Animal Nutrition 19 (2024) 287–300

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

KeAi CHINESE ROOTS GLOBAL IMPACT

Original Research Article

Interactions between rumen epithelium-associated microbiota and host immunological and metabolic adaptations in response to different milk replacer feeding intensities in dairy calves

Carolin B.M. Müller, Lisa-Maria Tümmler, Henry Reyer, Torsten Viergutz, Björn Kuhla*

Institute for Farm Animal Biology (FBN), Dummerstorf 18196, Germany

A R T I C L E I N F O

Article history:
Received 26 September 2023
Received in revised form
6 June 2024
Accepted 4 September 2024
Available online 7 September 2024

Keywords:
Milk replacer
Rumen microbe
Immune system
Host-microbe interaction

A B S T R A C T

The milk replacer feeding regime in dairy calves has a great impact on metabolic and immunological functioning and affects animal welfare and lifetime performance. The feeding regime influences the rumen microbial composition, and epithelium-associated microbes may interact with the immune system of the host. We examined the correlations between blood leukocyte counts and the rumen epithelium-associated microbiome in dairy calves fed 2 different milk replacer feeding intensities and if these factors related to metabolic traits. Fourteen newborn female dairy calves were allocated to a group receiving either 10% ($n = 7$) or 20% ($n = 7$) milk replacer of their body weight (on average 41 kg) and provided ad libitum access to grass hay and concentrate pellets. At 3 weeks of life, all calves were fitted with a rumen cannula. Calves were weaned at 12 weeks of life and received a total mixed ration for ad libitum intake. Pre- (8–10 weeks of life) and post-weaning (21–23 weeks of life), methane production was measured in respiration chambers, and rumen epithelium and blood were sampled for 16S rRNA sequencing and leukocyte analyses, respectively. Pre-weaning, the reduced milk replacer feeding intensity was accompanied with higher concentrate intake but lower growth performance ($P < 0.001$), a higher abundance of amylolytic and lower abundance of cellulolytic epimural microbes. The group fed a low milk replacer intensity had also greater portions of monocytes ($P = 0.031$), CD8$^+$ ($P < 0.001$), and CD14$^+$ ($P = 0.044$) leukocytes, suggesting elevated inflammatory conditions. Correlations between CD8$^+$ T cells and rumen methanogens, Ruminococcaceae, and Lachnospiraceae were recorded, but these were not consistent throughout maturation. Post-weaning, differences in feed intake and rumen microbial composition converged among milk replacer groups, while differences in growth performance ($P = 0.040$) and CD8$^+$ cells ($P < 0.001$) were still present. In conclusion, a reduced milk replacer feeding intensity in dairy calves compromised growth performance and immunity and this effect persisted in the long-term. Significant correlations between the proportion of leukocytes and distinct epimural microbe taxa indicated an interplay between rumen epimural colonization and immune functioning of the host. However, further research is required addressing this interplay between rumen epimural microbes and immune functioning in dairy calves.

© 2024 The Authors. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co. Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

The allowance of milk or milk replacer (MR) intake to dairy calves affects animal welfare and may have long-lasting effects on lifetime performance (Hammon et al., 2020; Kenez et al., 2018; Whalin and Weary, 2021). If not restricted, dairy calves consume voluntarily up to 14 L milk per day in the first 2 weeks of life (Borderas et al., 2009) and are naturally weaned between 7 and 14 months of age (Enriquez et al., 2011). Indeed, dairy calves fed MR for ad libitum intake achieved 63% to 80% higher daily weight gains

* Corresponding author.
E-mail address: b.kuhla@fbn-dummerstorf.de (B. Kuhla).

Peer review under the responsibility of Chinese Association of Animal Science and Veterinary Medicine.

https://doi.org/10.1016/j.aninu.2024.09.001
2405-6545/© 2024 The Authors. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co. Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

than their restriction-fed counterparts resulting in considerable differences in body weight (BW) and organ weights (Geiger et al., 2016; Hammon et al., 2018; Jasper and Weary, 2002). Moreover, Alimirzaei et al. (2020) found that plasma inflammatory markers decline with elevations in MR feeding and this decline may be associated with an increase in animal resilience. However, under conditions of practical farming, dairy calves are often weaned not later than 12 weeks of life. Therefore, an early stimulation of rumination activity preparing the calf for post-weaning feeding conditions is necessary. High MR feeding intensities delay rumen development and have adverse effects on digestibility of solid feed and a sufficient ruminal maturation before weaning (Quigley et al., 2018; Terre et al., 2007). Moreover, Terre et al. (2007) postulated that different MR feeding regimes are associated with differences in rumen microbial development and composition. In accordance, doubling the MR feeding intensity reduced the abundance of carbohydrate-degrading bacteria, while increasing the rumen microbial diversity in pre-weaned lambs (Huang et al., 2023).

Changes in the composition of microbiota attached to the rumen epithelium alter nutrient absorption (Li et al., 2023; Minuti et al., 2015) and rumen epithelial barrier function (Malmuthuge et al., 2019). As the nutrient supply and barrier function are critical for the activation of the host immune system, the rumen epimural microbial composition may exert indirect and direct effects on the immune and inflammatory responses of the rumen epithelium but also on systemic immune and inflammatory responses (Garcia et al., 2017; Luo et al., 2022; Na and Guan, 2022). Accordingly, the oral administration of postbiotic *Lactobacillus plantarum RG14* improved rumen papillae growth and lowered the number of leukocytes and platelets in the circulation of lambs (Izuddin et al., 2019). In dairy calves, *Enterococcus faecium* and *Clostridium butyricum* increased the peripheral numbers of cluster of differentiation (CD) 282 positive ($^+$) monocytes, CD3$^+$ T cells and CD4$^+$, CD8$^+$, and WC1$^+$ T cells (Qadis et al., 2014a,b). Conversely, it was found that forestomach walls express immune receptors and cytokines, that rumen fluid is infiltrated with leukocytes and immunoglobulins originating from saliva and potentially from forestomach walls, and that these immune variables were associated with inflammatory markers in blood (Filipe, 2018; Petryshak et al., 2020; Trevisi et al., 2014, 2018). Moreover, ruminal immune compounds are involved in the selection of rumen epimural and fluid microbiota and thus modify the overall microbial composition and fermentation processes (Filipe, 2018; Palma-Hidalgo et al., 2023). In line with the latter, the immunization with a 3-methanogen mix reduces enteric methane production and alters the ruminal archaeal population in sheep (Williams et al., 2009; Wright et al., 2004). In addition, Meese et al. (2020) found leukocyte proliferation rates were positively associated with methane emissions and thus ruminal fermentation intensities in dairy cows.

Cattle at critical physiological stages have a high susceptibility to inflammatory processes, and it is of great interest to uncover the underlying mechanisms of the interplay between nutrition and animal health during these stages (Garcia et al., 2017). The bidirectional host-microbe interaction between rumen epithelium-associated microbes and the host immune system as described above have not been studied in dairy calves fed different MR intensities. Therefore, the objective of the present study was to investigate correlations between the rumen epithelium-associated microbiome and the blood leukocyte pattern and to relate these factors also with metabolic traits of dairy calves fed different MR intensities. A further objective was to examine, if potential differences between feeding groups persist after weaning. Results of the present study shall contribute to understand the underlying bidirectional interactions between the rumen wall adherent microbes and the host immune system and to further define beneficial and adverse aspects of different MR feeding intensities in dairy calves.

2. Materials and methods

The present study was part of a larger animal experiment, which was evaluated and approved by the office of Agriculture, Food Security and Fishery Mecklenburg-Western Pomerania, Rostock, Germany (No. 7221.3-1.1-009/16) following the guidelines of the German Animal Welfare Act. The animal experiment was conducted between October 2016 and May 2018 at Research Institute for Farm Animal Biology (FBN), Dummerstorf, Germany.

2.1. Animals and grouping

Fourteen female Holstein calves were born at FBN or purchased from 2 cooperating dairy farms immediately after birth. Newborn calves were moved individually to a pen at FBN without initial colostrum feeding. After weighing, calves were randomly allocated to 2 different feeding groups either receiving 10% (10%-MR; $n = 7$) or 20% (20%-MR; $n = 7$) MR feeding of their respective BW per day. The 10%-MR feeding intensity represented conventional feeding programs (Khan et al., 2011), while 20%-MR corresponded to recommendations closely linked to ad libitum intake (Hammon et al., 2020). Initial BW of calves was comparable between MR groups (mean ± SD: 10%-MR: 41 ± 3 kg, 20%-MR: 40 ± 2 kg). Before the start of MR feeding, calves were provided pooled colostrum for 2.5 d at daily allowance of 10% (10%-MR) or 12% (20%-MR) of BW as described by Tümmler et al. (2021). Briefly, calves received their first meal within 2 to 8 h after birth and the second meal at least 4 h after the first meal. At 3 weeks of age (18 ± 3 d), all calves were fitted with a permanent rumen cannula on the left dorsolateral flank (3.5 cm o.d.) at the level of the dorsal ruminal part (*saccus dorsalis*). Milk replacer intake and BW fully recovered and continued to increase 3 ± 1 d after surgery. The first 5 animals entering the study were dehorned at the age of 7 ± 1 weeks (52 ± 5 d; 10%-MR: $n = 3$; 20%-MR: $n = 2$). Because the combined impact of rumen cannulation and dehorning on the calves' health status was not tolerable, and due to the presence of genetically hornless calves, the dehorning procedure was not applied for the remaining animals entering the study.

2.2. Feeding regime

The feeding trial lasted until the age of 23 weeks (Experimental design: Fig. S1). Animals had free access to water throughout the experimental schedule. After the colostrum feeding period, calves were fed MR (Bergin Milch LC 50; Bergophor Futtermittelfabrik Dr. Berger GmbH & Co. KG, Kulmbach, Germany; 140 g per kg water, 38 °C feeding temperature) by an automatic feeding system (Kälbermama Lifestart; Urban GmbH & Co. KG, Hude/Wüsting, Germany). Body weight was recorded weekly to adjust the amount of MR offered according to the respective feeding group (10%-MR vs. 20%-MR). Milk replacer was provided in 3 equal meals per day at 07:00, 13:00, and 18:00. Starting at 6 weeks of age, the number of meals was elevated from 3 to 4 at 07:00, 10:00, 13:00, and 18:00 for the 20%-MR feeding group. From week 9 to 10, the weaning procedure started with a gradual reduction of MR provision from 20% to 10% of BW in the 20%-MR group accounting for a 3.5% reduction in MR provision per day. Subsequently, the amount of MR was gradually reduced for both MR feeding groups accounting for a

daily reduction in MR provision of 3.5% in 20%-MR and of 7% in 10%-MR calves until weaning at week 13. Calves had free access to starter feed (0.3 mm × 14 mm pellets; BERGIN Kälberpellet; Bergophor Futtermittelfabrik Dr. Berger GmbH & Co. KG) and long-stemmed grass hay until end of week 12 and 14, respectively. The starter feed was limited to 2 kg/d in week 13 and 14 and was gradually reduced to 0 kg/d in week 15 and 16. A total mixed ration (TMR) was offered for ad libitum intake from week 11 onwards. Individual feed intake of grass hay, starter pellets, and TMR was measured daily. Data on feed constitutes and nutrient composition are presented in Table 1.

2.3. Body condition, growth performance, and rumen development

The average daily gain (ADG) was calculated from BW gain per week. Body condition score (BCS) was assessed weekly according to Raschke (2007). Rumen size was estimated weekly by ultrasonographic imaging (MicroMaxx UltrasoundSystem and WAMicroMaxx P10; Sonosite Inc., Bothel) as previously described by Tümmler et al. (2020). Thereby, the distance between dorsal and ventral margins of the rumen was identified in the 11th intercostal space at a frequency of 8 to 4 MHz and a depth of 7.2 cm (Braun et al., 2013).

2.4. Blood and rumen fluid sampling

At 8 and 21 weeks of age (55 ± 2 d; 148 ± 2 d), blood samples were collected from the jugular vein into 9-mL EDTA monovettes (catalog no. 02.267.001; Sarstedt, Nümbrecht, Germany) and placed on ice. Immediately after sampling, full blood samples were transferred to the laboratory for leukocyte analysis. The remaining blood was centrifuged at 1565 × *g* (20 min, 4 °C), and the obtained plasma was stored at −20 °C until further analysis. After blood sampling, approximately 30 mL of rumen fluid was gained from the ruminal *Saccus ventralis* by inserting a probe via the rumen cannula. The pH of rumen fluid was determined immediately. Subsequently, rumen fluid was passed through a 0.7-mm sieve, and 2.5 mL of the fluid was mixed with 1 mL of 0.5% isocaproic acid (internal standard), centrifuged at 13,000 × *g* (10 min, 4 °C) and stored at −20 °C until the analysis for short chain fatty acids (SCFA).

Table 1
Feed constitutes and nutrient composition of total mixed ration ($n = 8$), milk replacer ($n = 1$), starter pellets ($n = 1$), and grass hay ($n = 3$).

Item	Total mixed ration	Milk replacer[1]	Starter pellets[2]	Grass hay
Ingredients, g/kg of DM				
Grass silage	172			
Corn silage	469			
Grass hay	15			
Barley straw	10			
Corn meal	67			
Wheat meal	15			
Extracted soy meal	19			
Extracted rapeseed meal	49			
Soy oil	1			
Concentrate[3]	169			
Granulated feed supplement[4]	4			
Mineral feed[5]	7			
Limestone[6]	4			
Nutrients, g/kg of DM				
Crude ash	53	80	79	52
Crude fat	31	208	35	14
Crude protein	157	221	201	81
Crude fiber	156	1	93	317
ADF	185	–	126	364
aNDF	349	–	223	544
Starch	284	32	293	–
Sugar	15	300	112	139
ME[7], MJ/kg DM	11	17	12	9

ADF = acid detergent fiber; aNDF = neutral detergent fiber after amylase treatment.
[1] BERGIN Milch LC 50 (Bergophor Futtermittelfabrik Dr. Berger GmbH & Co. KG, Kulmbach, Germany): skimmed milk powder, whey powder, refined plant oil (palm and coconut), dextrose, egg powder. Additives per kilogram original substance: 25,000 IU vitamin A, 4000 IU vitamin D_3, 200 mg vitamin E, 200 mg vitamin C, 8 mg Cu, 100 mg Fe, 45 mg Zn, 30 mg Mn, 0.2 mg I, 0.2 mg Se. Composition: 1.8% Lys, 0.9 % Ca, 0.7% P, 0.5% Na.
[2] BERGIN Kälber pellet (Bergophor Futtermittelfabrik Dr. Berger FmbH & Co. KG, Kulmbach, Germany): extracted soy meal from peeled and steam-heated beans, wheat, dried sugar beet pulp, apple pomace, apple molasses, pulped corn, wheat bran, products and by-products from bakery and pasta industry, pulped linseed, $Ca(H_2PO_4)_2$, $CaCO_3$, dried yeast, dextrose. Additives per kilogram original substance: 16,000 IU vitamin A (rumen-protected), 4000 IU vitamin D_3, 200 mg vitamin E, 300 mg vitamin C, 4 mg vitamin K_3, 15 mg vitamin B_1, 8 mg vitamin B_2, 10 mg vitamin B_6, 50 μg vitamin B_{12}, 50 μg vitamin B_{12} (rumen-protected), 20 mg Ca-D-pantothenate, 40 mg niacin amide, 4 mg folic acid, 0.3 mg biotin, 150 mg Fe, 12 mg Cu, 60 mg Zn, 50 mg Mn, 1 mg I, 0.4 mg Se. Composition: 1.2% Ca, 0.6% P, 0.3% Na.
[3] MF 2000 (Vollkraft Mischfutterwerke GmbH, Güstrow, Germany): 30% extracted soy meal from peeled and steam-heated beans, 25.4% corn grit, 15% malt culms 6.9% extracted rapeseed meal, 5.1% wheat, 5% sugar beet pulp, 4.9% dried grain pulp, 4.1% beet molasses, 2% $NaHCO_3$, 1% $CaCO_3$, 0.2% NaCl. Additives per kilogram original substance: 10,000 IU vitamin A, 1125 IU vitamin D_3, 40 mg vitamin E, 0.6 mg I, 0.4 mg Co, 50 mg Mn, 75 mg Zn, 0.4 mg Se. Composition: 24% crude protein, 3.3% crude fat, 6.8% crude fiber, 8.4% crude ash, 0.7% Ca, 0.5 % P, 0.65% Na.
[4] Granulate 100532 FBN (FUGEMA GmbH, Malchin, Germany): dried sugar beet pulp, extracted soy meal from peeled and steam-heated beans, rye, NaCl, $NaCaPO_3$. Additives per kilogram original substance: 24,000 IU vitamin A, 4120 IU vitamin D_3, 100 mg vitamin E, 30 mg Cu, 90 mg Fe, 160 mg Zn, 100 mg Mn, 2 mg Co, 1 mg I, 1 mg Se, 14 g urea. Composition: 22.2% crude protein, 1.5% crude fat, 8.6% crude fiber, 8.5% crude ash, 1% Ca, 0.37% P, 0.42% Na.
[5] Panto-Mineral R 7609 (HL Hamburger Leistungsfutter GmbH, Hamburg, Germany): 32.8% $CaNaPO_3$, 19.3% $CaMg(CO_3)_2$, 16.7% NaCl, 15.3% $CaCO_3$, 6.1% MgO, 3% sugar cane molasses. Additives per kilogram original substance: 900,000 IU vitamin A, 200,000 IU vitamin D_3, 4.5 g vitamin E, 1.5 g Cu, 8 g Zn, 5 g Mn, 60 mg I, 70 mg Co, 50 mg Se. Composition: 20% Ca, 6% P, 8 % Na, 6% Mg.
[6] Kreidekalk (Spezialfutter Neuruppin GmbH und Co. KG, Neuruppin, Germany): $CaCO_3$. Composition: 37 % Ca.
[7] Metabolizable energy (ME) content of total mixed ration, starter and grass hay was calculated according to GfE (2001). Milk replacer ME content was calculated according to NRC (2001).

2.5. Methane measurement and indirect calorimetry

Well-adapted calves were individually transferred into an open-circuit respiration chamber (Derno et al., 2009) in week 9 (58 ± 2 d) before weaning and again in week 22 (150 ± 3 d) after weaning. The CO_2 gas recovery rate of the 4 chambers were 99.9% ± 0.96%. After a 12- to 16-h equilibration period of gas exchange, gas concentrations of oxygen, carbon dioxide, and methane were recorded over a period of 48 h. Respiratory measurements were conducted at 15 °C, 70% humidity, and an airflow rate of 10 m^3/h. Gas samples were drawn in 6 min intervals by a membrane pump (80 L/h; KNF Neuburger Laboport, Freiburg, Germany) and analyzed paramagnetically (oxygen) or by infrared absorption (carbon dioxide and methane) (SIDOR and GMS800; SICK MAIHAK GmbH, Reute, Germany). Body weight was determined before entering and after exiting the respiration chamber for calculating the average BW and metabolic BW (mBW). Throughout the respiratory measurements, calves had free access to water. In week 9, MR was fed in 3 equal portions at 07:00, 13:00, and 18:00 for 10%-MR calves and in 4 equal portions at 07:00, 10:00, 13:00, and 18:00 for 20%-MR calves. Additional hay and starter pellets in week 9 and TMR in week 22 were placed in the trough and back scaled every 24 h to determine daily hay, pellet, and TMR intake. For feeding and cleaning, staff entered the chambers by an air lock flushed with chamber air and wore facemasks connected to outdoor air preventing interference of respiratory measurements.

Heat production (HP) was calculated from average daily gas emissions (L/d) referred to the mBW according to Brouwer (1965):

HP/mBW (kJ/$kg^{0.75}$) = [16.18 × oxygen (L/d) + 5.02 × carbon dioxide (L/d) − 2.17 × methane (L/d) − 5.99 × N_{Urine} (g/d)]/mBW ($kg^{0.75}$), in which, daily urinary nitrogen excretion (N_{Urine}) was estimated according to Jentsch et al. (1996): N_{Urine} = 12 g/d for BW ≤ 83 kg, 20 g/d for BW ≤ 120 kg, and 25 g/d for BW > 120 kg.

Energy balance (EB) was calculated from the difference between HP and metabolizable energy intake (MEI) during respiratory measurements as:

EB (MJ/d) = HP (MJ/d) − MEI (MJ/d).

The energy conversion ratio (ECR) was calculated as the ratio between ADG and MEI.

2.6. Feces and rumen epithelium sampling

Feces were sampled in week 8 (d 59 ± 2) and week 22 (d 152 ± 3). Briefly, feces were grab sampled from the rectum in the morning and the afternoon for 4 consecutive days and on day 5 and 6 solely in the morning and stored at −20 °C. The rumen epithelium was sampled in week 9 (d 61 ± 2) and week 22 (d 155 ± 4). Rumen papillae from the left lateral site were picked by a long forceps via the rumen cannula. Obtained papillae were washed in saline solution, transferred to liquid nitrogen, and stored at −80 °C until analysis of the rumen epithelium-associated microbiota.

2.7. Analytics

2.7.1. Nutrient composition of feed

Milk replacer powder, starter pellets, and grass hay were sampled per batch, and TMR was sampled once a week. Dry matter (DM) content of feed samples was analyzed by air-drying at 60 °C for 24 h and repeated drying at 103 °C for 4 h before the analysis of nutrient composition according to VDLUFA (1976) (Landwirtschaftliche Untersuchungs- und Forschungsanstalt, Rostock; LUFA GmbH, Rostock, Germany) (Table 1). The nutrient analyses in MR, starter pellets, and grass hay involved the following methods: crude ash (burning at 550 °C; method 8.1); crude protein (Kjeldahl, CP = N × 6.25; method 4.1.1); crude fat (Soxhlet petrol ether extraction; method 5.1.1); crude fiber (treatment with sulphuric acid and sodium hydroxide; method 6.1.1); sugar (Luff-Schoorl method, reduction in Fehling's solution; method 7.1.3); starch (optical rotation after treating with hydrochloric acid and ethanol; method 7.2.1). The nutrient analyses of TMR samples were conducted using near infrared spectroscopy. Quantifications of acid detergent fiber (ADF; treatment with sulphuriccetyltrimethyl-ammonium bromide) and amylase-treated neutral detergent fiber (aNDF) in feed samples were performed according to van Soest (method 6.5.2 and 6.5.1; Vansoest et al., 1991). The metabolizable energy of roughage feed was calculated according to GfE (2001) and of MR according to NRC (2001).

2.7.2. Fecal pH and starch

Fecal samples were thawed and dried at 65 °C for 72 h. Subsequently, samples collected on day 1 to 6 were equally pooled and 1.1 g was agitated in 5 mL distilled water. After 30 min, the pH was measured via a pH electrode at 22 °C (WTW SenTix41; Xylem Analytics Germany Sales GmbH & Co, Weilheim, Germany). Dried, pooled fecal samples (300 mg) were incubated with 9 mL 16% hydrochloric acid for 60 min at 130 °C. After centrifugation (1565 × *g*, 15 min, 4 °C), the pH of the supernatant was adjusted to 6.5 by adding sodium hydroxide. The glucose concentration was determined colorimetrically (ABX Pentra C400 clinical chemistry analyzer; HORIBA Europe GmbH, Oberursel, Germany) using the kit A11A01-667 (HORIBA Europe GmbH). The starch concentration was calculated as glucose × 0.9 and was referred to the amount of fecal sample.

2.7.3. Blood leukocytes

Blood samples (100 μL) were incubated with VersaLyse (Beckman Coulter GmbH, Krefeld, Germany) for 20 min at room temperature and centrifuged at 250 × *g* for 5 min. Obtained cell pellets were washed twice by adding 700 μL phosphate-buffered saline (PBS) and subsequent centrifugation at 150 × *g* for 5 min. Staining of leukocytes was reached by adding monoclonal antibodies for marking CD4 (co-receptor primarily expressed on T-helper cells, monocytes, and macrophages; mouse anti-bovine CD4: +FITC; Bio-Rad, Hercules, USA), CD8 (co-receptor primarily expressed on cytotoxic T cells; mouse anti-bovine CD8: +RPE; Bio-Rad), CD14 (receptor expressed on monocytes and macrophages; mouse anti-bovine CD14: +RPE; Bio-Rad), and CD21 (co-receptor found on B cells; mouse anti-bovine CD21: +RPE; Bio-Rad). The mixture was incubated for 30 min at 4 °C in the absence of light. After centrifugation at 250 × *g* for 5 min at 4 °C, the gained cell pellet was resuspended in 600 to 1000 μL PBS. Granulocytes, monocytes, lymphocytes, and the respective CDs were quantified by fluorescence activated cell sorting analysis (Gallios; Beckman Coulter GmbH) counting at least 10,000 cells. Cells were gated on the forward and side scatter dot plot, and signals were analyzed by Kaluza Analysis software (Beckman Coulter GmbH) to obtain relative counts of leukocytes.

2.7.4. Plasma and rumen fluid metabolites

Thawed plasma samples were spectrophotometrically and potentiometrically analyzed (ABX Pentra C400; HORIBA Europe GmbH, Oberursel, Germany) using the following kits: A11A01-664 (albumin), -667 (glucose), -640 (triglycerides), -721 (L-lactate) (HORIBA ABX, Montpellier, France), LT-UR 0010 (urea; Labor + Technik Eberhard Lehmann GmbH, Berlin, Germany), 434–91,795/436–91,995 (nonesterified fatty acids (NEFA); FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany), and RB1007 (β-Hydroxybutyric acid [BHB]; Randox Laboratories, Crumlin, UK).

Rumen fluid samples containing the internal standard were thawed and 1 mL of sample was acidified with 5 μL of 37% hydrochloric acid. The concentrations of SFCA were analyzed in triplets by a gas chromatography coupled with a flame ionization detector (GC-FID, Series 17A; Shimadzu Corp., Kyoto, Japan) and equipped with a 25-m × 0.25-mm free fatty acid phase column (Roth, Karlsruhe, Germany).

2.7.5. Next generation sequencing of rumen epithelium associated microbes

Rumen epithelium samples were ground under liquid nitrogen. For DNA extraction 18 to 24 mg of grinded tissue was processed using the QIAamp cador Pathogen Mini Kit (no. 54106; QIAGEN, Hilden, Germany). The laboratory protocol followed the pre-described procedure from the associated handbook (no. 54106; QIAGEN), including the pretreatment step with reagent DX (no. 19088, Pretreatment B1; QIAGEN). Following DNA quantification, purified DNA was used for next generation 16S rRNA sequencing of the rumen epithelium associated microbes (Lamp et al., 2018). Library preparation targeted the variable V4 region of the 16S rRNA gene using the specific primers 515′F (forward: 5′-GTGBCAGCMGCCGCGGTAA-3′; Hugerth et al. (2014)) and 806R (reverse: 5′-GGACTACHVGGGTWTCTAAT-3′; Caporaso et al. (2011)). Polymerase chain reaction (PCR) was performed with the 5 Prime HotMasterMix (5 Prime, Hamburg, Germany) and conducted in duplicates. Cycling conditions of PCR included the initial denaturation (95 °C, 2 min), 35 cycles of amplification (95 °C for 30 s; 50 °C for 60 s; 72 °C for 90 s), and a final extension period (72 °C 10 min). Subsequently, duplicates of each sample were combined, all samples were mixed in equal amounts using SequalPrep Normalization plates (Thermo Fisher Scientific, Dreieich, Germany) and transferred to Illumina sequencing (HiSeq2500; Illumina, San Diego, CA, USA) to generate 2 × 250 base pair paired-end reads. Gained data are deposited in the NCBI database, accession number PRJNA1004089: https://www.ncbi.nlm.nih.gov/bioproject/1004089. Raw data were filtered, trimmed, and analyzed using the mothur software (version 1.44.1; Schloss et al. (2009)). Sequences were aligned to the Silva reference database (release 138; Quast et al. (2013)) and clustered into operational taxonomic units (OTU). In the clustering procedure, sequences that were at least 97% identical were assigned to the same OTU. A subsampling to 362,475 reads was performed based on the sample with the lowest number of observations. The taxonomic classification of cumulated microbial abundances was generated at a phylum and genus level (Silva reference database).

2.8. Statistical analyses

All data, except rumen epimural microbial composition, were statistically analyzed using the SAS software for Windows, version 9.4 (Copyright, SAS Institute Inc., Cary, NC, USA). The following time frames of feed intake (week 9 and 22), condition data (BW, BCS, rumen size: 61 ± 4 d and 152 ± 3 d (week 9 and 22), and ADG (week 8–10 and week 21–23) were considered in the statistical evaluation. In response to individual veterinary treatments of calves, all variables were tested for outliers by IQR function; no influence of individual medical treatments on obtained data was identified. The GLIMMIX procedure was used to respond to non-normal distributed unit-levels. The GLIMMIX model considered the fixed factors MR-group (10%-MR vs. 20%-MR), time point of sampling (pre- or post-weaning), the dehorning process (dehorned or not dehorned), and their interactions. The statistical influence of the dehorning process on variables was only considered if physiologically reasonable, such as in the analysis of the epimural microbiome and the leukocyte distribution pattern. Repeated measurements on both time points were considered by the RANDOM statement of the GLIMMIX procedure involving the SUBJECT = animal option and an unstructured covariance structure. Degrees of freedom approximation was performed by the Kenward-Roger method. The NOITER option of the PARMS statement was included into the model to obtain MIVQUE0 estimates. The distribution patterns of unit-levels were analyzed by comparing fitted distributions in the JMP software (version 14, SAS Institute Inc.) for each variable. The result of the best fitting distribution pattern defined the chosen distribution pattern in the GLIMMIX procedure and was specified in the results tables. Least squares means (LSM) and standard error (SE) were computed for each fixed effect and interactions. Differences between LSM were calculated by the Tukey–Kramer procedure. If data were not normally distributed, the ILINK function was included to back-transform LSM to the original data scale. The SLICE statement of the GLIMMIX procedure was used for the partitioned analysis of LSM for the two-way interaction MR intake × time point of sampling.

Metataxonomic profiles, describing differences in relative abundances on the phylum and genus level, were created with the R packages “mixOmics” (version 6.6.2) (Rohart et al., 2017), “DESeq2”, and the included nbinom Wald test (Love et al., 2014). Based on the principal component analysis (PCA), 2 outlier samples were identified on the genus level and excluded from further evaluations (excluded samples: 2 × 20%-MR pre-weaning; Fig. S2). Low abundant genera and phyla were filtered to allow at least 10 samples with 5 or more reads per taxa. Thus, 152 out of 965 genera (15.9%) and 19 out of 39 phyla (48.7%) were considered in the statistical evaluation. The model of DESeq2 involved MR groups as fixed effects and compared their metataxonomic profiles pre- and post-weaning. Because the number of dehorned calves were not equally distributed to both MR groups, results obtained from all calves were compared with results obtained from non-dehorned calves. If the distribution pattern of the specific genus or phylum was comparable between results considering all calves and only non-dehorned calves, it was included into the final evaluation. The alpha diversity on OTU level was calculated using the inverse Simpson function in the R package “vegan” (Oksanen et al., 2022). However, the statistical evaluation of alpha diversity was performed by the GLIMMIX procedure as described above. Correlations between the leukocyte distribution pattern, plasma urea and BHB concentrations and relative microbial abundances in rumen epithelium, nutrient intake, BW, and methane emissions were conducted by the CORR procedure of SAS software. Results were considered as statically significant at $P < 0.05$ and as tendencies at $0.05 \leq P < 0.10$. Data are presented as LSM ± SE, if not specified otherwise.

3. Results

3.1. Feed intake, animal characteristics, and respiratory measurements

Pre-weaning, 20%-MR calves consumed more than double the amount of MR than 10%-MR calves (2.0 vs. 0.9 kg DM/d; $P < 0.001$) (Table 2). However, the overall solid feed intake (SFI) was more than twice as high in 10%-MR as in 20%-MR calves (0.9 vs. 0.4 kg DM/d; $P < 0.001$) and was characterized by a thrice elevated consumption of starter pellets (0.6 vs. 0.2 kg/d; $P < 0.001$). Thus, the 20%-MR group ingested larger amounts of crude protein (49%, 511 vs. 344 g/d), crude fat (106%, 432 vs. 210 g/d), sugar (79%, 664 vs. 372 g/d), and metabolizable energy (56%, 39 vs. 25 MJ/d) ($P < 0.001$), whereas the 10%-MR group ingested greater amounts of crude fiber (60%, 144 vs. 90 g/d; $P = 0.011$) and starch (70%, 207 vs.

Table 2
Feed intake and nutrient intake by solid feed of calves fed 10% (10%-MR) or 20% (20%-MR) milk replacer of their body weight at 9 (pre-weaning) and 22 weeks of age (post-weaning) [1].

Item	Pre-weaning			Post-weaning			P-value[2]		
	10%-MR	20%-MR	P-value[3]	10%-MR	20%-MR	P-value[3]	MR	TIME	MR × TIME
Feed intake, kg DM/d									
SF[5]	0.9 ± 0.07[a]	0.4 ± 0.04[b]	<0.001	4.8 ± 0.22[bc]	5.4 ± 0.27[ac]	0.079	<0.001	<0.001	<0.001
MR[5]	0.9 ± 0.03[a]	2.0 ± 0.08[b]					<0.001		
Starter[4]	0.6 ± 0.05[a]	0.2 ± 0.05[b]					<0.001		
Hay[4]	0.3 ± 0.03	0.2 ± 0.04					0.420		
TMR[4]				4.8 ± 0.20	5.5 ± 0.31		0.080		
Nutrient intake by SF, g/d									
Crude ash[5]	62 ± 6.9[a]	26 ± 3.1[b]	0.002	258 ± 21.0[bc]	283 ± 25.3[ac]	0.461	0.023	<0.001	<0.001
Crude fat[5]	25 ± 3.0[a]	9 ± 1.2[b]	<0.001	150 ± 10.5[bc]	164 ± 12.5[ac]	0.425	0.003	<0.001	<0.001
Crude protein[5]	145 ± 17.5[a]	54 ± 7.2[b]	<0.001	775 ± 45.2[bc]	812 ± 51.8[ac]	0.608	0.005	<0.001	<0.001
Crude fiber[5]	143 ± 15.8[a]	87 ± 10.6[b]	0.010	757 ± 42.6[bc]	833 ± 51.4[ac]	0.268	0.096	<0.001	0.002
ADF[5]	171 ± 18.3[a]	99 ± 11.7[b]	0.005	904 ± 56.6[bc]	992 ± 68.1[ac]	0.334	0.075	<0.001	<0.001
aNDF[5]	275 ± 29.4[a]	154 ± 18.1[b]	0.004	1671 ± 83.1[bc]	1818 ± 98.9[ac]	0.277	0.041	<0.001	<0.001
Starch[5]	178 ± 25.1[a]	53 ± 8.1[b]	<0.001	1370 ± 92.4[bc]	1510 ± 111.5[ac]	0.353	<0.001	<0.001	<0.001
Sugar[4]	106 ± 7.9[a]	55 ± 8.6[b]	0.003	83 ± 10.5[bc]	72 ± 11.5[ac]	0.279	0.038	0.430	0.001
MEI[5], MJ/d	10 ± 1.1[a]	4 ± 0.5[b]	<0.001	55 ± 2.7[bc]	62 ± 3.3[ac]	0.134	0.006	<0.001	<0.001
Starch/crude fiber[5]	1.2 ± 0.13[a]	0.6 ± 0.08[b]	<0.001	1.8 ± 0.09[b]	1.8 ± 0.09[a]	0.955	<0.001	<0.001	<0.001
Starch/aNDF[5]	0.6 ± 0.05[a]	0.4 ± 0.03[b]	<0.001	0.8 ± 0.05[b]	0.8 ± 0.04[a]	0.998	<0.001	<0.001	0.001

SF = solid feed; MR = milk replacer; TMR = total mixed ration; ADF = acid detergent fiber; aNDF = neutral detergent fiber after amylase treatment; MEI = metabolizable energy intake.

[a-d] Different superscript letters within one row indicate $P < 0.05$ (Tukey slice test) showing the comparison between MR groups pre- and post-weaning, and between pre- and post-weaning for each MR group separately.

[1] Data are given as least squares means (LSM) ± standard error (SE).

[2] *P*-value from ANOVA analysis.

[3] *P*-value from Tukey slice test.

[4] Normal distributed pattern.

[5] Gamma distributed pattern. Least squares means were back-transformed to the original data scale by the ILINK function.

122 g/d; $P = 0.004$) (Table S1). Although 10%-MR calves ingested more nutrients by solid feed than 20%-MR calves, the ratio of starch to crude fiber intake as well as the ratio of starch to aNDF intake by solid feed was double as high in 10%-MR than 20%-MR calves (1.2 vs. 0.6; 0.6 vs. 0.4; $P < 0.001$) (Table 2). Body weight and BCS were 37% (97 vs. 71 kg) and 23% (2.7 vs. 2.2), respectively, higher in 20%-MR than in 10%-MR calves ($P < 0.001$) (Table S2) pre-weaning. Neither ADG nor ECR differed between groups. Rumen size was comparable among MR intensities, but if referring to the BW, it was 50% larger in 10%-MR than in 20%-MR calves. Respiratory measurements revealed that 10%-MR calves tended to release more than double the amount of methane compared to 20%-MR calves (23 vs. 10 L/d; $P = 0.084$) (Table S2). However, methane emissions normalized to SFI were not different between MR groups. Energy balance tended to be 2.5 times higher in 20%-MR than in 10%-MR calves (6.4 vs. 2.6 MJ/d; $P = 0.079$).

Except sugar intake, nutrient intake was greater in the post- compared to the pre-weaning period ($P < 0.001$; Table 2, Table S1). Post-weaning, 20%-MR calves tended to consume slightly more TMR than 10%-MR calves (5.5 vs. 4.8 kg DM/d; $P = 0.080$), but total nutrient and energy intakes were not different between groups (Table 2). Similar to the pre-weaning period, BW was greater (202 vs. 180 kg; $P = 0.040$) and rumen size normalized to BW smaller in 20%-MR compared to 10%-MR calves (0.23 vs. 0.26 cm/kg BW; $P = 0.030$; Table S2). Average daily gain was 50% higher in the 20%-MR than 10%-MR group (1.2 vs. 0.8 kg/d, $P = 0.029$), but was not accompanied by differences in ECR. The 20%-MR group emitted 18% more methane ($P = 0.027$), but methane emissions relative to SFI did not differ between groups (Table S2).

3.2. Ruminal epithelium-associated microbiota and short chain fatty acids

Evenness of rumen microbial community, as indicated by the inverse Simpson index, increased on average by 44% over time ($P = 0.026$), and this increase was more steep in 10%-MR (40% vs. 63%; $P = 0.045$) than in 20%-MR calves (52% vs. 69%; $P = 0.162$) (Fig. 1, Table S3). Taxonomic classification identified Bacteroidota, Firmicutes, Proteobacteria, and Spirochaetota to be the most abundant phyla in both groups, accounting for 82% of the whole sequence (Fig. S3). In the course of maturation, the relative abundance of Bacteroidota decreased from 44% to 35% ($P < 0.001$), causing a distinct shift in the ratio between Firmicutes and Bacteroidota from 0.61 to 0.89 ($P = 0.002$).

In the pre-weaning period, the phylum Verrucomicrobiota was more than twice as abundant in 20%-MR as compared to 10%-MR calves (2.3% vs. 0.8%; $P = 0.028$), whereas Synergistota was more prevalent in 10%-MR calves ($P = 0.020$) (Table 3). The *Methanosphaera* genus abundance was higher in 10%-MR compared to 20%-MR calves (0.054% vs. 0.029%; $P = 0.041$). Among differently abundant genera, only 4 genera accounted for more than 1% of the whole rumen microbial community. These 4 genera included the abundance of *F082*, *U29-B03*, and *Ruminococcus*, which were positively associated, and *Bacteroidales unclassified*, which was negatively associated with MR feeding intensity. Most significantly different abundances listed *Bacteroidales BS11 gut group*, *Syntrophococcus*, and *Bacteroidia unclassified* as 6 times more abundant in 10%-MR than in 20%-MR calves ($P < 0.001$). In contrast, *Prevotellaceae UCG-004* was found 6 times more abundant in 20%-MR than in 10%-MR calves ($P < 0.001$).

Post-weaning, the phyla Fusobacteriota and Elusimicrobiota were 5-fold ($P = 0.031$) and 2-fold ($P = 0.046$) more abundant in 20%-MR than in 10%-MR calves, respectively. Both *Anaerovorax* and *Succinivibrionaceae UCG-002* were the only genera with a relative abundance exceeding the 1% threshold, and both were negatively associated with MR consumption ($P = 0.009$ and $P = 0.015$, respectively). However, most significantly different abundances between MR groups listed *Bacilli unclassified*, *Lachnospiraceae NK4A136 group*, and *UCG-001*, all of them more abundant in 10%-MR, and unclassified genera of *Neisseriaceae* less abundant in 10%-MR than in 20%-MR calves ($P < 0.005$).

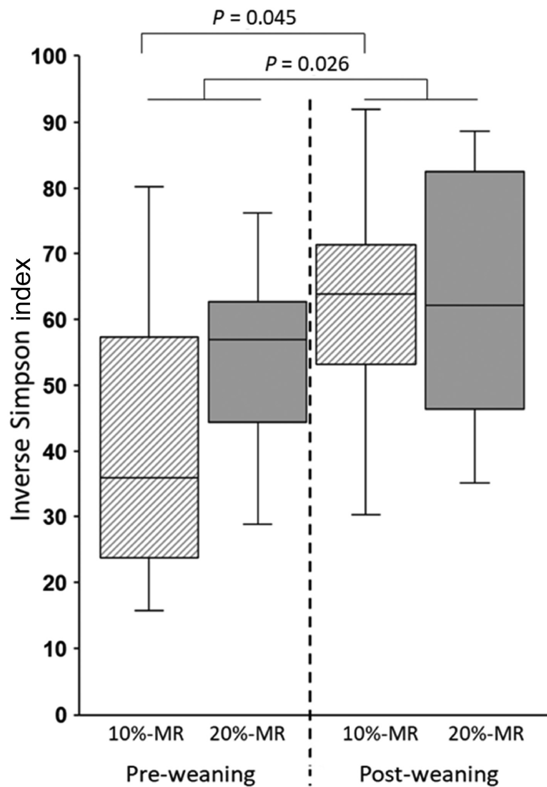

Fig. 1. Alpha diversity of rumen microbial community between calves fed 10% (10%-MR) or 20% (20%-MR) milk replacer of their respective body weight pre- and post-weaning (9 and 22 weeks of age). P-values display significant differences (P < 0.05) between pre- and post-weaning normal distributed alpha diversity of rumen microbial community independent of milk replacer feeding intensity (P = 0.026) and among 10%-MR (P = 0.045) and 20%-MR (P = 0.162) calves, respectively. MR = milk replacer.

Ruminal SCFA concentrations increased with growing feed intake and maturation, but the pattern and concentration of SCFA were not different among groups before weaning despite differences in nutrient intake (Table S4). However, post-weaning, n-valerate was slightly higher in 10%-MR than in 20%-MR calves (1.8 vs. 1.4 mM; P = 0.051).

3.3. Fecal pH and starch

The pH value of the feces and the fecal starch concentration did not differ between the MR groups (Table S5). Over time, fecal pH declined and fecal starch concentration increased from the pre- to the post-weaning period. However, this effect was mainly due to changes in the 20%-MR group showing a greater decrease in fecal pH (P = 0.004) and a greater increase in fecal starch concentrations (P = 0.011) over time.

3.4. Plasma metabolites and leukocyte distribution pattern

Pre-weaning, plasma urea (2.1 vs. 1.4 mM; P = 0.010) and NEFA (183 vs. 94 μM; P = 0.096) concentrations increased (Table 4), whereas the plasma BHB concentration decreased with higher MR feeding intensity (0.12 vs. 0.6 mM; P < 0.001). Post-weaning, plasma BHB was found 38% (P < 0.001), L-lactate 34% (P = 0.049), and glucose 10% (P = 0.058) higher in 10%-MR than in 20%-MR calves.

Flow cytometry revealed whole blood monocytes (P = 0.031), $CD8^+$ (P < 0.001), and $CD14^+$ cells (P = 0.044) being less abundant in 20%-MR than in 10%-MR calves before weaning (Table 4). The reduction in $CD8^+$ abundance with intensified MR feeding allowed for a tendency towards a greater ratio between $CD4^+$ and $CD8^+$ in 20%-MR than in 10%-MR calves (P = 0.055). However, the effect of dehorning on the $CD4^+$:$CD8^+$ ratio and $CD21^+$ abundance was stronger than the overall influence of the MR feeding intensity. Post-weaning, differences in plasma metabolites and leukocyte distribution pattern between MR groups largely converged, except for $CD8^+$, which was still more than 2.5 times more abundant in the 10%-MR than 20%-MR group (P < 0.001).

3.5. Correlations between leukocytes, epimural microbes, and metabolic traits

To identify the correlations between the leukocytes and the rumen epithelium-associated microbes, as well as between leukocytes, nutrient intakes, and methane emissions, correlation studies were performed (Fig. 2, Table S6). Pre-weaning, $CD8^+$ abundance correlated positively with the abundance of *Bacteroidetes BD2-2*, *Bacteroidia unclassified*, Chloroflexi, *P-251-o5*, *Ruminobacter* and *Ruminococcaceae unclassified*. Although $CD8^+$ correlated positively with methane emissions, there were no relationships between $CD8^+$ and the abundance of methanogens. While the abundance of $CD8^+$ correlated negatively, the $CD4^+$:$CD8^+$ ratio correlated positively with various parameters of nutrient and energy intake. In addition, positive correlations were found between the $CD4^+$:$CD8^+$ ratio and *Prevotellaceae NK3B31 group, Prevotellaceae UCG-004*, *Rikenellaceae unclassified*, *Ruminococcus* and some unclassified genera. Both $CD14^+$ and $CD21^+$ correlated negatively with NK4A214 but positively with the abundance of Elusimicrobiota or *Succinivibrionaceae unclassified*, respectively. Post-weaning, we found $CD8^+$ correlated negatively with methane emission and *Methanomassillicoccales unclassified, Bacteroidetes BD2-2* and *Neisseriaceae unclassified*, but positively with *Lachnospiraceae UCG-008*. Furthermore, $CD14^+$ cells were positively correlated with *Acetitomaculum*, *Agathobacter*, *Catenisphaera*, and *Desulfobulbus*, whereas $CD21^+$ cells revealed positive correlations with *Bacteria unclassified* and the *Bacteriodales RF16 group*.

We next examined the correlations between metabolic traits and the abundance of rumen epithelium-associated microbes (Fig. 3). Before weaning, plasma urea concentrations were inversely correlated with the abundance of Euryarchaeota, *Family XIII AD3011 group*, *Lachnospiraceae* and *Methanobrevibacter*. Plasma BHB concentrations were negatively correlated with the occurrence of *Bacteroidetes BD2-2*, *CAG 352* and *UCG-010*, but positively with *Lachnospiraceae, Oscillospiraceae unclassified*, and *Succinivibrionaceae UCG-001.* After weaning, only the positive correlation between plasma BHB and the negative correlation between plasma urea and Lachnospiraceae remained existent. However, plasma BHB concentrations were also positively correlated with *Clostridia UCG-014* and *Succinivibrionaceae unclassified*, but negatively with *U29-B03*.

4. Discussion

4.1. Feed intake, growth performance, and metabolism

Similar to previous findings (Chapman et al., 2017; Jasper and Weary, 2002; Quigley et al., 2018), 20%-MR calves in the current study consumed less solid feed, in particular less starter pellets, than 10%-MR calves pre-weaning. Lower SFI was associated with smaller rumen sizes relative to BW and lower plasma urea and BHB

Table 3
Relative abundances of phyla (p.) and genera (g.) in the rumen microbial community of calves fed 10% (10%-MR) or 20% (20%-MR) milk replacer of their body weight at 9 (pre-weaning) and 22 weeks of age (post-weaning)[1].

Item	Relative abundances[2], %		Log2 Fold change[3]	*P*-value[4]
	10%-MR	20%-MR		
Pre-weaning				
p. Verrucomicrobiota	**0.773**	**2.272**	−1.75	0.028
p. Synergistota	0.196	0.075	1.44	0.020
g. Bacteroidales BS11 gut group	0.262	0.003	6.92	<0.001
g. Prevotellaceae UCG-004	0.160	0.807	−2.54	<0.001
g. Syntrophococcus	0.135	0.021	2.38	<0.001
g. Bacteroidia unclass.	0.134	0.018	2.92	0.001
g. Lachnospiraceae UCG-010	0.249	0.036	2.35	0.003
g. Agathobacter	0.047	0.004	3.57	0.004
g. Mycoplasma	0.006	0.025	−2.24	0.005
g. F082	**2.096**	**5.138**	−1.67	0.009
g. U29-B03	**0.184**	**1.171**	−2.55	0.014
g. Selenomonadaceae unclass.	0.014	0.002	2.62	0.018
g. Ruminococcus	**2.037**	**3.790**	−1.00	0.019
g. Erysipelotrichales unclass.	0.021	0.004	2.38	0.019
g. Bacteroidales unclass.	**1.144**	**0.443**	1.22	0.019
g. Anaerolineae unclass.	0.029	0.023	−1.71	0.027
g. Mogibacterium	0.188	0.076	1.18	0.028
g. UCG-004	0.172	0.060	1.35	0.029
g. Roseburia	0.047	0.008	2.25	0.029
g. Rhodocyclaceae unclass.	0.996	0.036	4.76	0.031
g. Howardella	0.047	0.017	1.63	0.038
g. Methanosphaera	0.054	0.029	0.77	0.041
g. UCG-001	0.133	0.674	−2.50	0.044
g. Anaerovoracaceae	0.831	0.242	1.50	0.047
Post-weaning				
p. Fusobacteriota	0.006	0.046	−2.74	0.031
p. Elusimicrobiota	0.097	0.222	−1.35	0.046
g. Neisseriaceae unclass.	0.025	0.257	−3.30	<0.001
g. Bacilli unclass.	0.071	0.031	1.26	0.004
g. Lachnospiraceae NK4A136 group	0.291	0.081	2.09	0.005
g. UCG-001	0.166	0.030	2.55	0.005
g. Lachnospiraceae FCS020 group	0.062	0.022	1.65	0.007
g. Anaerovorax	**1.097**	**0.247**	2.16	0.009
g. vadinBE97	0.027	0.093	−1.71	0.010
g. Succinivibrionaceae UCG-002	**1.964**	**1.727**	−2.11	0.015
g. Succinivibrio	0.020	0.084	−2.04	0.017
g. CAG-352	0.373	0.976	−1.31	0.044
g. Endomicrobium	0.020	0.074	−1.90	0.044

[1] Only significantly different abundances of taxa between milk replacer (MR) groups are presented. Bold written numbers belong to microbial taxa accounting for more than 1% of rumen epimural microbial community in 10%-MR or 20%-MR calves.
[2] Given as group mean.
[3] Log2 Fold change derives from DESeq2 analysis describing the comparison between 10%-MR and 20%-MR groups.
[4] Displays significant differential relative abundances of phyla and genera between both MR groups.

concentrations. The latter has been suggested to account for reduced maturation of rumen epithelium (Deelen et al., 2016; Suarez-Mena et al., 2017). Relative to the 20%-MR group, 10%-MR calves were not able to ingest the same level of dietary energy, protein, and fat despite unlimited provision of starter pellets. The level of protein and energy intake is most critical in taping the full growth potential in dairy calves as emphasized previously (Chapman et al., 2017; Welboren et al., 2019). As a consequence, 10%-MR calves tended to be in a lower EB and had less BW and BCS than 20%-MR calves pre-weaning.

Post-weaning, 10%-MR calves were not able to fully compensate for the differences in BW, not until 22 weeks of age, as studied herein, and not by 34 weeks of age, as investigated by Liermann et al. (2023). One reason for this is the lower TMR intake, although the rumen size normalized to BW was still higher in 10%-MR than in 20%-MR calves. Interestingly, we found plasma BHB concentrations still higher in 10%-MR calves compared to their counterparts. Both rumen- and liver-derived BHB contribute to plasma BHB concentrations. However, in dairy calves older than 70 d of age, plasma BHB concentrations decline and their relevance as specific marker for rumen development and rumen-derived BHB is questionable (Schwarzkopf et al., 2019). The higher plasma BHB concentrations in 10%-MR calves in the present study may originate from hepatic metabolic processes yielding BHB, which acts as an energy carrier and cellular signaling compound. Accordingly, BHB has been shown to serve as a fuel during acute inflammations and to reduce appetite (Laeger et al., 2010). Therefore, differences in BHB concentrations may contribute to group differences in TMR intake observed in the present study. Besides, plasma L-lactate concentrations were higher in 10%-MR than in 20%-MR calves. Hepatic gluconeogenesis from lactate is two- to threefold greater in pre-weaned than in post-weaned calves and thus becomes less relevant for energy metabolism after weaning (Baldwin et al., 2004). However, under hypoxic conditions, L-lactate is increasingly produced by the skeletal muscle and indicates a lower efficiency in glucose utilization and cellular energy production in 10%-MR than 20%-MR calves. Thus, besides greater BHB concentrations, a lower glucose use efficiency as indicated by higher L-lactate concentrations might contribute to prevent BW compensation post-weaning. It has been reported that differences in the

Table 4
Plasma metabolite concentrations and flow cytometric leukocyte counts of calves fed 10% (10%-MR) or 20 % (20%-MR) milk replacer of their body weight at 8 (pre-weaning) and 21 weeks of age (post-weaning).[1].

Item	Pre-weaning			Post-weaning			*P*-value[2]			
	10%-MR	20%-MR	*P*-value[3]	10%-MR	20%-MR	*P*-value[3]	MR	TIME	HORN[4]	MR × TIME
Urea[5], mM	1.4 ± 0.14[a]	2.1 ± 0.16[b]	0.010	2.9 ± 0.28[b]	2.4 ± 0.30[ab]	0.233	0.764	0.001	0.601	0.016
Glucose[5], mM	7 ± 0.3	6 ± 0.3	0.497	6 ± 0.2	5 ± 0.2	0.058	0.087	0.018	0.087	0.667
NEFA[6], μM	94 ± 22.3[ab]	183 ± 47.8[a]	0.096	99 ± 9.8[ab]	91 ± 8.5[b]	0.572	0.172	0.118	0.661	0.080
Triglycerides[5], mM	0.3 ± 0.04[ac]	0.3 ± 0.04[ab]	0.499	0.2 ± 0.02[bd]	0.2 ± 0.02[cd]	0.647	0.706	<0.001	0.081	0.362
BHB[6], mM	0.1 ± 0.01[a]	0.1 ± 0.00[b]	<0.001	0.5 ± 0.02[b]	0.4 ± 0.02[a]	<0.001	<0.001	<0.001	0.356	0.007
L-Lactate[6], mM	0.7 ± 0.04	0.7 ± 0.05	0.413	0.9 ± 0.08	0.7 ± 0.07	0.049	0.075	0.422	0.833	0.115
Albumin[5], g/L	27 ± 0.8[ac]	26 ± 0.8[ab]	0.524	31 ± 0.5[bd]	31 ± 0.5[cd]	0.603	0.472	0.004	0.904	0.679
Leukocyte counts[7], %										
Granulocytes[5]	47 ± 7.5	60 ± 7.0	0.235	44 ± 4.2	50 ± 4.6	0.387	0.120	0.378	0.125	0.605
Monocytes[5]	12 ± 1.1[a]	9 ± 1.0[b]	0.031	11 ± 0.8[ab]	10 ± 0.9[ab]	0.749	0.061	0.891	0.472	0.090
Lymphocytes[5]	31 ± 6.2	27 ± 5.6	0.622	40 ± 4.5	34 ± 4.9	0.385	0.320	0.203	0.125	0.884
CD4^{+}[6]	9 ± 1.8	8 ± 1.7	0.854	12 ± 2.9	12 ± 3.2	0.968	0.909	0.234	0.237	0.900
CD8^{+}[6]	1.5 ± 0.22[a]	0.4 ± 0.07[b]	<0.001	0.8 ± 0.09[b]	0.3 ± 0.04[a]	<0.001	<0.001	<0.001	0.001	0.002
CD14^{+}[5]	11 ± 0.9[a]	9 ± 0.8[b]	0.044	9 ± 0.7[ab]	10 ± 0.8[ab]	0.737	0.222	0.524	0.223	0.035
CD21^{+}[5]	13 ± 2.9	12 ± 2.7	0.899	20 ± 3.2	17 ± 3.5	0.578	0.606	0.111	0.034	0.734
CD4^{+}:CD8^{+}[6]	8 ± 2.3	20 ± 5.3	0.055	17 ± 3.9	30 ± 10.4	0.212	0.017	0.134	0.003	0.632

NEFA = non-esterified fatty acids; BHB = β-hydroxybutyric acid; CD = cluster of differentiation.
[a-d] Different superscript letters within one row indicate $P < 0.05$ (Tukey slice test) showing the comparison between milk replacer (MR) groups pre- and post-weaning, and between pre- and post-weaning for each MR group separately.
[1] Data are given as least squares means (LSM) ± standard error (SE).
[2] *P*-value from ANOVA analysis.
[3] *P*-value from Tukey slice test.
[4] Dehorning process as fixed effect.
[5] Normal distributed pattern.
[6] Gamma distributed pattern. Least squares means were back-transformed to the original data scale by the ILINK function.
[7] $n = 7$ (10%-MR and 20%-MR groups post-weaning), $n = 6$ (20%-MR group pre-weaning), $n = 5$ (10%-MR group pre-weaning).

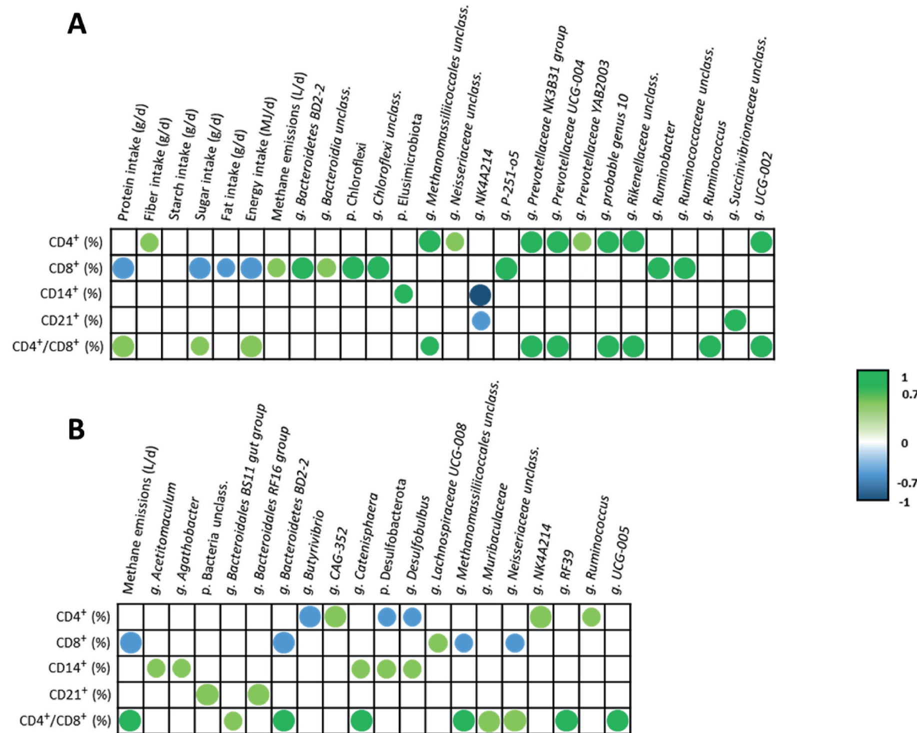

Fig. 2. Pearson correlations between the percentage of leukocytes, methane emission, nutrients intake, and rumen epimural microbiota abundances of calves fed 10% (10%-MR) or 20% (20%-MR) milk replacer of their body weight pre- (A) and post- (B) weaning. Taxa abundances were considered if the mean relative abundance of at least one milk replacer (MR) group was representing more than 0.1% of the microbial community. All correlations shown were statically significant ($P < 0.05$) with strong correlations ($P < 0.025$) indicated by large circles and weaker correlations ($0.025 < P < 0.05$) by weaker circles. The colored scale indicates a positive (closer to 1, green) or negative (closer to −1, blue) correlation.

metabolic profile and energy expenditure of dairy calves fed ad libitum or restrictively before weaning persist until first lactation (Kenez et al., 2018; Leal et al., 2021). Conversely, Kesser et al. (2017) did not find any differences in BHB plasma concentrations or glucose metabolism between first lactating dairy cows fed restrictively or ad libitum MR during the rearing period. Therefore, further research is demanded to address the long-term effects of MR feeding intensity on energy metabolism of ruminants later in life.

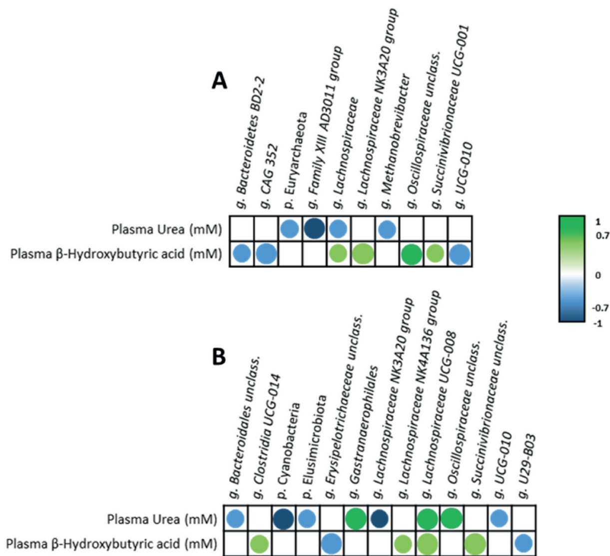

Fig. 3. Pearson correlations between plasma urea and β-hydroxybutyric acid concentrations and rumen epimural microbiota abundances of calves fed 10% (10%-MR) or 20% (20%-MR) milk replacer of their body weight pre- (A) and post- (B) weaning. Taxa abundances were considered if the mean relative abundance of at least one milk replacer (MR) group was representing more than 0.1% of the total microbial community. All correlations shown were statically significant ($P < 0.05$) with strong correlations ($P < 0.025$) indicated by large circles and weaker correlations ($0.025 < P < 0.05$) by weaker circles. The colored scale indicates a positive (closer to 1, green) or negative (closer to −1, blue) correlation.

4.2. Epimural microbiome and its correlations with plasma metabolites

Microbes adherent to the rumen epithelium make up only a small portion of whole rumen microbes (1% to 2%) (Carballo, 2019). However, these microbes play an essential role in microbiome-host communication and rumen maturation (Chai et al., 2021; Filipe, 2018). Although relative rumen size was greater in 10%-MR calves, the alpha diversity of the rumen-epithelium associated microbiome did not differ between feeding groups. By contrast, the microbial diversity of the rumen fluid increased with higher MR feeding level in lambs (Huang et al., 2023). These results suggest that the epimural microbiome develops differently from the rumen fluid microbiome and the epimural microbial diversity does not play a dominant role in rumen maturation. However, the increase in microbial alpha diversity from the pre- to the post-weaning period was steeper in 10%-MR compared to 20%-MR calves, likely due to the lower microbial diversity in 10%-MR calves ingesting more starter pre-weaning. It has been shown earlier that the microbial diversity and richness in rumen fluid decrease with increasing concentrate level in dairy cows (Khafipour et al., 2016) and in MR fed lambs (Huang et al., 2023). The decrease in microbial diversity and richness is associated with a reduction in microbial stability and detrimental effects on rumen health (Khafipour et al., 2016).

The Verrucomicrobiota phylum and the *Ruminococcus* genus belong to the major hemicellulose and cellulose degraders within the rumen microbial community (Gharechahi et al., 2021; Yi et al., 2022). The abundance of both ruminal Verrucomicrobiota and *Ruminococcus* increase with the declining concentrate-to-forage ratio in feed rations fed to yaks and cattle (Carballo, 2019; Wang et al., 2020). The abundances of Verrucomicrobiota and *Ruminococcus*, accounting for a large proportion among differently abundant microbes between MR groups pre-weaning, were higher in 20%-MR calves, likely because this group revealed a lower starch/crude fiber intake ratio. Interestingly, members of the Ruminococcaceae family have been assigned an important role in rumen maturation and the establishment of host immunity in lambs (Yin et al., 2023). The abundance of the *F082* genus, which is associated with starch degradation and propionate production (Jize et al., 2022; Ma et al., 2020), was also higher in the 20%-MR group, suggesting that other microbes play a more dominant role in starch degradation in the 10%-MR group. The Synergistota phylum and the genera *Bacteroidales BS11 gut group*, *Roseburia, Syntrophococcus, Erysipelotrichales unclassified,* and Anaerovoracaceae are involved in the degradation of polysaccharides but not clearly allocated to an amylolytic or saccharolytic degradation pattern (Liu et al., 2017; Ren et al., 2019; Solden et al., 2017; Wang et al., 2022). The latter phylum and genera abundances were greater in 10%-MR calves, suggesting that these microbes play a dominant role in the starch degradation in these animals. This conclusion corresponds to the finding by Wang et al. (2022), who found Synergistota more abundant in the rumen content of yak calves supplemented with starter pellets than without starter pellets. Similarly, Zhang et al. (2018) found the abundance of *Roseburia* in the feces of Holstein heifers increases with elevation in concentrate level. Liu et al. (2017) reported that ruminal *Syntrophococcus* was more abundant in lambs receiving MR and starter pellets than in lambs receiving MR solely. Finally, Carballo (2019) and Mao et al. (2012) reported ruminal Erysipelotrichales more abundant in concentrate versus forage fed calves. Taken together, a high MR feeding level seem to increase the rumen microbial alpha diversity and the relative abundance of cellulolytic bacteria, while it decreased the relative abundance of many amylolytic bacteria. These differences between MR feeding intensities were found no matter if these genera are attached to the rumen epithelium, as in the present study, or occur in rumen fluid (Huang et al., 2023).

The greater SFI resulted in tending higher methane emissions in 10%-MR compared to 20%-MR calves. Higher methane production was accompanied with greater abundances of rumen epithelium associated *Methanosphaera.* This result contrasts the finding of an earlier study reporting an increase in fecal *Methanosphaera* with reduced concentrate intake (Zhang et al., 2018). However, *Methanosphaera* produce methane from methanol, which is a degradation product of pectin-rich feedstuffs such as soybeans, dried beet pulp, and apples with peel (Bailoni et al., 2003; Söllinger et al., 2018), but was also present in starter pellets. Thus, an elevated intake of starter pellets may result in a greater abundance of *Methanosphaera* in 10%-MR calves. However, one limitation of the present study is that we only studied the epimural microbial composition despite the majority of methane is produced by methanogens residing in rumen fluid. Although a first study analyzed the relationship between luminal and epimural microbial composition (Mao et al., 2015), we can only speculate if our present findings about the epimural microbial composition can be transferred to the situation in the lumen. Recently, epimural *Methanobrevibacter* were found in significant DNA abundances, but their methanogenic activity was limited (Tan et al., 2021). On the other hand, Pacífico et al. (2021) postulated that the contribution of epimural methanogens to the overall methane production has been underestimated so far. Therefore, the contribution of the epimural methanogens to the overall methane production deserves future research. Interestingly, Euryarchaeota including the *Methanobrevibacter* genera correlated inversely with plasma urea concentrations. It is conceivable that high plasma urea concentrations increase ammonia production at the ruminal epithelium and thereby reduce epimural methanogens. It has been shown that high ammonia concentrations diminish the abundance of methanogens and alter the microbial composition in vitro (Shen et al., 2023). Shen et al. (2023) reported that ammonia concentrations correlated

negatively with Lachnospiraceae, and this finding corresponds to the inverse correlation between plasma urea concentrations and Lachnospiraceae genera. Furthermore, we found plasma BHB concentrations directly correlated with Lachnospiraceae, a butyrate producing family. Ruminal butyrate is oxidized in the rumen wall forming BHB (Deelen et al., 2016; Suarez-Mena et al., 2017), indicating a direct link between post-absorptive metabolism and epimural microbes.

Post-weaning, the number of microbial differences declined between MR groups, likely because animals ingested comparable amounts of nutrients from the same feed, at least for 5 weeks before sampling. However, Lachnospiraceae was more abundant in 10%-MR calves still after weaning. Interestingly, the higher Lachnospiraceae abundance was accompanied with higher plasma BHB concentrations in 10%-MR calves, again underscoring their strong relationship described above.

4.3. Leukocyte distribution pattern and correlations with the epimural microbiome

Monocytes and associated $CD14^{+}$ cells have a central role in bovine innate immunity with effector function during inflammatory processes and in detecting bacterial lipopolysaccharides (LPS) (Hussen and Schuberth, 2017). Subacute acidic conditions in the lumen of the digestive tract may lead to lysis of gram negative bacteria causing a dramatic increase in LPS concentration in dairy cows (Khafipour et al., 2009; Monteiro and Faciola, 2020; Stefanska et al., 2018). Furthermore, acidic conditions damage the rumen epithelium integrity, favoring the translocation of pathogenic substances, such as LPS, into the blood stream and finally whole body inflammation (Monteiro and Faciola, 2020). These LPS-induced inflammatory processes have been proven by increased blood concentrations of serum amyloid A as inflammatory marker, LPS-binding proteins, and $CD14^{+}$ in dairy cows (Monteiro and Faciola, 2020; Stefanska et al., 2018). In dairy calves, similar responses have been shown in monocytes and monocyte-derived macrophages after LPS stimulation (Guo et al., 2009). Moreover, Alimirzaei et al. (2020) found that fecal *Escherichia coli* producing endotoxins were enriched in 10%-MR compared with 20%-MR calves. Interestingly, despite group differences in starch intake, our experimental groups did not differ in rumen SCFA, rumen pH, fecal pH, and fecal starch excretion. The latter indicates the level of starch hydrolysis and hindgut acidosis in dairy calves (Eckert et al., 2015; Steele et al., 2016). According to McCurdy et al. (2019), the lack of differences in rumen pH is attributable to buffering effects of hay consumption. However, since the pre-weaning rumen is not fully developed, the lack of differences in rumen SCFA pattern may indicate an incomplete ruminal starch fermentation. The amount of starch entering the small intestine account for up to 60% of starch intake (Theurer, 1986). Approximately 61% (Moharrery et al., 2014) of this entering starch is digested in the small intestine by fermentation (43%) and enzymatic hydrolysis (18%) (Gilbert et al., 2015). Thus, increasing amounts of starch may cause higher concentrations of SCFA in the small intestine and local acidotic induced inflammatory processes (Bissell and Hall, 2010). Taken together, increased abundances of monocytes and $CD14^{+}$ in 10%-MR calves, who had a greater starch-fiber intake ratio, might be explained by acidotic induced epithelium lesions in the gastrointestinal tract and associated inflammatory responses to LPS or other endotoxins pre-weaning.

A sufficient energy supply by MR feeding is most crucial for the development of the immune function (Khan et al., 2011). A non-sufficient energy supply may cause inflammatory responses as characterized by increased cytokine and acute phase protein concentrations in dairy calves (O'Loughlin et al., 2012; Reuter, 2007; Schäff et al., 2016). Nonnecke et al. (2003) reported a more than 2-fold elevated interferon-γ synthesis by mononuclear leukocytes in dairy calves fed a low plane of energy and MR (1.4% vs. 2.5% of BW of DM per day). The cytokine interferon-γ can be produced by CD8 T cells to exhibit immunomodulatory functions and to promote immune cell migration to the site of inflammation (Bhat et al., 2017). In our study, we found $CD8^{+}$ cells more abundant in 10%-MR than in 20%-MR calves. Moreover, $CD8^{+}$ cells negatively correlated with energy and protein intake suggesting an increased inflammatory processes due to a non-sufficient energy supply of pre-weaned 10%-MR calves.

Post-weaning, differences in the proportion of monocytes and $CD14^{+}$ among MR groups canceled out, because the abundances of monocytes and $CD14^{+}$ decreased in 10%-MR calves but increased in 20%-MR calves from the pre- to post-weaning period. These results might be explained by comparable energy intakes post-weaning. However, since the increase in fecal starch concentration and decrease in fecal pH from pre- to post-weaning was more steep in 20%-MR than in 10%-MR calves, one might assume a lower ruminal and intestinal starch digestion but stronger acidic conditions in the hindgut of 20%-MR calves post-weaning. The latter may have accounted for higher monocyte and $CD14^{+}$ portions in these animals. Nevertheless, $CD8^{+}$ was still more abundant in 10%-MR than in 20%-MR calves, indicating long-term effects of a non-sufficient energy supply on the immune status of 10%-MR calves.

A couple of correlations found between the abundance of leukocytes and microbial phyla and genera suggest an interplay between host immune functioning and commensal colonization (Taschuk and Griebel, 2012). The correlation between the $CD4^{+}$:$CD8^{+}$ ratio and *Methanomassillicoccales* pre-weaning and the indirect correlation between $CD8^{+}$ or methane emission with *Methanomassillicoccales* post-weaning indicates that a high level of inflammation is accompanied with a low level of epimural methanogenesis (Söllinger et al., 2018). This finding adds to a recent study by Meese et al. (2020), who reported that cows emitting low levels of methane have a lower immune response and thus a greater disease susceptibility. On the other hand, we found CD21 B lymphocytes positively correlated with *Succinivibrionaceae unclassified* pre-weaning. This genus was shown to compete with methanogens for hydrogen utilization via succinate formation (McCabe et al., 2015), suggesting that not only *Succinivibrionaceae* but also CD21 cells may affect methane production. However, we found no relationship between CD21 B lymphocytes and methane emission or epimural methanogens. These results contrast an earlier study reporting that the activation of B lymphocytes by immunization with methanogens reduces ruminal archaeal population and methane production in sheep (Williams et al., 2009; Wright et al., 2004). We further found correlations between the abundances of *Ruminococcaceae unclassified* and *Ruminobacter* with the blood $CD8^{+}$ portion pre-weaning and Lachnospiraceae UCG-008 *and* blood $CD8^{+}$ portion post-weaning. By contrast, Yin et al. (2023) reported an inverse relationship between various fecal Ruminococcaceae or *Lachnospiraceae* families with the serum concentration of the pro-inflammatory cytokines, such as interleukine (IL)-1β, -6 and -12 and tumor necrosis factor-alpha (TNFα) in lambs. Due to these inconsistencies, future studies are needed to further elucidate the influence of early-life nutritional interventions on the interactions between rumen epithelium-associated microbes and the immune system of calves.

5. Conclusion

A reduction in MR feeding intensity elevated starter consumption in dairy calves and facilitated rumen size development and the enrichment of a more amylolytic and less cellulolytic pronounced

rumen microbial community. Differences in the leukocyte distribution pattern among MR groups suggest the occurrence of inflammatory processes in 10%-MR calves compared with 20%-MR calves. These inflammatory processes can be ascribed to differences in energy intake and starch digestion between groups. Correlations between rumen methanogens, Ruminococcaceae, Lachnospiraceae and $CD8^{+}$ cells in blood were found, but these were not consistent throughout maturation. Post-weaning, previous differences in epimural microbial composition and SFI between MR groups converged, while differences in BW and relative abundance of $CD8^{+}$ cells in blood were still observed. It can be concluded that a reduced MR feeding intensity in dairy calves seems to have long-term negative effects on animal health and growth performance. However, the higher MR feeding intensity requires a more gradual weaning process to ensure a sufficient rumen maturation. Further experimental investigations are required addressing the interplay between microbial colonization and immunity considering potential effects of early-life nutritional interventions in dairy calves.

Credit Author Statement

Carolin B.M. Müller developed the statistical models, performed statistical analysis, interpreted and visualized the data, and wrote the manuscript; **Lisa-Maria Tümmler** conducted the animal experiment and provided medical care; **Henry Reyer** performed 16S rRNA sequencing and supported the development of statistical models; **Torsten Viergutz** performed fluorescence activated cell sorting; **Björn Kuhla** conceptualized and supervised the animal experiment, interpreted the data, and wrote the manuscript. All authors contributed to the article and approved the final manuscript.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgments

We gratefully thank H. Brandt, C. Arlt, A. Schwirk-Kulack, R. Fürstenberg, V. Röttgen, A. Vernunft, M. Derno, and the staff at the “Tiertechnikum” and the Experimental Animal Facility Cattle at the FBN, Dummerstorf, Germany, for assistance in respiration chamber measurements, rumen cannula manufacturing and surgeries, sonographic measurements, animal care and handling, sample collection and preparation. This project was conducted in frame of the FACCE-JPI program and received funding from the German Federal Ministry of Food and Agriculture (BMEL) through the Federal Office for Agriculture and Food (BLE; Bonn, Germany), grant number 2814ERA04A. The work related to the epimural microbiome analysis was financially supported the FBN seed funding program.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.09.001.

References

Alimirzaei M, Alijoo YA, Dehghan-Banadaky M, Eslamizad M. The effects of feeding high or low milk levels in early life on growth performance, fecal microbial count and metabolic and inflammatory status of holstein female calves. Animal 2020;14:303–11.

Bailoni L, Bonsembiante M, Schiavon S, Pagnin G, Tagliapietra F. Estimation of the content of pectins in feeds: fractional extraction and quantitative determination. Vet Res Commun 2003;27:249–51.

Baldwin RL, Mcleod KR, Klotz JL, Heitmann RN. Rumen development, intestinal growth and hepatic metabolism in the pre- and postweaning ruminant. J Dairy Sci 2004;87:E55–65.

Bhat P, Leggatt G, Waterhouse N, Frazer IH. Interferon-gamma derived from cytotoxic lymphocytes directly enhances their motility and cytotoxicity. Cell Death Dis 2017;8.

Bissell HA, Hall MB. Cattle differ in ability to adapt to small intestinal digestion of starch. J Dairy Sci 2010;93:845.

Borderas TF, De Passille AMB, Rushen J. Feeding behavior of calves fed small or large amounts of milk. J Dairy Sci 2009;92:2843–52.

Braun U, Kruger S, Hassig M. Ultrasonographic examination of the reticulum, rumen, omasum and abomasum during the first 100 days of life in calves. Res Vet Sci 2013;95:326–33.

Brouwer E. Report of sub-committee on constants and factors. Energy metabolism of farm animals. London: Academic Press; 1965.

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R. Global patterns of 16s rrna diversity at a depth of millions of sequences per sample. P. Natl. Acad. Sci. USA 2011;108:4516–22.

Carballo OC. The effect of early life nutrition on rumen microbial community development and impact on lifetime performance in ruminants. Massey University; 2019.

Chai JM, Lv XK, Diao QY, Usdrowski H, Zhuang YM, Huang WQ, Cui K, Zhang NF. Solid diet manipulates rumen epithelial microbiota and its interactions with host transcriptomic in young ruminants. Environ Microbiol 2021;23: 6557–68.

Chapman CE, Hill TM, Elder DR, Erickson PS. Nitrogen utilization, preweaning nutrient digestibility, and growth effects of holstein dairy calves fed 2 amounts of a moderately high protein or conventional milk replacer. J Dairy Sci 2017;100:279–92.

Deelen SM, Leslie KE, Steele MA, Eckert E, Brown HE, Devries TJ. Validation of a calf-side beta-hydroxybutyrate test and its utility for estimation of starter intake in dairy calves around weaning. J Dairy Sci 2016;99:7624–33.

Derno M, Elsner HG, Paetow EA, Scholze H, Schweigel M. Technical note: a new facility for continuous respiration measurements in lactating cows. J Dairy Sci 2009;92:2804–8.

Eckert E, Brown HE, Leslie KE, Devries TJ, Steele MA. Weaning age affects growth, feed intake, gastrointestinal development, and behavior in holstein calves fed an elevated plane of nutrition during the preweaning stage. J Dairy Sci 2015;98: 6315–26.

Enriquez D, Hotzel MJ, Ungerfeld R. Minimising the stress of weaning of beef calves: a review. Acta Vet Scand 2011;53.

Filipe J. Immune response in dairy cow forestomachs. [PhD]. University of Milan; 2018.

Garcia M, Bradford BJ, Nagaraja TG. Invited review: ruminal microbes, microbial products, and systemic inflammation1, 21presented as a part of the arpas symposium: understanding inflammation and inflammatory biomarkers to improve animal performance at the adsa–asas joint annual meeting, salt lake city, Utah, July 2016. Funding was provided by the arpas foundation.2 contribution no. 17-366-j from the Kansas agricultural experiment station. Prof Anim Sci 2017;33:635–50.

Geiger AJ, Parsons CLM, James RE, Akers RM. Growth, intake, and health of holstein heifer calves fed an enhanced preweaning diet with or without postweaning exogenous estrogen. J Dairy Sci 2016;99:3995–4004.

Gharechahi J, Vahidi MF, Bahram M, Han JL, Ding XZ, Salekdeh GH. Metagenomic analysis reveals a dynamic microbiome with diversified adaptive functions to utilize high lignocellulosic forages in the cattle rumen. ISME J 2021;15:1108–20.

Gilbert MS, Pantophlet AJ, Berends H, Pluschke AM, Van Den Borne JJGC, Hendriks WH, Schols HA, Gerrits WJJ. Fermentation in the small intestine contributes substantially to intestinal starch disappearance in calves. J Nutr 2015;145:1147–55.

Guo YJ, Zhao GQ, Tanaka S, Yamaguchi T. Differential responses between monocytes and monocyte-derived macrophages for lipopolysaccharide stimulation of calves. Cell Mol Immunol 2009;6:223–9.

Hammon HM, Frieten D, Gerbert C, Koch C, Dusel G, Weikard R, Kuhn C. Different milk diets have substantial effects on the jejunal mucosal immune system of pre-weaning calves, as demonstrated by whole transcriptome sequencing. Sci. Rep.-Uk 2018;8.

Hammon HM, Liermann W, Frieten D, Koch C. Review: importance of colostrum supply and milk feeding intensity on gastrointestinal and systemic development in calves. Animal 2020;14:S133–43.

Huang YL, Wang GX, Zhang Q, Chen ZY, Li C, Wang WM, Zhang XX, Wang XJ, Zhang DY, Cui PP, Ma ZW. Effects of milk replacer feeding level on growth performance, rumen development and the ruminal bacterial community in lambs. Front Microbiol 2023;13.

Hugerth LW, Wefer HA, Lundin S, Jakobsson HE, Lindberg M, Rodin S, Engstrand L, Andersson AF. Degeprime, a program for degenerate primer design for broad-taxonomic-range pcr in microbial ecology studies. Appl Environ Microbiol 2014;80:5116–23.

Hussen J, Schuberth HJ. Heterogeneity of bovine peripheral blood monocytes. Front Immunol 2017;8.

Izuddin WI, Loh TC, Foo HL, Samsudin AA, Humam AM, Postbiotic l. Plantarum rg14 improves ruminal epithelium growth, immune status and upregulates the intestinal barrier function in post-weaning lambs. Sci Rep 2019;9.
Jasper J, Weary DM. Effects of ad libitum milk intake on dairy calves. J Dairy Sci 2002;85:3054–8.
Jentsch W, Beyer M, Derno M, Franz H. Untersuchungen zum energie- und stickstoffumsatz von kälbern bei unterschiedlichen umgebungstemperaturen. Arch Tierz 1996;103:571–87.
Jize Z, Zhuoga D, Xiaoqing Z, Na T, Jiacuo G, Cuicheng L, Bandan P. Different feeding strategies can affect growth performance and rumen functions in gangba sheep as revealed by integrated transcriptome and microbiome analyses. Front Microbiol 2022;13.
Kenez A, Koch C, Korst M, Kesser J, Eder K, Sauerwein H, Huber K. Different milk feeding intensities during the first 4 weeks of rearing dairy calves: Part 3: plasma metabolomics analysis reveals long-term metabolic imprinting in holstein heifers. J Dairy Sci 2018;101:8446–60.
Kesser J, Korst M, Koch C, Romberg FJ, Rehage J, Muller U, Schmicke M, Eder K, Hammon HM, Sadri H, Sauerwein H. Different milk feeding intensities during the first 4 weeks of rearing dairy calves: Part 2: effects on the metabolic and endocrine status during calfhood and around the first lactation (vol 100, pg 3109, 2017). J Dairy Sci 2017;100:4195-.
Khafipour E, Krause DO, Plaizier JC. A grain-based subacute ruminal acidosis challenge causes translocation of lipopolysaccharide and triggers inflammation. J Dairy Sci 2009;92:1060–70.
Khafipour E, Li S, Tun HM, Derakhshani H, Moossavi S, Plaizier JC. Effects of grain feeding on microbiota in the digestive tract of cattle. Anim. Front. 2016;6:13–9.
Khan MA, Weary DM. Von Keyserlingk MaG. Invited review: effects of milk ration on solid feed intake, weaning, and performance in dairy heifers. J Dairy Sci 2011;94:1071–81.
Laeger T, Metges CC, Kuhla B. Role of beta-hydroxybutyric acid in the central regulation of energy balance. Appetite 2010;54:450–5.
Lamp O, Reyer H, Otten W, Nurnberg G, Derno M, Wimmers K, Metges CC, Kuhla B. Intravenous lipid infusion affects dry matter intake, methane yield, and rumen bacteria structure in late-lactating holstein cows. J Dairy Sci 2018;101:6032–46.
Leal LN, Doelman J, Keppler BR, Steele MA, Martin-Tereso J. Preweaning nutrient supply alters serum metabolomics profiles related to protein and energy metabolism and hepatic function in holstein heifer calves. J Dairy Sci 2021;104:7711–24.
Li KA, Shi BL, Na RH. The colonization of rumen microbiota and intervention in pre-weaned ruminants. Animals 2023;13.
Liermann W, Tümmler L-M, Kuhla B, Viergutz T, Hammon HM. Effects of rumen cannulation combined with different pre-weaning feeding intensities on the intestinal, splenic and thymic immune system in heifer calves several month after surgery. Front Immunol 2023;14.
Liu J, Bian G, Sun D, Zhu W, Mao S. Starter feeding altered ruminal epithelial bacterial communities and some key immune-related genes' expression before weaning in lambs. J Anim Sci 2017;95:910–21.
Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for rna-seq data with deseq2. Genome Biol 2014;15.
Luo T, Li YT, Zhang WY, Liu JX, Shi HB. Rumen and fecal microbiota profiles associated with immunity of young and adult goats. Front Immunol 2022;13.
Ma T, Wu W, Tu Y, Zhang N, Diao Q. Resveratrol affects in vitro rumen fermentation, methane production and prokaryotic community composition in a time- and diet-specific manner. Microb Biotechnol 2020;13:1118–31.
Malmuthuge N, Liang GX, Guan LL. Regulation of rumen development in neonatal ruminants through microbial metagenomes and host transcriptomes. Genome Biol 2019;20.
Mao SY, Zhang ML, Liu JH, Zhu WY. Characterising the bacterial microbiota across the gastrointestinal tracts of dairy cattle: membership and potential function. Sci Rep 2015;5.
Mao SY, Zhang RY, Wang DS, Zhu WY. The diversity of the fecal bacterial community and its relationship with the concentration of volatile fatty acids in the feces during subacute rumen acidosis in dairy cows. BMC Vet Res 2012;8.
Mccabe MS, Cormican P, Keogh K, O'connor A, O'hara E, Palladino RA, Kenny DA, Waters SM. Illumina miseq phylogenetic amplicon sequencing shows a large reduction of an uncharacterised succinivibrionaceae and an increase of the methanobrevibacter gottschalkii clade in feed restricted cattle. PLoS One 2015;10.
Mccurdy DE, Wilkins KR, Hiltz RL, Moreland S, Klanderman K, Laarman AH. Effects of supplemental butyrate and weaning on rumen fermentation in holstein calves. J Dairy Sci 2019;102:8874–82.
Meese S, Ulbrich SE, Bollwein H, Bruckmaier R, Wellnitz O, Kreuzer M, Rontgen M, Gimsa U, Schwarm A. Methane emission, metabolism, and performance of holstein dairy cows with low, medium, and high lymphocyte proliferation during transition. J Dairy Sci 2020;103:4367–77.
Minuti A, Palladino A, Khan MJ, Alqarni S, Agrawal A, Piccioli-Capelli F, Hidalgo F, Cardoso FC, Trevisi E, Loor JJ. Abundance of ruminal bacteria, epithelial gene expression, and systemic biomarkers of metabolism and inflammation are altered during the peripartal period in dairy cows. J Dairy Sci 2015;98:8940–51.
Moharrery A, Larsen M, Weisbjerg MR. Starch digestion in the rumen, small intestine, and hind gut of dairy cows - a meta-analysis. Anim Feed Sci Technol 2014;192:1–14.
Monteiro HF, Faciola AP. Ruminal acidosis, bacterial changes, and lipopolysaccharides. J Anim Sci 2020;98.
Na SW, Guan LL. Understanding the role of rumen epithelial host-microbe interactions in cattle feed efficiency. Anim. Nutr. 2022;10:41–53.
Nonnecke BJ, Foote MR, Smith JM, Pesch BA, Van Amburgh ME. Composition and functional capacity of blood mononuclear leukocyte populations from neonatal calves on standard and intensified milk replacer diets. J Dairy Sci 2003;86: 3592–604.
GfE. (gesellschaft für ernährungsphysiologie; society of nutrition physiology) recommended energy and nutrient supply for dairy cows and growing cattle. 8 ed. Frankfurt am Main, Germany: DLG-Verlag; 2001.
Nrc. (National research council) nutrient requirements of dairy cattle. Washington DC. 2001. United States.
O'loughlin A, Lynn DJ, Mcgee M, Doyle S, Mccabe M, Earley B. Transcriptomic analysis of the stress response to weaning at housing in bovine leukocytes using rna-seq technology. BMC Genom 2012;13.
Oksanen J, Simpson GL, Guillaume Blanchet F, Kindt R, Legendre P, Minchin PR, O'hara RB, Solymos P, Henry M, Stevens H, Szoecs E, Wagner H, Barbour M, Bedward M, Bolker B, Borcard D, Carvalho G, Chirico M, De Caceres M, Durand S, Evangelista HBA, Fitzjohn R, Friendly M, Fureaux B, Hannigan G, Hill MO, Lahti L, Mgclinn D, Ouellette M-H, Ribeiro Cunha E, Smith T, Stier A, Ter Braak CJF, Weedon J. Vegan: community ecology package. R package version 2.6-2. 2022. https://CRAN.R-project.org/package=vegan.
Pacífico C, Petri RM, Ricci S, Mickdam E, Wetzels SU, Neubauer V, Zebeli Q. Unveiling the bovine epimural microbiota composition and putative function. Microorganisms 2021;9.
Palma-Hidalgo JM, Belanche A, Jiménez E, Newbold CJ, Denman SE, Yáñez-Ruiz DR. Multi-omics in vitro study of the salivary modulation of the goat rumen microbiome. Animal 2023;17.
Petryshak M, Seniv R, Seniv T, Zukow W. Relationships between immune parameters of saliva and blood. Phys Educ Sport Pedagog 2020;6:84–98.
Qadis AQ, Goya S, Yatsu M, Kimura A, Ichijo T, Sato S. Immune-stimulatory effects of a bacteria-based probiotic on peripheral leukocyte subpopulations and cytokine mrna expression levels in scouring holstein calves. J Vet Med Sci 2014a;76:677–84.
Qadis AQ, Goya S, Yatsu M, Yoshida YU, Ichijo T, Sato S. Effects of a bacteria-based probiotic on subpopulations of peripheral leukocytes and their cytokine mrna expression in calves. J Vet Med Sci 2014b;76:189–95.
Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glockner FO. The silva ribosomal rna gene database project: improved data processing and web-based tools. Nucleic Acids Res 2013;41:D590–6.
Quigley JD, Hill TM, Dennis TS, Suarez-Mena FX, Schlotterbeck RL. Effects of feeding milk replacer at 2 rates with pelleted, low-starch or texturized, high-starch starters on calf performance and digestion. J Dairy Sci 2018;101:5937–48.
Raschke K. Erstellung eines schemas zur beurteilung der körperkondition von kälbern der rassen “deutsches fleckvieh” und “holstein friesian” (development of a protocol to assess the body condition of german simmental and holstein calves). Faculty of Veterinary Medicine Ludwig Maximillian Univ.; 2007.
Ren ZH, Yao RJ, Liu Q, Deng YT, Shen LH, Deng HD, Zuo ZC, Wang Y, Deng JL, Cui HM, Hu YC, Ma XP, Fang J. Effects of antibacterial peptides on rumen fermentation function and rumen microorganisms in goats. PLoS One 2019;14.
Reuter RR. Nutritional modulation of the immune system of beef cattle. Texas Tech University; 2007.
Rohart F, Gautier B, Singh A, Le Cao KA. Mixomics: an r package for 'omics feature selection and multiple data integration. PLoS Comput Biol 2017;13.
Schäff CT, Gruse J, Maciej J, Mielenz M, Wirthgen E, Hoeflich A, Schmicke M, Pfuhl R, Jawor P, Stefaniak T, Hammon HM. Effects of feeding milk replacer ad libitum or in restricted amounts for the first five weeks of life on the growth, metabolic adaptation, and immune status of newborn calves. PLoS One 2016;11.
Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 2009;75:7537–41.
Schwarzkopf S, Kinoshita A, Kluess J, Kersten S, Meyer U, Huber K, Danicke S, Frahm J. Weaning holstein calves at 17 weeks of age enables smooth transition from liquid to solid feed. Animals 2019;9:1132. 2021;11.
Shen JS, Zheng WJ, Xu YX, Yu ZT. The inhibition of high ammonia to in vitro rumen fermentation is ph dependent. Front Vet Sci 2023;10.
Solden LM, Hoyt DW, Collins WB, Plank JE, Daly RA, Hildebrand E, Beavers TJ, Wolfe R, Nicora CD, Purvine SO, Carstensen M, Lipton MS, Spalinger DE, Firkins JL, Wolfe BA, Wrighton KC. New roles in hemicellulosic sugar fermentation for the uncultivated bacteroidetes family bs11. ISME J 2017;11:691–703.
Söllinger A, Tveit AT, Poulsen M, Noel SJ, Bengtsson M, Bernhardt J, Hellwing ALF, Lund P, Riedel K, Schleper C, Hojberg O, Urich T. Holistic assessment of rumen microbiome dynamics through quantitative metatranscriptomics reveals multifunctional redundancy during key steps of anaerobic feed degradation. mSystems 2018;3.
Steele MA, Penner GB, Chaucheyras-Durand F, Guan LL. Development and physiology of the rumen and the lower gut: targets for improving gut health. J Dairy Sci 2016;99:4955–66.
Stefanska B, Czlapa W, Pruszynska-Oszmalek E, Szczepankiewicz D, Fievez V, Komisarek J, Stajek K, Nowak W. Subacute ruminal acidosis affects fermentation and endotoxin concentration in the rumen and relative expression of the cd14/tlr4/md2 genes involved in lipopolysaccharide systemic immune response in dairy cows. J Dairy Sci 2018;101:1297–310.
Suarez-Mena FX, Hu W, Dennis TS, Hill TM, Schlotterbeck RL. Beta-hydroxybutyrate (bhb) and glucose concentrations in the blood of dairy calves as influenced by

age, vaccination stress, weaning, and starter intake including evaluation of bhb and glucose markers of starter intake. J Dairy Sci 2017;100:2614–24.
Tan RSG, Zhou M, Li F, Guan LL. Identifying active rumen epithelial associated bacteria and archaea in beef cattle divergent in feed efficiency using total rna-seq. Curr. Res. Microb. Sci. 2021;2:100064.
Taschuk R, Griebel PJ. Commensal microbiome effects on mucosal immune system development in the ruminant gastrointestinal tract. Anim Health Res Rev 2012;13:129–41.
Terre M, Devant M, Bach A. Effect of level of milk replacer fed to holstein calves on performance during the preweaning period and starter digestibility at weaning. Livest Sci 2007;110:82–8.
Theurer CB. Grain processing effects on starch utilization by ruminants. J Anim Sci 1986;63:1649–62.
Trevisi E, Amadori M, Riva F, Bertoni G, Bani P. Evaluation of innate immune responses in bovine forestomachs. Res Vet Sci 2014;96:69–78.
Trevisi E, Riva F, Filipe JFS, Massara M, Minuti A, Bani P, Amadori M. Innate immune responses to metabolic stress can be detected in rumen fluids. Res Vet Sci 2018;117:65–73.
Tümmler L-M, Derno M, Tuchscherer A, Kanitz E, Kuhla B. Effects of 2 liquid feeding rates over the first 3 months of life on whole-body energy metabolism and energy use efficiency of dairy calves up to 5 months. J Dairy Sci 2021;104:10399–414.
Tümmler LM, Derno M, Rottgen V, Vernunft A, Tuchscherer A, Wolf P, Kuhla B. Effects of 2 colostrum and subsequent milk replacer feeding intensities on methane production, rumen development, and performance in young calves. J Dairy Sci 2020;103:6054–69.
Vansoest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci 1991;74:3583–97.
Vdlufa Methodenbuch. band iii: Die chemische untersuchung von futtermitteln. In: Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VD LUFA) Verlag, Darmstadt, Germany; 1976.
Wang LJ, Li Y, Zhang YG, Wang LH. The effects of different concentrate-to-forage ratio diets on rumen bacterial microbiota and the structures of holstein cows during the feeding cycle. Animals 2020;10.
Wang Y, Xia HZ, Yang Q, Yang DY, Liu SJ, Cui ZH. Evaluating starter feeding on ruminal function in yak calves: combined 16s rrna sequencing and metabolomics. Front Microbiol 2022;13.
Welboren AC, Leal LN, Steele MA, Khan MA, Martin-Tereso J. Performance of ad libitum fed dairy calves weaned using fixed and individual methods. Animal 2019;13:1891–8.
Whalin L, Weary DM. Von Keyserlingk MaG. Understanding behavioural development of calves in natural settings to inform calf management. Animals 2021;11.
Williams YJ, Popovski S, Rea SM, Skillman LC, Toovey AF, Northwood KS, Wright ADG. A vaccine against rumen methanogens can alter the composition of archaeal populations. Appl Environ Microbiol 2009;75:1860–6.
Wright ADG, Kennedy P, O'neill CJ, Toovey AF, Popovski S, Rea SM, Pimm CL, Klein L. Reducing methane emissions in sheep by immunization against rumen methanogens. Vaccine 2004;22:3976–85.
Yi SM, Dai DW, Wu H, Chai ST, Liu SJ, Meng QX, Zhou ZM. Dietary concentrate-to-forage ratio affects rumen bacterial community composition and metabolome of yaks. Front Nutr 2022;9.
Yin XJ, Ji SK, Duan CH, Tian PZ, Ju SS, Yan H, Zhang YJ, Liu YQ. The succession of fecal bacterial community and its correlation with the changes of serum immune indicators in lambs from birth to 4 months. J Integr Agric 2023;22:537–50.
Zhang J, Shi HT, Wang YJ, Cao ZJ, Yang HJ, Li SL. Effect of limit-fed diets with different forage to concentrate rations on fecal bacterial and archaeal community composition in holstein heifers. Front Microbiol 2018;9.