal supply of rumen-protected protein to Holstein Friesian cows during the dry period on the transfer of passive immunity and colostral microbial composition

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ABSTRACT

The objective of this study was to analyze if maternal supply of rumen-protected protein during the dry period can affect the IgG concentration and microbial composition of colostrum and the IgG absorption and fecal microbial composition in the calf. Seventy-four multiparous Holstein Friesian (HF) dairy cows were stratified per parity and randomly assigned to one of 2 different dry period diets, a diet with a low crude protein (CP) level (LP) and a diet with a high CP level (HP) by addition of rumen-undegraded protein (RUP; formaldehyde-treated soybean meal, Mervobest, Nuscience, Drongen, Belgium). Colostrum was collected within 1 h after calving and IgG concentration was quantified by radial immunodiffusion analysis. Forty-nine calves (23 female and 26 male) were enrolled in the trial with a 2 × 2 factorial design, with prenatal and postnatal treatment as the 2 independent variables. This led to 4 experimental groups: LPLP, LPHP, HPLP, and HPHP, in which the first 2 letters refer to the prenatal treatment (diet of the dam) and the last 2 refer to the postnatal treatment (diet of the colostrum-producing cow). Calves received 3× 2 L of colostrum within 2, 6, and 24 h after birth. Meconium and feces were collected solely from female calves (n = 18) by digital palpation of the rectum, immediately after birth and before colostrum administration and at d 3 of age. Microbial DNA was extracted from meconium (n = 9), feces (n = 15), and colostrum (n = 49). Amplicon sequencing of the bacterial V3-V4 region of the 16S rRNA gene was performed for characterization of the bacterial communities. Colostrum IgG concentration was higher in cows that were supplemented with RUP, especially in cows entering their second lactation (LSM ± SEM 61.3 ± 2.3 vs. 55.2 ± 2.8 g of IgG/L). Calves born out of LP cows that received colostrum from HP cows (LPHP) had a lower serum IgG level compared with HPHP and LPLP calves (LSM ± SEM 14.2 ± 1.3 vs. 18.8 ± 1.2 and 20.9 ± 1.3 g of IgG/L in HPHP and LPLP, respectively). The most abundant phyla in colostrum were *Proteobacteria* (48.2%), *Firmicutes* (24.8%), *Bacteroidetes* (9.5%), and *Actinobacteria* (5.0%). The most abundant phyla in calf meconium and feces were *Firmicutes* (42.5 and 47.5%), *Proteobacteria* (21.7% and 33.7%), *Bacteroidetes* (16.8% and 15.7%), and *Actinobacteria* (2.9% and 3.1%). There was no difference in the overall microbial communities between colostrum from HP and LP cows. However, 2 genera (both members of the family *Lachnospiraceae*) were more abundant in colostrum from HP cows compared with LP cows. The microbial composition of meconium, feces and colostrum differed from each other. Fecal samples were more similar to each other and are characterized by a lower intersample diversity compared with colostrum and meconium samples. To conclude, increasing the CP level by addition of RUP in the dry period diet affected the colostrum IgG concentration and the transfer of passive immunity, but did not change the overall microbial composition of colostrum nor of meconium and feces in the calf.
Key words: bovine colostrum, dry period diet, microbial composition, transfer of passive immunity

INTRODUCTION

Colostrum production, or colostrogenesis, starts approximately 3 to 4 wk before calving and ends abruptly after giving birth (Brandon et al., 1971). For multiparous cows, this means that colostrum production occurs during the dry period. Simultaneously, at the end of gestation nutrient requirements for the growing prenatal calf are at its maximum (Bell et al., 1995; Quigley and Drewry, 1998; Lean et al., 2013). The NRC (2001) nutrient requirements were recently revised and for pregnant dairy cows protein requirements for gesta-

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tion are set at 125 g of MP (or true digested protein in the small intestines (**DVE**; Tamminga et al., 1994) per day at 200 d of gestation and gradually increase to a daily need of 320 and 489 g of MP at 250 and 275 d of gestation (NASEM, 2021) to meet for the needs of the exponential growth of the gravid uterus at the end of gestation (Bell et al., 1995). However, these recently updated NASEM (2021) recommendations on prepartum protein supply to late-pregnant cows do not take protein requirements for colostrum production into account because data on the importance of dietary protein supply for colostrum production is lacking. In the past, several studies showed little effect of supplementing dietary protein in the prepartum diet on the milk and protein yield in the early lactation (Bell et al., 2000; Lean et al., 2013). Feeding additional protein prepartum can, however, improve the health status of fresh cows (Lean et al., 2013). While in the past several researchers focused on the effect of prepartum protein supplementation on the performance in the subsequent lactation (Huyler et al., 1999; Santos et al., 2001; Doepel et al., 2002), less attention was paid to the effect on colostrum production or calf performances. Furthermore, an inadequate MP supply has a negative effect on the immune system and is linked to an increased risk of peripartal diseases (Drackley and Cardoso, 2014).

Other studies reported that the prepartum diet can influence colostrum production and composition. Salehi et al. (2016) showed a positive effect of feeding dairy cows sunflower seeds (that are high in linoleic acid) at the end of gestation on colostral CP and brix values and conjugated linoleic acid concentrations. Elevating the CP level of the prepartum diet in Holstein Friesian (**HF**) cows lowered the colostrum density without affecting the IgG content (Toghyani and Moharrery, 2015).

In addition to effects on colostrum production and composition, maternal nutrition in the dry period may also affect the health of the neonatal calf. It has been demonstrated that maternal nutrition has both short- and long-term effects on the newborn's health and development (Godfrey and Barker, 2000). Previous research showed a dietary effect on the transfer of passive immunity in beef (Blecha et al., 1981; Hough et al., 1990) and dairy cows (Burton et al., 1984; Toghyani and Moharrery, 2015) without affecting colostral IgG concentration. Calves born out of cows fed a protein restricted diet at the end of gestation, showed lower absorption efficiency of IgG (Blecha et al., 1981; Burton et al., 1984; Hough et al., 1990). Because the IgG concentration of colostrum seems to be unaffected by prepartum CP intake (Blecha et al., 1981; Burton et al., 1984; Hough et al., 1990), the question remains how to explain the lower absorption capacity of these calves. There are 2 possible hypotheses: either maternal nutrition affects the intrauterine development of the fetal intestines impairing the absorption of IgG, or maternal nutrition affects colostrum composition in a way that IgG absorption in the intestines is altered, without influencing the colostral IgG concentration. In the present study the focus was on the last hypothesis.

In addition to the essential source of antibodies and nutrients for the neonatal calf, colostrum is very rich in bioactive molecules (Godhia and Patel, 2013; Van Hese et al., 2020), and it harbors a rich and diverse microflora (Lima et al., 2017; Van Hese et al., 2022). These colostrum components contribute as well to the maturation of the gastrointestinal tract (**GIT**; Blum, 2006; Hammon et al., 2013). Additionally, colostrum is one of the calf's first inoculation sources of bacteria and affects the bacterial colonization of the GIT in the calf (Godden, 2008; Malmuthuge et al., 2015; Fischer et al., 2018). We furthermore recently showed that the abundance of some bacteria in bovine colostrum is associated with both the colostral IgG concentration as well as the serum IgG concentration in the recipient calves (Van Hese et al., 2022). For example, a higher abundance of *Enterobacteriaceae* and *Pseudomonas* in colostrum was associated with lower IgG levels in serum from HF cows (Van Hese et al., 2022). Moreover, maternal nutrition can influence the microbial composition of milk. In human breast milk, a negative correlation was found between the abundance of *Streptococcus* and the maternal consumption of unsaturated fatty acids (Babakobi et al., 2020). In rats, a higher maternal protein intake led to a higher abundance of *Lactobacillus* in their milk (Warren et al., 2019). Whether this is as well the case in bovine colostrum, has to the best of our knowledge never been analyzed before

The hypothesis of this study is that prepartum supplementation of RUP improves colostrum quality at both the level of the IgG concentration and at the level of the microbial composition which leads to higher IgG absorption in the calf. The main objective of the present study was to evaluate the effect of RUP (formaldehyde-treated soybean meal, Mervobest, Nuscience, Drongen, Belgium) supplementation in the dry period on the colostrum IgG concentration in HF dairy cows and IgG absorption in calves. The secondary objective was to analyze the effect of prepartum RUP supplementation on the microbial composition of colostrum and associate the latter with the IgG absorption in the calf. In addition, the effect of prepartum RUP supplementation on birthweight and growth rate in the calf, as well as associations between the microbial composition of colostrum and that of the calves' feces were analyzed.

MATERIALS AND METHODS

Ethics Statement

The experimental protocol was approved by the Flanders Research Institute for Agriculture, Fisheries and Food (**ILVO**) ethical committee (case number EC2019/337). All methods were carried out in accordance with the approved guidelines.

Experimental Design

A schematic overview of the experimental design is represented in Figure 1. A random list of treatment (high protein; **HP**) and control (low protein; **LP**) was generated (Haahr, 2019) and cows were assigned chronologically to this list based on expected calving date. Cows within each parity class (parity 2 vs. parity >2) were assigned to either the HP (n = 38) or LP (n = 34) dry period diet, hence obtaining stratified randomization by parity. The dry period was divided in 2 periods, cows started in the far-off period approximately 45 d before expected calving date and switched to the close-up period approximately 14 d before expected calving date. Cows in the LP group received a typical Flemish dry period diet consisting of maize silage and straw throughout the far-off period and switched to the close-up diet during the close-up period (ILVO, 2011). Cows in the HP group received the standard dry period far-off and close-up diet supplemented with RUP to achieve a 2% point increase in CP. All cows were equally supplemented with minerals and vitamins according to recommendations of the Dutch Centraal Veevoeder Bureau (**CVB**, 2016). The calves born from the cows entered a 2 × 2 factorial experimental design, because of the possible interaction between the prenatal (maternal nutrition during the dry period) and postnatal (diet of the colostrum-producing cow) treatment. In this way, both calves born from LP and HP cows were assigned to one of the 2 postnatal treatments, resulting in 4 treatment groups: **HPHP**, **HPLP**, **LPLP**, and **LPHP**, with the first 2 letters referring to the mother's diet during the dry period and the last 2 to the diet of the cow when producing colostrum.

Animals, Feed, and Housing

Holstein Friesian cows from the ILVO experimental dairy herd entering their first or greater dry period were enrolled in the study between March 2019 and September 2020. Data from 5 cows (2 in the HP and 3 in the LP group) was removed because they were identified as outliers having a too short or too long dry period (<20 and >60 d, respectively), using the 'boxplot.stats' function of the R package grDevices (R Core Team, 2022). Data from another 3 cows (1 in the HP and 2 in the LP group) was excluded because they failed to produce colostrum. Eventually, 35 HP and 29 LP cows remained for statistical analysis of the descriptive statistics.

Cows were dried off approximately 45 d before expected calving date and treated with intramammary antibiotics (Virbactan DC, Virbac, Carros, France) and a teat-sealer (OrbeSeal, Zoetis, Parsippany, NJ). Dried-off cows were group-housed in freestall barns with slatted floors and cubicles covered with rubber mattresses and were fed a far-off ration until 14 d before expected calving. The LP far-off diet was a TMR composed of maize silage mixed with chopped straw and a dry cow mix (including a dry cow mineral premix). The HP far-off diet was the LP diet supplemented with RUP (formaldehyde-treated soybean meal, Mervobest, Nuscience). The far-off TMR was formulated assuming a daily DMI of approximately 11.6 kg and with an energy and protein content of approximately 800 feed unit lactation (**VEM**) and 48 g of DVE per kg of DMI in the LP far-off diet and 820 VEM and 75 g of DVE in the HP far-off diet. Cows switched to the close-up diet at 14 d before expected calving. The close-up diet was a TMR formulated with a balanced concentrate (LP close-up diet) or with a protein-rich concentrate containing RUP (formaldehyde-treated soybean meal, Mervobest, Nuscience; HP close-up diet). The TMR was formulated assuming a daily DMI of approximately 10.5 kg and with an energy and protein content of approximately 1,048 VEM and 93 g of DVE per kg of DMI in the LP close-up diet and 1,044 VEM and 116 g of DVE per kg of DMI in HP close-up diet, according to the Dutch energy and protein system (Van Es, 1975; Tamminga et al., 1994). Rations in both the far-off and close-up period were administered as a TMR and cows were fed once a day between 0800 and 0900 h. All dry period rations were supplemented with minerals and vitamins according to CVB recommendations (CVB, 2016). Diet composition changed slightly during the course of the experiment (running over 1.5 yr) due to changes in quality and nutritional values of the silages used. Samples of individual forages were taken every 2 wk and pooled for a period of 3 mo. Concentrates were sampled every 4 wk and pooled for a period of 6 mo. Chemical composition of individual feedstuffs was determined as described by de Boever et al. (2017). Briefly, rumen degradability characteristics of OM, CP, starch, and NDF of Mervobest (formaldehyde-treated soybean meal), rolled barley, beet pulp, and soybean meal were determined with the nylon bag technique using 3 rumen cannulated cows (for details, see de Boever et al., 2017). Nutrient content of concentrates, dry cow

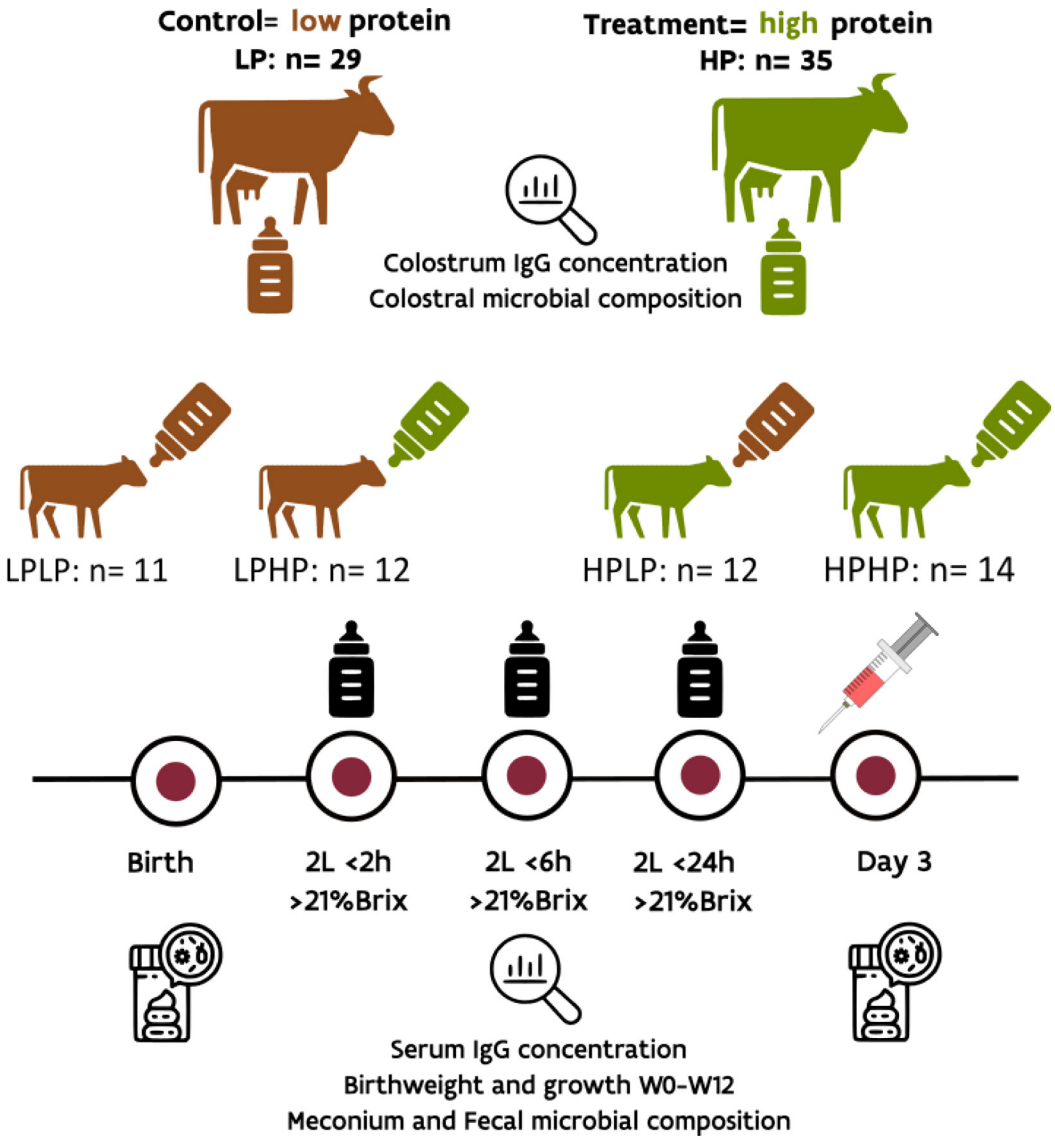

Figure 1. Schematic overview of the experimental design. A 2 × 2 factorial design was set up to analyze both the pre- and postnatal effect of maternal supplementation with RUP. In this way 4 treatment groups were created in the calves: LPLP, LPHP, HPLP, and HPHP, in which the first 2 letters refer to the treatment of the dam and the last 2 letters refer to the treatment of the colostrum-producing cow (LP = low crude protein; HP = high crude protein).

mix, maize silage, grass silage, and wheat straw was obtained with chemical analyses. Samples of all individual feeds were oven-dried at 65°C and ground to pass a 1-mm screen (Whiley, Rheotec). Thereafter, samples were dried at 103°C to determine residual moisture (EC, 1971; dry matter content of silages was corrected for fermentation losses during drying) and further incinerated at 550°C to obtain crude ash content (ISO, 2002). Crude protein (N × 6.25) was determined according to Kjeldahl (ISO, 2005). Crude fat was extracted with petroleum ether after hydrolysis with hydrochloric acid (ISO, 1999). Neutral detergent fiber was determined with the Ankom Fiber Analyzer using α-amylase and sodium sulfite and expressed on ash-free basis (Van Soest et al., 1991). Starch was determined after autoclaving and hydrolysis with amyloglucosidase (NEN, 1974). True protein digested in the small intestine and degradable protein balance content of the feeds were calculated based on the degradation characteristics of the nutrients or estimated with regression equations based on chemical composition and protein solubility (adapted from de Boever et al., 2004, after expansion of the data set to 41 grass silages) according to the Dutch protein evaluation system (Van Duinkerken et

al., 2011). To estimate the NE_L content of the feeds according to Van Es (1975), regression equations (de Boever et al., 1999) based on the chemical composition and the in vitro cellulase digestibility of the OM were used. To analyze the in vitro cellulase digestibility of the OM, samples were first treated with a pepsin solution and incubated at 40°C for 24 h. Next, the mixtures were heated at 80°C for 45 min. Finally, a cellulase-buffer mixture was added and samples were incubated at 40°C for 24 h (for details see de Boever et al., 1986). The detailed composition and nutritional value of the 4 rations are represented in Table 1. Detailed information on chemical composition of each feed ingredient is given in Supplemental Table S1 (https://doi.org/10.6084/m9.figshare.21821163; Van Hese et al., 2023). Individual feed intake of the TMR was registered with the Insentec Roughage Intake Control (**RIC**) system (Insentec B.V., Marknesse, the Netherlands). Cows had ad libitum access to multiple RIC bins (maximum 1.7 cows per bin) to control for a bin effect on the feed intake of the cow. Average DMI per treatment during far-off and close-up dry period are represented in Table 2. The individual nutrient intake was calculated by combining individual feed intake registration and the chemical composition analyses of the different feedstuffs over the complete trial (Table 2). Body temperature was monitored with an intravaginal thermometer (Vel'Phone, Medria, Chateaubourg, France), placed approximately 5 d before expected calving by a trained personnel, according to the company's instructions. When the cows' body temperature experienced the typical prepartum drop, the Vel'Phone system sends an alert of expected calving in 24 to 48 h. When the thermometer was pushed out by the amniotic membranes, an alert of expulsion was sent. Cows were moved to maternity pens with deep straw bedding after receiving an alert of expected calving within 24 to 48 h, in combination with external signs of impending calving (relaxation of the pelvic ligaments and strutting of the teats). Immediately after birth, calves were housed in individual indoor pens and fed colostrum according to the standard procedure described below. After their third meal of colostrum (around 24 h after birth), female calves were transferred to individual outdoor hutches until 16 wk of age, male calves were housed in a separate stall in individual indoor pens until they left the farm (at approximately 2 wk of age). From then on, all calves received replacer milk according to their birthweight (see Supplemental Table S2; https://doi.org/10.6084/m9.figshare.21821163; Van Hese et al., 2023). Female calves were weighed on Thursdays, every 2 wk from birth until 12 wk of age. Daily growth was calculated by dividing weight (in kg) by the interval (in days).

Collection of Colostrum Samples

A detailed description of the colostrum sampling protocol was published in Van Hese et al. (2022). Briefly, cows were milked with a portable milking machine within 1 h after parturition. Before milking, the udders were cleaned with a dry cloth and the teats were wiped with a 70% ethyl alcohol gauze. Before usage, the milk bucket was washed with hot water (>70°C). After each usage, the teatcups and tubes were cleaned with the cleaning system at the milking parlor, the milk bucket was washed with hot water and an acid descaler (Parlor Cleaner, DeLaval BV, Steenwijk, the Netherlands). Immunoglobulin G concentration of colostrum was assessed immediately after milking with the brix refractometer. Only colostrum with at least 21% Brix and a volume of >6 L was sampled and stored in the freezer to feed the calves enrolled in this trial (53 samples in total, 28 HP and 25 LP). Colostrum samples for subsequent analyses were collected straight from the milk bucket (3 sterile centrifuge tubes of 50 mL), to retrieve representative samples of the colostrum that was administered to the calf. Colostrum was aliquoted in micro centrifuge tubes of 2.0 mL and stored at −80°C until microbial DNA extraction and radial immunodiffusion (**RID**) analysis. Per cow, 6 L of colostrum was divided in 3 × 2 L bags and stored in the freezer (−20°C) awaiting to be fed to the calves enrolled in the trial.

Colostrum IgG Concentration Measurement

Colostral IgG concentration was measured with a commercial RID assay (Bovine IgG RID kit, Triple J Farms, Bellingham, WA). Colostrum samples were thawed at room temperature (20°C–24°C), thoroughly mixed, diluted (1:1) with a saline solution (0.9% NaCl) and again mixed well. Following the manufacturer's instructions, each well of the RID test plate was filled with 5 μL of the diluted colostrum samples. Colostrum samples were tested alongside the manufacturer's reference sera containing 180, 1,472, and 2,803 mg of IgG/dL, respectively. RID plates were incubated for 24 h at room temperature and the precipitating ring diameter was measured with a stereomicroscope (Olympus SZX7, Olympus Corporation, Tokyo, Japan) and an ocular micrometer (magnification 10×).

Colostrum Administration and Serum and Fecal Collection

A total of 49 calves (26 male and 23 female) enrolled in the trial and were divided in 4 treatment groups: LPLP (n: 11), LPHP (n: 12), HPLP (n: 12), HPHP

Table 1. Diet composition (mean ± SD) during far-off and close-up dry period throughout the entire trial period (weighted average based on days before calving and number of animals)

	Dry-period diet			
	Far-off		Close-up	
Item	HP	LP	HP	LP
Feed component (% of DM)				
Maize silage	61.2 ± 3.8	65.8 ± 1.3	33.9 ± 4.5	34.1 ± 3.3
Grass silage	—	—	30.8 ± 5.0	30.6 ± 3.3
Beet pulp	—	—	9.2 ± 1.6	9.0 ± 1.1
Wheat straw	25.1 ± 3.2	27.2 ± 1.0	1.2 ± 3.6	0.6 ± 1.9
Maize meal	—	—	7.2 ± 2.6	7.2 ± 2.2
Soybean meal	—	—	4.0 ± 2.1	4.8 ± 1.6
Rolled barley	—	—	1.2 ± 0.5	1.4 ± 0.4
Concentrate	—	—	7.4 ± 1.4[1]	7.5 ± 1.5[2]
Prolacta[3]	—	—	1.9 ± 0.3	1.9 ± 0.3
Dry cow mix[4]	6.2 ± 0.8	6.7 ± 0.3	—	—
Mervobest[5]	6.1 ± 0.9	—	—	—
Nutritional component,[6] % of DM (unless noted otherwise)				
DM, %	47.9 ± 1.4	46.9 ± 0.8	39.2 ± 2.0	38.9 ± 1.4
CP	11.5 ± 0.9	8.9 ± 0.7	16.4 ± 0.9	14.5 ± 0.5
MP[7]	8.6 ± 0.4	6.2 ± 0.3	10.2 ± 0.5	8.2 ± 0.4
OEB[8] (1/kg of DM)	−3.0 ± 0.8	−3.4 ± 0.8	1.1 ± 0.8	0.8 ± 0.5
NDF	44.9 ± 2.6	46.8 ± 0.8	35.9 ± 2.1	36.6 ± 1.3
NE_L[9] (mL/kg of DM)	5.9 ± 0.2	5.8 ± 0.2	6.7 ± 0.2	6.7 ± 0.2

[1]High crude protein (HP) close-up diet included a protein-rich concentrate (meal) containing Mervobest (89.5%), molasses (5%), sodium chloride (1.2%), feed phosphate (1%), trace elements (1%), lignin sulfonate (1%), feed chalk (0.8%), magnesium oxide (0.5%).

[2]Low crude protein (LP) close-up diet included a balanced concentrate (meal) containing beet pulp (37%), soybean meal (21%), wheat (18.5%), maize (12%), molasses (5%), salt (1.2%), soybean oil (1%), feed phosphate (1%), microminerals (1%), lignin sulfonate (1%), chalk (0.8%), magnesium oxide (0.5%).

[3]Prolacta (Aveve BV, Wilsele, Belgium) is a mineral premix with 2.2 g of Ca, 39.5 g of P, 81.6 g of Mg, 30.1 g of Na, 2000 mg of choline chloride, 20 mg of calcium iodate, 15 mg of cobalt sulfate, 1,000 mg of copper sulfate, 1,250 mg of manganese oxide, 2,500 mg of zinc sulfate, 40 mg of sodium selenite, 1,000,000 IU of vitamin A, 200,000 IU of vitamin D_3, 4,400 mg of vitamin E.

[4]Dry cow mix contains soybean meal (58%), Prolacta (30%), molasses (5%), lucerne (alfalfa) meal (5%), and urea (2%).

[5]Mervobest (Nuscience) is formaldehyde-treated soybean meal to bypass rumen degradation.

[6]The chemical composition was calculated based on the average intake by the cows.

[7]Calculated with the DVE-system to predict true protein digested in the small intestine (Tamminga et al., 1994).

[8]OEB = degradable protein balance (Tamminga et al., 1994).

[9]Estimated by regression equations in the Belgian-Dutch net energy evaluation system (de Boever et al., 1999) and then recalculated (i.e., 1,000 VEM = 6.9 MJ of NE_L; Van Es, 1978).

(n: 14; Figure 1). Calves were stratified per parity of the dam, calves born from cows with parity 2 received colostrum from a cow with parity 2, idem for calves born from cows with parity >2. Calves received a total of 6 L of colostrum of the same cow (other than their mother) in the first 24 h of life (3 × 2 L within 2, 6 and 24 h after birth). Colostrum was thawed at 50°C for 20 to 30 min in a thawing water bath of the Store and Thaw colostrum management system (Pyon Products, Hereford, UK). Colostral IgG concentration of all feedings was monitored with a digital brix refractometer (Obione, Macon, France). Blood samples were taken from calves at the age of 3 d from the jugular vein with a vacutainer without coagulant (BD vacutainer SST II advance, BD, Franklin Lakes, NJ) and a 20-G needle, and were centrifuged at 2,564 × *g* for 10 min at 4°C for serum separation. An aliquot of serum was sent to an external laboratory (Dierengezondheidszorg Vlaanderen VZW, Lier, Belgium), to perform serum protein electrophoresis to measure serum total protein (g/L) and serum IgG (g/L). Meconium and fecal samples were collected before colostrum administration and at d 3 after birth, respectively, through digital palpation of the rectum. Before sampling, the calf's perineum was wiped with a 70% ethyl alcohol gauze. Fecal samples were aliquoted in 2.0-mL cryotubes, snap frozen in liquid nitrogen, and stored at −80°C until microbial DNA extraction.

DNA Extraction and 16s rRNA Gene Sequencing

Colostrum that contained at least 50 g of IgG/L and was stored for administration to the calves (53 samples in total: 28 from the HP and 25 from the LP group),

Table 2. Individual daily DMI (LSM ± SEM) and daily nutrient intake (LSM ± SEM) calculated based on the observed individual intake of the TMR during the far-off and close-up dry period for high crude protein (HP) and low crude protein (LP) diet

	Dry-period diet			
	Far-off		Close-up	
Item	HP	LP	HP	LP
Total daily DMI (kg)	14.0 ± 0.3[a]	13.5 ± 0.3[a]	15.9 ± 0.3[b]	15.3 ± 0.3[b]
Nutrient intake, g/cow per d (unless noted otherwise)				
CP	1,605.0 ± 32.5[a]	1,211.6 ± 35.2[b]	2,626.7 ± 35.9[c]	2,227.1 ± 38.5[d]
Starch	2,851.2 ± 83.6[ab]	2,949.8 ± 90.7[b]	2,581.9 ± 87.9[c]	2,689.3 ± 94.8[ac]
Crude fat	313.0 ± 6.6[a]	300.8 ± 7.2[a]	451.0 ± 7.2[b]	440.7 ± 7.8[b]
Crude ash	728.6 ± 15.6[a]	683.7 ± 16.9[a]	1,241.1 ± 17.3[b]	1,163.9 ± 18.5[c]
NDF	6,288.3 ± 114.3[a]	6,335.9 ± 123.9[a]	5,706.7 ± 124.4[b]	5,635.7 ± 133.5[b]
MP[1]	1,203.3 ± 22.5[a]	845.7 ± 24.4[b]	1,631.0 ± 24.7[c]	1,258.0 ± 26.5[a]
OEB[2] (1/cow per d)	−30.0 ± 0.9[a]	−33.2 ± 1.0[a]	9.6 ± 1.0[b]	7.1 ± 1.1[b]
NE_L[3] (MJ/cow per d)	82.7 ± 1.7[a]	78.4 ± 1.9[a]	106.4 ± 1.9[b]	102.5 ± 2.0[b]

[a–d]Different superscripts represent significant differences at a P-value < 0.05 in nutritional component intake within dry period diet.

[1]Calculated with the DVE-system to predict true protein digested in the small intestine (Tamminga et al., 1994).

[2]OEB = degradable protein balance (Tamminga et al., 1994).

[3]Estimated by regression equations in the Belgian-Dutch net energy evaluation system (de Boever et al., 1999) and then recalculated (i.e., 1,000 VEM = 6.9 MJ NE_L; Van Es, 1978).

was analyzed for microbial composition. A detailed description of the microbial DNA extraction protocol from colostrum was published previously (Van Hese et al., 2022). Briefly, microbial DNA was extracted from 1.5 mL of colostrum of the first milking with the Powerfood microbial kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. After DNA extraction, an additional purification step was performed with the NucleoSpin gDNA Clean-up kit (Macherey-Nagel GmBH and Co. KG, Düren, Germany). Microbial DNA was extracted from 250 mg of meconium and feces with the Qiamp Powerfecal Pro DNA kit (Qiagen), following the manufacturer's protocol. DNA concentration was measured with the Quantus Fluorometer (Promega Benelux, Leiden, the Netherlands) and A260/280 and A260/230 absorption ratios were measured with the NanoPhotometer N50 (Implen, München, Germany). Library preparation (including quality control) was performed for the V3-V4 region of the 16S rRNA gene using primers 344F (CCTACGGGNGGCWGCAG) and 806R (GACTACHVGGGTATCTAATCC; Kittelmann et al., 2013) with a 2-step PCR protocol using the Nextera XT index kit following the Illumina protocol (Illumina 2013, San Diego, CA) by Macrogen (Seoul, South Korea).

Indexed libraries were pooled and sequenced using Illumina MiSeq V3-technology (2 × 300 bp; Macrogen). The raw sequence data are stored in the NCBI Short Read Archive, under BioProject PRJNA915010.

Sample Categories

Colostrum samples with a quality ≤50 g/L, >50 and <100 g/L, and ≥100 g/L were categorized as bad, good, and excellent quality, respectively (Quigley et al., 2013). Calf serum IgG concentrations <10 g/L, ≥10 and <18 g/L, ≥18 and <25 g/L, and ≥25 g/L were categorized as poor, fair, good, and excellent, respectively, based on Lombard et al. (2020). Differential abundance analysis of the sequencing data were performed between different categories of colostrum quality and serum IgG concentration as explained below. Calves with an IgG level ≤10 g/L were categorized as calves with failure of passive transfer (**FPT**; Beam et al., 2009). The prevalence of FPT was calculated as the number of FPT cases divided by the total number of calves.

Microbiome Data Analysis

All statistical analyses were performed using R statistical software, version 3.6.2 (R Core Team, 2022). Data analysis of the microbial composition was described in detail and published previously in Van Hese et al. (2022). Briefly, the amplicon sequencing data set was demultiplexed by the sequence provider. Removal of primer sequences and filtering as well as trimming were performed using the DADA2 pipeline in R (version 3.6.2; Callahan et al., 2016). Forward and reverse reads were trimmed at a length of 280 and 210 bp,

respectively. Forward and reverse estimated errors were set to 2 and 4, respectively. Taxonomy was assigned using the RDP naïve Bayesian classifier method (Wang et al., 2007) and the SILVA database, version 138 (Quast et al., 2013). Reads were classified at multiple taxonomic levels: phylum (p), class (c), order (o), family (f), and genus (g). Amplicon sequence variants (**ASV**) corresponding to the orders *Mitochondria*, *Chloroplast*, *Chloroflexi*, and *Cyanobacteria* were removed (Kuehn et al., 2013; Lundberg et al., 2013). Rarefaction analyses were performed using the R-package vegan (Oksanen et al., 2007) and rarefaction curves (see additional file 1) reached a plateau which suggests that saturation in sequencing was achieved (Zaheer et al., 2018). Colostrum samples containing more than 40% of ASV that could not be affiliated to any domain were removed (6 in total, 3 in both HP and LP) before downstream data analysis. This means that the statistical analysis of the microbial composition of colostrum included 47 samples (25 HP and 22 LP cows).

Alpha-diversity (the microbial variation within samples) was measured in unfiltered data using the Shannon diversity index, Chao1 richness, and inverse Simpson diversity calculated with the Phyloseq package (McMurdie and Holmes, 2013). Due to a lack of normality, the Wilcoxon rank sum test was used to define statistical difference in α diversity measures (Shannon, inverse Simpson, and Chao1) between the parameters of interest: treatment, gestation length, parity, colostrum quality, serum IgG, and, season. Bray-Curtis distances were calculated to assess β-diversity (the microbial variation between samples), with the nonmetric multidimensional scaling (**NMDS**) method. For subsequent data analysis, only ASV with at least 20 counts across all samples were retained. Read counts were transformed to relative abundances per sample. A Betadisper analysis was performed to check for homogeneity of variances of compared variables. Statistical differences between communities were analyzed by PERMANOVA using the Adonis function (Oksanen et al., 2007). Pairwise MANOVA was performed when factors contained more than 2 levels with the RVAideMemoire package (Hervé and Hervé, 2020). Differentially abundant ASV between treatment, colostrum quality, and serum level categories were identified using the DESeq2 package with the Wald test (Love et al., 2014). Before the differential abundance analysis, we summed all ASV belonging to the same taxonomic group (i.e., genus) instead of testing each ASV individually, allowing to study general trends for the complete genus. *P*-values were adjusted for multiple testing using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995).

Statistical Testing

The effect of treatment on DMI and intake of nutritional components (summarized in Table 2) was examined with following linear mixed model in R:

$$Y_{si} = \beta_0 + S_{0s} + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_3 X_4 + \varepsilon,$$

with β_0 as the intercept, S_{0s} as the random intercept of the cow, β_{1-5} as the estimated effect of variable X, X_1 as the effect of days ante partum, X_2 as the parity effect (with 2 levels, 2 and >2), X_3 as the treatment effect (with 2 levels, HP and LP), X_4 as the ration effect (with 2 levels, far-off and close-up), and ε as the random error. An interaction between treatment and ration was included in the model. Before modeling, outliers in DMI per individual cow per ration were identified and excluded with the boxplot method using the rstatix package in R (Kassambara, 2022). To check the assumption of normality, a quantile-quantile plot (Wilk and Gnanadesikan, 1968) and histogram were drawn of the linear mixed models' residuals in R. Treatment means were compared using the LSM procedure with pairwise comparisons of treatment by ration with a Tukey correction for multiple comparisons (Searle et al., 1980). To examine the effect of treatment (HP and LP diet) on the colostrum IgG concentration (measured with Brix), ANOVA was performed with linear models in R using the following model:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1 X_2 + \varepsilon,$$

with β_0 as the intercept; β_1, β_2, and β_3 as the estimated effect of variable X; X_1 as the treatment effect (with 2 levels, HP and LP); X_2 as the parity effect (with 2 levels, 2 and >2); and ε as the random error. An interaction between treatment and parity was included in the model. To check the assumption of normality, a quantile-quantile plot (Wilk and Gnanadesikan, 1968) and histogram were drawn of this linear model's residuals in R. Treatment means were compared using the LSM procedure with pairwise comparisons of treatment by parity (Searle et al., 1980), results were interpreted one-sided. Apparent efficiency of absorption (**AEA**) was calculated as serum IgG (g/L) × birthweight (kg) × 0.07 (estimated % blood volume) ÷ total amount of IgG administered (g; Halleran et al., 2018) in female calves only (due to missing birthweight records in male calves). To examine the effect of treatment on the serum IgG concentration in the calf, ANOVA was performed with linear models in R using the following model:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \varepsilon,$$

with β_0 as the Y-intercept, β_1 and β_2 as the population slopes, X_1 as the treatment effect (with 4 levels: HPHP, HPLP, LPHP, and LPLP), X_2 as the parity effect (with 2 levels, 2 and >2), X_3 as the effect of total IgG administered in 3 feedings, and ε as the random error. A forward selection procedure was performed to build the model for the associations between the recorded variables (treatment, total IgG, parity, sex, and birthweight) and serum IgG level. There was no significant interaction ($P > 0.1$) between treatment and parity, and therefore the interaction term was not included in the final model. Likewise, there was no interaction between birthweight and serum IgG level. The following nonsignificant fixed effects ($P > 0.1$) were not included in the final model: birthweight and sex. Treatment means were compared with the estimated marginal means procedure, Tukey adjusted P-values were calculated (Lenth, 2022). This last mentioned model was also used to analyze the effect of treatment on the AEA in female calves, with the exception that the effect of total IgG administered was not included in the model as it was already used to calculate the AEA. The same method was used to analyze the effect of prepartum maternal diet on the birthweight of the calf, with the exception that the treatment effect had only 2 levels (prepartum-LP and prepartum-HP). The association between serum IgG level and growth between birth and 12 wk of age was evaluated with the Pearson correlation test (Benesty et al., 2009). To analyze the effect of treatment on the calves' growth, ANOVA was performed with linear models in R, using the following model:

$$Y = \beta_0 + \beta_1 X_1 + \varepsilon,$$

with β_0 as the Y-intercept, β_1 as the population slope, X_1 as the treatment effect (with 4 levels: HPHP, HPLP, LPHP, and LPLP), and ε as the random error. Because there was no correlation between serum IgG level in calves and their growth, serum IgG level was not included as a random effect in this model. Treatment means were compared with the estimated marginal means procedure, Tukey adjusted P-values were calculated (Lenth, 2022).

Significance and tendencies were declared at a P-value below 0.05 and 0.10, respectively.

Power Analysis

A post hoc power analysis was conducted to determine whether the number of calves enrolled in the trial provided the present study with sufficient statistical power to reject the null hypothesis that treatment did not affect serum IgG absorption in the calf. The variables were estimated from the data and included the observed treatment effect estimated from the model (serum IgG = treatment + total IgG administered in 24 h). The significance level was chosen to be 0.05, sample size was set at 12. This resulted in a statistical power of 71%. Moreover, the post hoc power analysis showed that a sample size of 15 calves per treatment would provide sufficient statistical power of 80%. This was originally the intended sample size, however due to practical reasons (trial running for 1.5 yr) inclusion of more animals was not possible.

RESULTS

Descriptive Statistics on Feed Intake, Colostrum Quality, Passive Transfer of Immunity, and Growth

Feed intake data are represented in Table 2. Total dry matter intake was higher during the close-up compared with the far-off ($P < 0.0001$), but was similar between treatments (far-off HP vs. LP: $P = 0.603$; close-up HP vs. LP: $P = 0.459$). Crude protein and MP intake were highest in the HP group, which was intended with our experimental design. Results of the descriptive statistics are summarized in Table 3 and Supplemental Table S3 (https://doi.org/10.6084/m9.figshare.21821163; Van Hese et al., 2023) for cow and calf data. Parity 2 cows (cows that completed their first dry period) that consumed the HP dry period diet (HP, n = 22) had higher colostral IgG concentrations compared with parity 2 cows on the LP dry period diet (LP, n = 15), (LSM ± SEM 61.3 ± 2.3 g of IgG/L vs. 55.2 ± 2.3 g of IgG/L; $P = 0.046$). For cows with parity >2, no difference in colostral IgG concentration was found between HP (n = 13) and LP cows (n = 14; 58.4 ± 3.0 vs. 56.8 ± 2.9 g of IgG/L, respectively; $P = 0.356$). Considering 50 g of IgG/L as a threshold for bad versus good colostrum quality, 15 cows produced colostrum of bad quality of which 8 HP and 7 LP cows. Three cows failed to produce colostrum (1 HP and 2 LP).

Calves received on average 336.8 ± 7.5 g of IgG in 3 feedings. Average serum IgG level in calves was 17.7 ± 7.5 g/L. Calves in the LPHP group had lower serum IgG levels compared with calves from the HPHP and LPLP group ($P = 0.005$; Figure 2). Calves born out of cows with parity >2 had higher serum IgG levels compared with calves born out of cows with parity 2 (LSM ± SEM was 19.6 ± 1.0 g of IgG/L vs. 15.8 ± 1.0 g of IgG/L, respectively; $P = 0.008$). Linear regression modeling showed that total amount of IgG administered, parity, and treatment were predictors of serum IgG levels in the calves with an R^2 equal to 0.36 (serum

Table 3. Summary of cow descriptive statistics between cows receiving the low crude protein (LP) and high crude protein (HP) diet

Item	IgG concentration first milking colostrum, g/L (LSM ± SEM)	Dry period length, d (LSM ± SEM)	Sample count
Treatment × parity[1]			
HP 2	61.3 ± 2.3[a]	43.1 ± 1.5	22
LP 2	55.2 ± 2.8[b]	41.1 ± 1.8	15
HP >2	58.4 ± 3.0	43.6 ± 1.9	13
LP >2	56.8 ± 2.9	41.4 ± 1.9	14
Season of calving			
Spring	57.0 ± 2.4	41.9 ± 1.6	20
Summer	58.1 ± 2.1	42.1 ± 1.3	28
Autumn	60.2 ± 4.1	43.0 ± 2.6	7
Winter	60.5 ± 3.6	44.0 ± 2.3	9

[a,b]Different superscripts represent significant differences at a P-value < 0.05 in first milking colostrum IgG concentration within parity 2.

[1]One-sided post hoc test within parity.

IgG (g/L) = 5.88 + 0.04 × total IgG fed − 3.09 × parity2 − 1.75 × treatment HPLP − 4.59 × treatment LPHP + 2.14 × treatment LPLP; $P = 0.0002$). Because there was no difference between treatments in total amount of IgG administered to the calves (Supplemental Table S3), these 2 parameters were not correlated to each other. This was intentional due to the experimental setup which exclusively allowed administration of good quality colostrum (i.e., >50 g of IgG/L). Despite a balanced and strict colostrum management, 5 calves suffered from FPT (serum IgG level ≤10 g of IgG/L) of which 4 belonged to the LPHP and 1 to the HPLP group. AEA was only calculated in female calves as only for those calves birthweight was recorded. Calves born out of cows with parity 2 had a lower AEA compared with calves born out of cows with parity >2 (10.9% ± 2.3% vs. 12.6% ± 3.0%, respectively; $P = 0.238$). Calves in the LPLP group had the highest mean AEA of all treatment groups (17.9% ± 4.0%; $P = 0.360$; Supplemental Table S3).

Birthweight and growth for a period of 12 wk were only monitored in female calves (n = 21). The overall mean ± SE birthweight was 39.4 ± 1.1 kg and was not affected by parity nor prenatal treatment of the mother (39.5 ± 1.4 kg in HP vs. 39.2 ± 1.8 kg in LP). Calves grew between birth and 12 wk of age on average 0.76 ± 0.02 kg/d. Average daily gain was not affected by parity of the mother, pre- and postnatal treatment, nor serum IgG level. Birthweight and growth results are summarized in Supplemental Table S3.

The Microbial Composition of Colostrum Was Not Affected by Protein Supplementation in the Dry Period

Sequencing Results. After preprocessing of the raw sequence data 5,137,353 reads remained for downstream data analysis. Average number of reads per samples was 73,390 with a range between 25,500 and 124,255. All rarefaction curves reached a plateau (see Supplemental Figure S1; https://doi.org/10.6084/m9.figshare.21821163; Van Hese et al., 2023) which suggests that saturation in sequencing was achieved (Li et al., 2018).

Phylogenetic Profile. *Proteobacteria* was the most abundant phylum in colostrum overall, followed by *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*, with an overall mean abundance of 48.2%, 24.8%, 9.5%, and 5.0%, respectively. Mean relative abundances per treatment of the top 10 most abundant phyla, are represented in Figure 3A. *Pseudomonas*, *Acinetobacter*,

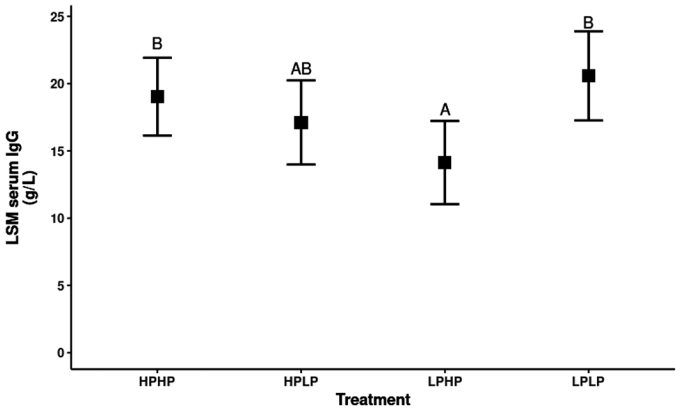

Figure 2. The graph represents the difference in 3 calves' serum IgG level between treatment groups; serum was taken at d 3 of age. Calves were assigned to 4 treatment groups: LPLP, LPHP, HPLP, and HPHP, in which the first 2 letters refer to the treatment of the dam and the last 2 letters refer to the treatment of the colostrum-producing cow (LP = low crude protein; HP = high crude protein). Boxes indicate the LSM calculated from the following linear model: serum IgG = treatment + parity of the dam + total IgG administered in 24 h. Error bars indicate the 95% confidence interval of the LSM; LSM sharing a letter are not different ($P \geq 0.05$; Tukey-adjusted comparisons).

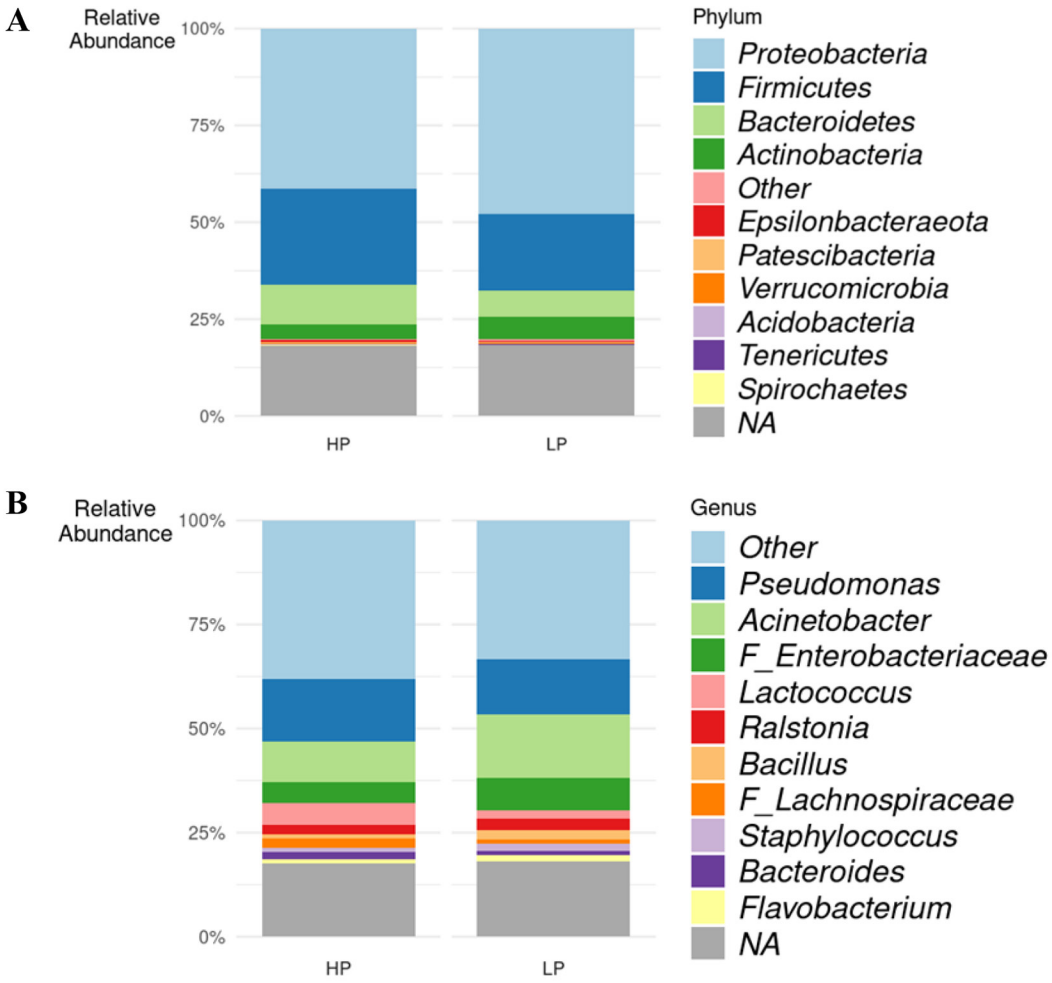

Figure 3. Bacterial composition of colostrum from cows receiving the low-protein dry-period diet (LP) and the high-protein dry-period diet (HP), showing (A) top 10 phyla and (B) genera that were most abundant in both LP and HP colostrum, all remaining genera are assigned to the category "other." Sequences that could not be annotated are assigned to the category "NA."

F_Enterobacteriaceae, and *Lactococcus* were the most abundant genera with an overall mean abundance of 15.9%, 13.6%, 6.9%, and 4.0%, respectively. Mean relative abundances per treatment of the top 10 most abundant genera are represented in Figure 3B.

Alpha Diversity. There was no difference in the average colostral bacterial diversity (inverse Simpson), evenness (Shannon) nor richness (Chao1) between HP and LP cows. Furthermore, α diversity measures did not differ between colostrum of different quality (good [n = 46] vs. excellent [n = 9]) either. However, in cows with parity 2, bacterial richness differed between colostrum of good (n = 21) and excellent (n = 2) quality ($P = 0.047$). Bacterial evenness was different between colostrum from cows that calved in summer versus winter, however only in cows entering their third or higher lactation ($P = 0.04$; see Supplemental Figure S3; https://doi.org/10.6084/m9.figshare.21821163; Van Hese et al., 2023).

Beta Diversity. Beta-diversity gives an indication about the similarity or difference between bacterial communities, for example, between colostrum from HP versus LP cows. Parameters of interest were treatment (HP vs. LP), parity (2 vs. >2), treatment within parity (HP 2 vs. LP 2 and HP >2 vs. LP >2), colostrum quality (good vs. excellent), serum IgG level of the calves (poor vs. fair vs. good vs. excellent), and season of calving (all comparisons). Solely the microbial composition of cows with parity 2 versus cows with parity >2 was different ($P = 0.042$) and between winter and the other calving seasons. Microbial communities did not differ between HP and LP cows, nor between colostrum of good versus excellent quality. In addition, the microbial communities of colostrum did not differ when colos-

trum was categorized based on the obtained IgG level in the recipient calves.

Differential Abundance Analysis. Instead of analyzing differences or similarities at community level, differential abundance analysis explores differences at taxonomic level. In the present study, differentially abundant (**DA**) genera in colostrum were studied between treatments (HP or LP diet of colostrum-producing cow), colostrum quality groups, serum IgG level in the calf, and colostrum parity groups. Results from the differential abundance analysis are summarized in Table 4. Between colostrum from HP and LP cows, 2 genera belonging to the family *Lachnospiracea* (*Eubacterium eligens*, $P = 6.6e\text{-}17$, and an unidentified genus from the family *Lachnospiraceae*, $P = 0.059$) were DA and both were higher abundant in colostrum from HP compared with colostrum from LP cows. Between colostrum from good versus excellent quality, 12 genera were DA, with 4 and 8 genera lower and higher abundant, respectively, in colostrum from good compared with colostrum from excellent quality. Six genera were DA in colostrum from cows with parity 2 compared with colostrum from cows with parity >2. Only *Pseudomonas* was lower abundant, whereas the other 5 genera were higher abundant in colostrum from cows with parity 2. When analyzing the DA genera in colostrum in function of the serum IgG level of the recipient calf, colostrum that resulted in a poor IgG level (n = 4) showed a higher and lower abundance of 25 and 28 genera, respectively, compared with colostrum that resulted in an excellent IgG level (n = 3). Top 10 genera (highest baseMean) that were DA in colostrum based on the serum IgG level are represented in Table 4.

The Microbial Composition of Meconium and Feces Differed from That of Colostrum.

Phylogenetic Profile. Most abundant phyla in meconium and feces were *Firmicutes* (42.5% and 47.5%), *Proteobacteria* (21.7% and 33.7%), *Bacteroidetes* (16.8% and 15.7%), and *Actinobacteria* (2.9% and 3.1%), which were as well most abundant in colostrum, albeit in different proportions (Figure 4A). Most abundant genera in meconium and feces were genera belonging to the families *Enterobacteriaceae*, *Bacteroides*, *Streptococcus*, a genus belonging to the family *Lachnospiraceae*, and *Clostridium sensu stricto 1* (Figure 4B).

Alpha Diversity. Between meconium and feces and between colostrum and feces, there was a difference in the average bacterial diversity (inverse Simpson), evenness (Shannon), and richness (Chao1; see Supplemental Figure S2; https://doi.org/10.6084/m9.figshare.21821163; Van Hese et al., 2023). Remarkably, fecal samples show very little between-sample variation in α diversity compared with meconium and colostrum samples. There was no difference in α diversity metrics between meconium and colostrum. Treatment of the dam did not affect α diversity in calves' meconium. Alpha diversity in calves' feces was not affected by treatment of the dam, nor of the colostrum-producing cow, nor of the combination of both (e.g., HPHP vs. HPLP; data not shown).

Beta Diversity. Colostrum, meconium, and feces were different to each other for their microbial community ($P = 0.001$), which is visualized in a Bray Curtis-based NMDS plot (Figure 5). However, there was no homogeneity of variances between colostrum and meconium samples and between colostrum and fecal samples, meaning that this statistical difference in β diversity could as well be due to the nonhomogeneity of variances. There was no difference in β diversity between meconium from calves born from LP compared with HP cows. Neither did the prenatal, nor postnatal, nor combination of both treatments affect β diversity in calves' feces (data not shown).

Differential Abundance Analysis. Between meconium and colostrum, 60 genera were DA, with 57 lower and 3 higher abundant in meconium compared with colostrum. Higher abundant genera in meconium versus colostrum were *Bacteroides*, *Turicibacter*, and *Ruminococcaceae UCG-014*. *Pseudomonas*, *Acinetobacter* and *Bacillus* were the 3 most prominent genera (highest baseMean) that were lower abundant in meconium versus colostrum. Between feces and colostrum, 284 genera were DA, with 281 lower and 3 higher abundant in feces compared with colostrum. Higher abundant genera in feces versus colostrum were *Escherichia-Shigella*, *Butyricicoccus*, and *Clostridium sensu stricto 2*. *Pseudomonas*, *Acinetobacter*, and an unidentified genus were the 3 most prominent genera (highest baseMean) that were lower abundant in feces versus colostrum. Between meconium and feces, 143 genera were DA, with 2 lower and 141 higher abundant in meconium compared with feces. The 3 most prominent genera (highest baseMean) that were higher abundant in meconium versus feces were *Acinetobacter*, an unidentified genus, and *Bacteroides*. *Shewanella* and *Nubsella* were the 2 genera that were lower abundant in meconium versus feces.

DISCUSSION

Maternal Supplementation of RUP Affects IgG Concentration in Colostrum

In multiparous HF cows, CP requirement for conceptus growth during late gestation is estimated at 117 g/d, respectively (Bell et al., 1995). When combined

Table 4. Summary of differentially abundant genera in colostrum related to the treatment and parity of the colostrum-producing cow, colostrum quality (good vs. excellent), and calf's serum IgG level (poor vs. excellent) analyzed with DESeq2 in R[1]

Genus[2]	Log baseMean	Log 2 FC	LfcSE	Adjusted *P*-value
Treatment (HP vs. LP)				
F_Lachnospiraceae	3.23	3.26	0.88	5.93E-02
Eubacterium eligens group	1.01	24.94	2.91	6.68E-15
Parity (2 vs. >2)				
Pseudomonas	4.91	−3.97	1.01	8.63E-03
Prevotella 2	0.68	23.13	2.86	6.94E-14
Microvirgula	1.09	23.23	2.84	4.61E-14
C_Mollicutes	0.90	23.56	2.64	2.48E-16
Parabacteroides	0.69	23.93	2.92	4.61E-14
Holdemanella	0.79	24.54	2.92	1.23E-14
Colostrum quality (good vs. excellent)[3]				
Prevotellaceae NK3B31 group	6.05	−8.76	2.90	2.55E-02
Clostridium sensu stricto 1	7.03	−7.35	2.35	1.95E-02
Lactobacillus	8.02	−5.90	1.35	2.94E-04
F_Lachnospiraceae	8.87	−5.39	1.30	5.27E-04
Acinetobacter	9.89	3.25	1.15	4.62E-02
Chryseobacterium	7.54	5.87	1.47	9.30E-04
Pseudomonas	11.31	6.29	1.46	3.20E-04
Pedobacter	4.68	7.00	2.03	7.11E-03
Lactococcus	9.01	9.35	2.10	2.49E-04
Nubsella	3.14	23.45	3.79	2.22E-08
Aeromonas	4.34	25.18	3.13	4.70E-14
Delftia	4.92	25.85	2.27	4.75E-28
Serum IgG level (poor vs. excellent)[4,5]				
Escherichia-Shigella	8.77	−30.00	4.53	9.14E-09
Butyricicoccus	7.44	−30.00	7.30	9.04E-04
F_Enterococcaceae	7.75	−17.61	3.43	1.48E-05
Clostridium sensu stricto 1	7.05	−16.77	4.08	9.04E-04
Bacteroides	9.65	−9.81	2.55	2.43E-03
Streptococcus	7.96	−9.56	2.46	2.18E-03
F_Enterobacteriaceae	10.74	−7.43	2.25	1.54E-02
F_Lachnospiraceae	8.71	−7.37	2.10	7.83E-03
Lactobacillus	7.48	−6.07	2.10	3.78E-02
Pseudomonas	10.26	10.49	2.30	1.75E-04

[1]The baseMean is the average of the normalized count values, divided by size factors, taken over all samples and was log-transformed. The log 2 fold change (FC) is the effect size estimate and indicates how much the abundance seems to have changed between the compared groups (group A vs. group B); a positive or negative FC indicates a higher or lower abundance, respectively, in group A versus group B. LfcSE is the standard error estimate for the log 2 FC estimate. *P*-values were adjusted for multiple testing with the Benjamini and Hochberg method (Benjamini and Hochberg, 1995).

[2]The most specific taxonomy level available was assigned if taxonomy could not be assigned on genus level indicated by preceding of the first letter of that taxonomy level (C = class; F = family).

[3]Colostrum was categorized as good and excellent if the IgG concentration exceeded 50 and 99.9 g/L, respectively (Quigley et al., 2013).

[4]Calf serum IgG level was categorized as poor and excellent if the serum IgG concentration was below 10 and exceeded 24.9, respectively (Lombard et al., 2020).

[5]Only top 10 genera with highest baseMean are included in the table.

with maintenance requirements, the total CP intake in late gestation should be at least 12% of DMI (Quigley and Drewry, 1998). Recently, NASEM (2021) recommendations for energy and protein in the dry period diet were revised to meet the needs of the exponential growth of the fetus at the end of gestation. The dry period diet should provide 11.9% of CP between 60 and 21 d prepartum and 14.3% of CP less than 21 d prepartum (NASEM, 2021). This means that the LP far-off diet in the present study did not meet the recommended CP content by 2%, with a mean CP level of only 9.0% ± 0.3% (vs. 11.4% ± 0.2% of CP in the HP far-off diet). Nevertheless, this maize silage based far-off diet is representative for the dry period feeding strategies in Flanders' dairy farms (ILVO, 2011). During the close-up period dietary CP levels were increased to a mean of 14.6% ± 0.3% and 16.5% ± 0.2% in the LP and HP diet, respectively, and meet or exceed the recommended CP level (NASEM, 2021). However, important to note is that these recommendations do not take into account the needs for mammary gland development and colostrum production because data are sparse. Several

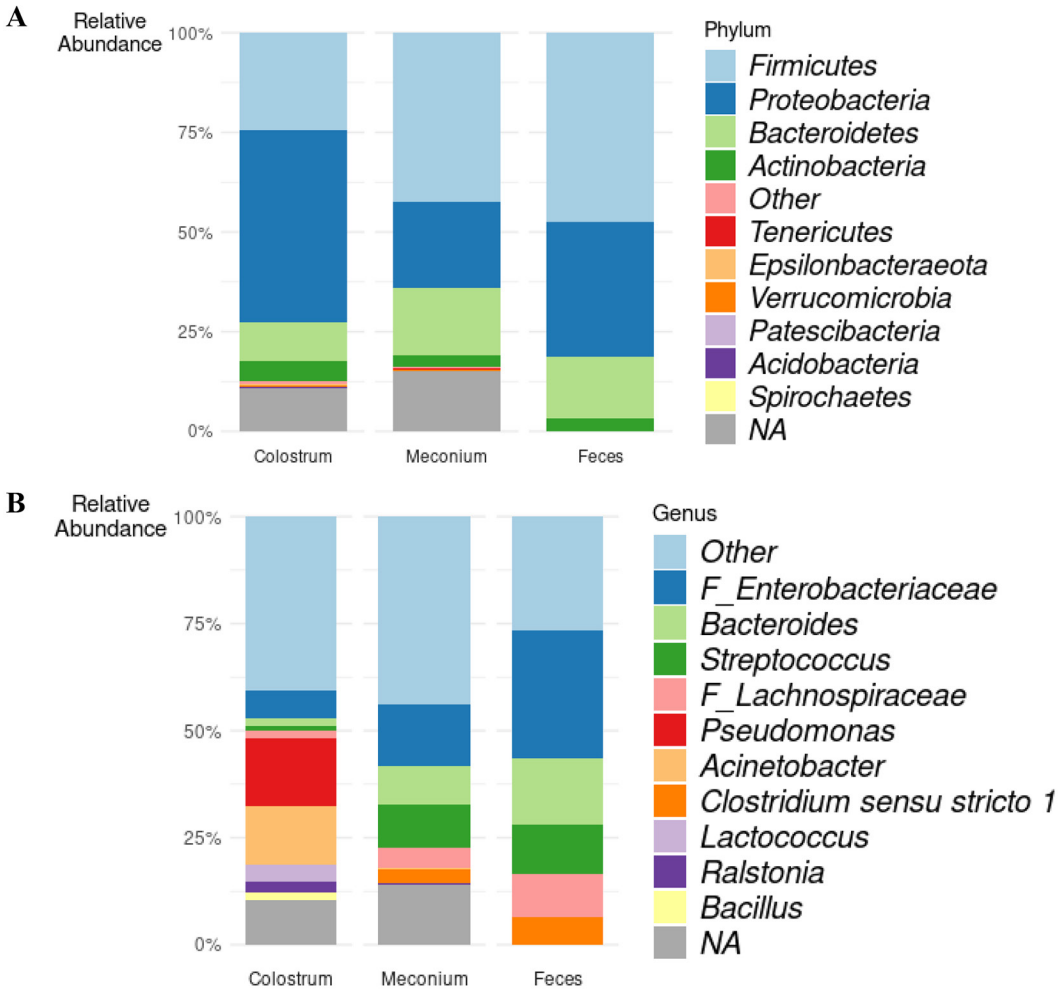

Figure 4. Bacterial composition of colostrum and calves' meconium and feces taken at d 3, showing (A) top 10 phyla and (B) top 10 genera that were most abundant in maternal colostrum and calves' meconium and feces. Sequences that could not be annotated are assigned to the category "NA."

studies have focused on the effect of the protein level in the prepartum diet on the milk yield in the subsequent lactation with variable and sometimes contradictory results (Bell et al., 2000). Feeding sheep a medium or HP diet in late pregnancy (116 and 157 g of CP/ kg DM, respectively), led to higher weights of the mammary gland, fetus, and uterine tissues compared with late-pregnant ewes that were fed a low protein diet (79 g of CP/kg DM; McNeill et al., 1997). The study of Vandehaar et al. (1999) showed a decrease in hepatic lipid content at parturition by increasing the dietary energy and protein density up to 1.6 Mcal of NE_L/kg and 16% CP in the last 3wk of gestation. These density levels are in line with the close-up HP diet in the present study. It has been suggested that higher CP levels in the prepartum diet increase labile protein reserves and therefore minimize metabolic disorders in the early subsequent lactation (Lean et al., 2013). Whereas previous research focused on the effect of the dry period diet on production performances in the subsequent lactation, little attention was paid on the effect of prepartum protein on the colostrum production (yield, nutritional composition, and IgG content) and calf performances in the dairy breed. Studies reporting on the effect of dietary CP levels on the colostrum quality and transfer of passive immunity are outdated (Burton et al., 1984; Hough et al., 1990) and recent studies on the effect of MP supply in the dry period on colostrum production and the transfer of passive immunity are scarce (Toghyani and Moharrery, 2015; Farahani et al., 2017). Metabolizable or true digested protein is the combination of microbial protein produced by fermentation in the rumen and dietary protein which escaped proteolysis by ruminal microbiota (Van Saun, 1993). Despite the fact that CP

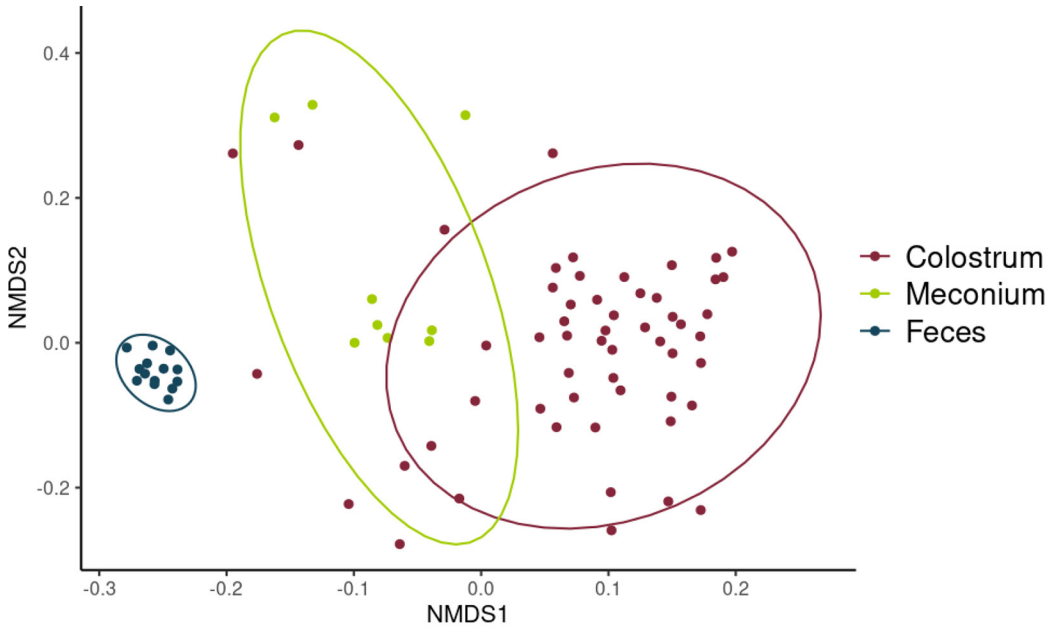

Figure 5. Bray-Curtis–based nonmetric multidimensional scaling (NMDS) plot showing a clear difference in microbial composition between calves' feces and meconium and maternal colostrum. Moreover, fecal samples cluster close together, meaning that they have a very similar microbial composition.

levels in the LP far-off diet were lower than recommended by NASEM (2021), MP supply of both the HP and LP diets used in the present study exceeded the NASEM (2021) recommendations of 5.2% MP for dry cows between 60 and 21 d prepartum and 6.7% MP for cows less than 21 d prepartum (Table 1). The study of Farahani et al. (2017) showed no effect on produced weight of first colostrum between cows supplemented with 849, 1,200, or 1,387 g/d of MP in the close-up period. Beef cows fed at 133% of their requirements for MP in the last 2 mo of gestation produced colostrum with higher fat levels compared with cows fed to meet their requirements for MP (Hare et al., 2019).

In the present study, cows that received RUP supplements (formaldehyde-treated soybean meal) during the far-off and close-up dry period, produced colostrum with higher IgG concentrations compared with cows that received the basic dry period diet. This effect was especially pronounced in cows completing their first dry period (61.3 ± 2.3 in HP vs. 55.2 ± 2.8 g of IgG/L in LP). In our previous study, colostrum from cows with parity 1 and 2 had a lower IgG concentration in comparison to cows with parity 3 or more (Van Hese et al., 2022). Other studies reported as well lower IgG levels in colostrum from second compared with third or greater lactation cows (Moore et al., 2005; Gulliksen et al., 2008). Cows entering their second lactation are not yet full grown (Enevoldsen and Kristensen, 1997), which could explain why younger cows may benefit more from protein supplementation during the dry period on colostrum quality. Several studies have analyzed colostrum composition in cows, but so far, no dietary influence on the IgG concentration could be demonstrated (Quigley and Drewry, 1998; Kehoe et al., 2007; Dunn et al., 2017a,b). However, a recent study showed a positive effect on colostrum IgG production and serum IgG absorption in the calves by supplementing the dry period diet with rumen-protected lysine and methionine, 2 limiting amino acids (AA) in cow diets (Wang et al., 2021). In the present study, RU soybean meal (Mervobest) was used to elevate the supply of MP in the dry period diet. This formaldehyde-treated product contains 5.7 and 25.0 g/kg DM metabolizable methionine and lysine, respectively (Vandaele et al., 2013). This led to an increase of 0.34 and 1.5 g/kg DM of metabolizable methionine and lysine, respectively, in the HP diet in this trial. Compared with the study of Wang et al. (2021), HP cows in our trial received per kg DM an equal amount of metabolizable methionine (0.35 vs. 0.34 g/kg DM intake, respectively), but a higher amount of metabolizable lysine (1.1 vs. 1.5g/kg DM intake, respectively). According to the results of Wang et al. (2021), supplementing dry cows with especially methionine seems beneficial for colostrum quality (i.e., IgG concentration). Fehlberg et al. (2020) reported as well no effect of feeding prepartum rumen-protected lysine on colostrum IgG concentration, nor volume. Supplying dry cows with canola meal (especially high

in His and Lys, but lower in Met; Acharya et al., 2015) did not affect colostrum IgG concentration, but tended to improve colostrum yield of first milking colostrum (Akhtar et al., 2022). In contrast to the present study, cows in the study of Wang et al. (2021) were not randomized based on parity. Therefore, it does not explain why we only saw this effect in the younger cows and not in cows with more than 2 parities and consequently it is uncertain whether the improvement of colostrum quality we observed in cows completing their first dry period, can be attributed to the elevation of these essential AA.

Important to state is that colostrogenesis is a complex process and controlled by several factors of which the working mechanism is not yet fully understood (Barrington et al., 2001; Castro et al., 2011; Quesnel and Farmer, 2019). Future research analyzing the effect of maternal nutrition on the IgG concentration in colostrum in dairy cows could include blood sampling of the cow to look for example at immune cell activity (Mann et al., 2016a,b).

Calves born out of LP cows, receiving colostrum from HP cows had the lowest serum IgG levels.

Results showed that calves that were born out of low protein cows and received colostrum from high protein cows (LPHP), had lower serum IgG levels compared with LPLP and HPHP calves, notwithstanding the fact that all the calves received on average the same amount of IgGs (Supplemental Table S3). As the calves in the present study all received colostrum of good quality and on average the same number of antibodies distributed over 3 feedings within an identical interval, colostrum quality, total amount of IgGs administered, and timing of colostrum feeding, all can be ruled out as the cause for the lower serum IgG levels in the LPHP group. Four out of the 5 calves that developed FPT (serum IgG level <10 g/L) belonged to this LPHP treatment group. A maternal dietary protein restriction has been shown to negatively affect IgG absorption in beef (Blecha et al., 1981; Hough et al., 1990) and dairy calves (Burton et al., 1984). This is inconsistent with our findings, because calves born out of LP cows that received colostrum from LP cows had on average the highest serum IgG levels. Important to note, our specific experimental design (2x2 factorial design) offers us the possibility to analyze both pre- and postnatal effects of the maternal diet on the IgG absorption in the calves. Before comparing our results to previous research, it should be noted that the studies referred to have a different approach compared with the present study. Blecha et al. (1981) solely analyzed the intrauterine effect on the calf of maternal crude protein restriction. In their trial, beef heifers received a restricted protein diet during the last 100 d of gestation. Calves born out of these heifers received colostrum from multiparous dairy cows (Blecha et al., 1981). This could, in a sense, be compared with our findings in the LPHP group having the lowest serum IgG levels. It is possible that these observations are linked to a mismatch between the pre- and postnatal conditions. A similar mismatch was as well noticed in the study of Blecha et al. (1981). In the study of Hough et al. (1990), also a 2x2 factorial design was applied in which calves received either colostrum from their own dam, or colostrum from a cow belonging to the reciprocal nutritional treatment group. Moreover, in the study of Hough et al. (1990), treatment cows received a diet restricted in both protein and energy, whereas in the present study, energy intake between treatments was kept identical. Lastly, in the present study, the statistical analysis was performed between the 4 treatment groups, in which the combination of pre-and postnatal treatment was preserved, whereas in the study of Hough et al. (1990) pre- and postnatal treatments were analyzed separately, probably due to a smaller sample size (22 calves in total). Finally, in the study of Burton et al. (1984) all calves received colostrum from their own dam, making it impossible to separate pre-and postnatal effects of maternal dietary CP levels. Although former studies performed on this topic confirm an effect of the maternal CP level prepartum on the IgG absorption in the calf, we have no clear explanation so far why the effect was only present in the LPHP group and not in the LPLP group. A possible hypothesis could be that these calves are performing worse due to a mismatch between the prenatal environment (cfr. low CP level at the end of gestation) and the postnatal resources (cfr. colostrum from protein supplemented cows). This concept was well described in studies analyzing the short- and long-term effects of the Dutch famine (of 1944–45) on the health of babies born during or shortly after this period (Stein et al., 1975, Roseboom et al., 2000; de Rooij et al., 2006). It is believed that the mother already prepares the fetus for the postnatal environment, the so called 'fetal programming' (Barker et al., 1993). When this prenatal forecast does not truly predict the postnatal situation (cfr. a mismatch), it may dysregulate the metabolic system which could lead to disorders later in life (Painter et al., 2005). Moreover, the timing during gestation at which an 'intervention' occurs can have separate outcomes. Micke et al. (2010) analyzed the effect of high and low levels of CP intake in pregnant beef heifers during the first or second trimester of gestation on the performance of their progeny. In male progeny, the postweaning BW was higher when they were born from heifers receiving a low CP diet in the first trimester and no difference in postweaning BW was observed when CP level of the maternal diet differed in the second trimester (Micke et al., 2010).

Subsequent profound research is however warranted to find out whether the results in the present study can be explained by a mismatch between the pre- and postnatal environment. More extensive research on the colostrum composition of LP and HP cows on the one hand and on the calves' intestinal morphology and physiology, however, can perhaps shed some light on this remarkable finding.

Birthweight, nor postnatal growth (0–12 wk) were affected by maternal nutrition or transfer of passive immunity.

In the present study, supplementing RUP in late gestation did not affect birthweight of the calf (39.5 ± 4.5 kg in HP vs. 39.2 ± 5.6 kg in LP). These findings are similar to other studies performed in dairy cows (Holland and Odde, 1992; Nowak et al., 2012; Dunn et al., 2017a; Moonsan et al., 2018). In beef cows, however, a positive effect of higher dietary CP levels in late gestation on birthweight was found (Larson et al., 2009; Kennedy et al., 2019). In addition to the genetic differences between dairy and beef cows, there is as well a distinct difference in management between the present study and the referred studies performed in beef cows. In the present study, dairy cows received throughout the entire dry period a constant diet with high quality feeds, whereas in the studies on beef cows, animals were held on pasture (Larson et al., 2009) or fed low quality diets (Larson et al., 2009; Kennedy et al., 2019). In addition, in our study total energy intake did not differ between the HP and LP group. The study of Salehi et al. (2017) showed that elevating energy intake by supplementing oilseeds at the end of gestation, positively affected calf birthweight. Perhaps, in dairy cows, the effect of the energy intake on the calf's birthweight is higher compared with the protein intake. Moreover, when rumen-protected (RP) methionine was supplemented to dairy cows at the end of gestation, a positive effect on the calves' birthweight, hip height and wither height was observed (Elolimy et al., 2019). Although, as already mentioned above, cows in the HP group also received a higher amount of metabolizable methionine, it is not clear why this did not positively affect calves' birthweight in the present study.

In addition to a similar birthweight among the calves of the different treatment groups, neither prenatal nor postnatal treatment did affect daily growth in the first 12 wk of life. This is comparable with the results of Dunn et al. (2017a) who showed no effect of maternal concentrate supplementation in the dry period on BW gain in their calves. On the contrary, calves born out of beef cows supplemented with protein had higher ADG until weaning compared with calves born out of unsupplemented cows (Stalker et al., 2006). The same consideration as with the effect on birthweight can be made, namely that there probably is an influence of the differences in breed and management in comparison to the present study, that may explain these contradictory results.

Supplementing RUP During the Dry Period Did Not Affect Colostral Microbial Composition

The most abundant phyla identified in colostrum were *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* (Figure 3A). The most abundant genera were *Pseudomonas*, *Acinetobacter*, *F_Enterobacteriaceae*, and *Lactococcus* (Figure 3B). This was similar to previous results recently reported by Van Hese et al. (2022), a study in which the microbial composition of colostrum in HF and Belgian Blue cows was analyzed. Furthermore, the study of Van Hese et al. (2022) showed that the abundance of some bacteria in colostrum (e.g., an unidentified *Lachnospiraceae*) is associated with a higher serum IgG level in dairy calves (Van Hese et al., 2022). Whether the abundance of these seemingly beneficial bacteria in colostrum could be influenced by managerial factors, such as nutrition, was in the scope of the present research. Even though there was no difference in the overall microbial composition between colostrum from HP and LP cows, 2 genera were higher abundant in colostrum from HP compared with LP cows. These 2 genera are members of the family *Lachnospiraceae*. This family was previously identified to be highly abundant in bovine colostrum (Van Hese et al., 2022) and therefore regarded as a member of the core microbiome of bovine colostrum (Lima et al., 2017). *Lachnospiraceae* is as well highly abundant in the rumen of cattle (Kittelmann et al., 2013) and associations were found between the abundance of *Lachnospiraceae* and feed efficiency in beef cows (Li and Guan, 2017). In the study of Chen et al. (2021) colostrum from dairy cows shared more than 50% of the detected OTUs with those found in rectal feces, including several strict anaerobes which are unlikely to enter colostrum through the teat canal. This can be explained by the entero-mammary pathway hypothesis that states that bacteria can be transported from the intestines to distal organs such as the mammary gland. According to the hypothesis, bacteria can be picked up by dendritic cells from the intestinal lumen and enter the lymphoid system where they can travel around inside lymphocytes or macrophages and end up in the mammary gland (Martín et al., 2004). Hypothesizing that the supplementation of RUP may lead to shifts in the microflora of the gastrointestinal tract, it is possible that this might affect the colostral microbial composition as well via this entero-mammary pathway. However, more evidence is needed to substantiate this hypothesis, for example, by also

analyzing the intestinal microflora of the cow, which was not performed in the present study.

Although in women, for example, the microbial composition of milk was affected by maternal diet when a vegetable protein and fiber based diet was compared with an animal protein and lipid based diet (Cortes-Macías et al., 2021). An important remark on this contradictory finding is that diets that were compared in women, differed greatly, whereas in the present study, the basic diet of the cows was identical, the only difference was the supplementation of RUP in the HP group. In addition to the extreme differences in the diets that were compared in the human studies, also the overall approach and statistical analysis differed. In these studies, groups were made by clustering based on the nutritional intake of the mothers (Cortes-Macías et al., 2021), or based on the microbial composition of the milk (Padilha et al., 2019). In addition, clustering based on nutritional intake (for example more fiber vs. more lipids) could be confounded by other lifestyle habits in addition to diet, for example a specific dietary pattern could be linked to more physical activity (Manz et al., 2019). It is not clear in which way these other lifestyle habits could contribute to the differences in microbial composition of human milk. Although we do not talk about 'lifestyle' but rather management of our cows, we can state that in addition to a differing dietary CP level at the end of gestation, management was identical.

The Abundance of Bacterial Genera in Colostrum Differed in Relation to the Parity of the Cow and Colostral and Serum IgG Concentration

Six genera were DA between colostrum of cows with parity 2 versus >2. *Pseudomonas* was lower abundant in colostrum from cows with parity 2. Lima et al. (2017) showed a lower abundance of this genus in colostrum from primiparous versus multiparous cows. However, our previous study did not confirm that *Pseudomonas* was DA between colostrum from primiparous versus multiparous cows (unpublished results, I. Van Hese, K. Goossens, B. Ampe, A. Haegeman, and G. Opsomer). It is thus far not clear if age or parity is related to the abundance of *Pseudomonas* in bovine colostrum.

Twelve genera were DA between colostrum of good and excellent quality. Similar to the previous results of our research group (Van Hese et al., 2022), a higher abundance of *Pseudomonas* and *Delftia* was related to a lower IgG concentration in colostrum. On the contrary, in the present study *Prevotellaceae* NK3B31 group, *Clostridium sensu stricto 1*, *Lactobacillus*, and an unidentified genus from *Lachnospiraceae* were higher abundant in colostrum of excellent versus good quality, which were not shown to be DA in colostrum of different quality in the study of (Van Hese et al., 2022). *Lactobacillus* is the most important genera of the lactic acid bacteria and is an important probiotic that is naturally present in raw milk (Coeuret et al., 2003). *Clostridium sensu stricto 1* was previously found to be a predominant genus in bovine colostrum (Xie et al., 2021). Moreover, this genus was a member of the core bacterial taxa in feces of 24-h-old calves, but not in feces from newborn (before colostrum ingestion) or 7-d-old calves (Alipour et al., 2018). Remarkably, in neonatal HF calves, a negative correlation between serum total protein and the abundance of *Clostridium sensu stricto 1* in feces was shown (Kumar et al., 2021; Castillo-Lopez et al., 2023). When analyzing the abundance of bacteria in colostrum related to the serum IgG level in the recipient calf, a higher abundance of *Clostridium sensu stricto 1* in colostrum was associated with a higher serum IgG level in the calf. Similar to the results of Van Hese et al. (2022), *Pseudomonas* was lower and an unidentified genus from *Lachnospiraceae* was higher abundant in colostrum that led to higher IgG serum levels in the calf.

The Microbial Composition of Cow Colostrum Differed from That of Calf Meconium and Feces

Because of the presence of microbes in meconium, it is thought that the fetal gut is already colonized in utero (Alipour et al., 2018; Klein-Jöbstl et al., 2019; Husso et al., 2021; Zhu et al., 2021). In the present study, the dominant microbial phyla in meconium were *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* (Figure 4A), which is comparable to other reports on the microbial composition of bovine meconium (Alipour et al., 2018; Husso et al., 2021; Owens et al., 2021; Zhu et al., 2021). The study of Zhu et al. (2021) stated that the calf's meconial microflora had resemblances with microflora from multiple maternal sites, such as the umbilical cord, placenta, colostrum, and amniotic fluid. Owens et al. (2021) showed that the colostrum microbiome was a significant predictor of the calf's fecal microbiome. This correlation decreased gradually in time and was highest for meconium and lowest for calves' feces at 60 d of age.

Alpha diversity in meconium was much greater than in calves' fecal samples. Klein-Jöbstl et al. (2019) observed as well a decrease in α diversity in early to later sampling time points, samples taken at 0.5 and 6 h postnatum had a higher Chao1 index compared with samples taken at 12, 24, and 48 h after birth. Moreover, the NMDS plot (Figure 5) shows only little variation in the microbial composition between fecal samples taken at d 3 of age. This suggests that the fecal microflora stabilizes rapidly after birth (Alipour et al., 2018). In

addition to a high similarity between fecal samples, the NMDS plot also illustrates a large difference between the microbial composition of colostrum and that of feces (Figure 5), which is consistent with results reported in earlier studies (Klein-Jöbstl et al., 2019; Hang et al., 2021).

In the study of Elolimy et al. (2019), maternal prepartum supplementation with RP methionine influenced the abundance of several bacteria in calves' meconium. Because of the low number of meconial and fecal samples in the present study, differential abundance analysis between different treatments was not performed. In the future, large-scale experiments on the effect of maternal nutrition on the calf's hindgut microbiota are needed to reveal possible interventions that might improve gut health in newborn calves.

CONCLUSIONS

Supplementing cows with RUP (formaldehyde-treated soybean meal) during the dry period positively affected the colostral IgG concentration, especially for cows in their first dry period. Because these younger cows have not yet reached their mature BW, it is possible that they benefit more from protein supplementation during the dry period compared with older cows. Neither calves' birthweight nor growth were affected by pre- and postnatal protein supplementation. Calves born out of LP cows and receiving colostrum from HP cows had the lowest serum IgG levels and the highest incidence of FPT. It is possible that a mismatch between the pre- and postnatal treatments is responsible for this observation. However, profound research is needed to confirm this hypothesis. The overall microbial composition of colostrum was not affected by RUP supplementation; solely the family *Lachnospiraceae* was more abundant in colostrum from HP compared with LP cows. In our previous research, the higher abundance of this family in colostrum was associated with higher serum IgG levels in calves. Finally, the microbial composition of colostrum differed from that of calves' meconium and feces. We observed, however, a high similarity of the microbial composition within calves' fecal samples.

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