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Dorbek-Kolin, Elisabeth

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Faecal microbiota in two-week-old female dairy calves during acute cryptosporidiosis outbreak – Association with systemic inflammatory response

Elisabeth Dorbek-Kolin [a,*], Aleksi Husso [b], Mikael Niku [b], Marina Loch [c], Tiina Pessa-Morikawa [b], Tarmo Niine [a], Tanel Kaart [d], Antti Iivanainen [b], Toomas Orro [c]

[a] Veterinary Biomedicine and Food Hygiene, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Kreutzwaldi 62, 51006 Tartu, Estonia
[b] Veterinary Biosciences, Faculty of Veterinary Medicine, University of Helsinki, Agnes Sjöbergin katu 2, P.O. Box 66, Helsinki, Finland
[c] Clinical Veterinary Medicine, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Kreutzwaldi 62, 51006 Tartu, Estonia
[d] Animal Breeding and Biotechnology, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Kreutzwaldi 62, 51006 Tartu, Estonia

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ABSTRACT

In the present study, relationships between the intestinal microbiota and innate immunity response, acute cryptosporidiosis, and weight gain in female dairy calves were investigated. A total of 112 calves born during a natural outbreak of cryptosporidiosis on one dairy farm was included in the study. Microbiota composition was analysed by means of 16S ribosomal RNA gene amplicon sequencing from faecal samples collected during the second week of life, while the status of *Cryptosporidium* spp. infection was determined using immunofluorescence. Serum samples from the second week of life were colourimetrically analysed for the following markers of acute inflammation: acute-phase proteins (serum amyloid A and haptoglobin) and pro-inflammatory cytokines (interleukin-1 beta, interleukin-6, and tumour necrosis factor-alpha). Statistical analyses were performed using random forest analysis, variance-partitioning, and negative binomial regression. The faecal microbiota of the two-week old calves was composed of the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and *Actinobacteria* (in order of decreasing abundance). Microbial diversity, measured in terms of the Shannon index, increased with the age of the calves and decreased if a high count of *Cryptosporidium* spp. oocysts was found in the faeces. *Fusobacterium* was positively associated with *Cryptosporidium* spp. oocyst count and serum amyloid A concentration. *Peptostreptococcus* was positively associated with haptoglobin and serum amyloid A concentrations, and negatively associated with average daily weight gain at 9 months of age. The markers of innate immunity, in combination with age, explained 6% of the microbial variation. These results suggest that some components of the intestinal microbiota may have a long-lasting negative effect on animal growth through the stimulation of the systemic innate immune response.

1. Introduction

Every dairy farmer's dream is to have a farm full of productive and healthy animals (Hansson and Lagerkvist, 2016). The neonatal period is critical because it is during this time that the immune system interacts with the outside world for the first time, and these early responses of the immune system may have long-term effects on future weight gain, production, and health (Seppä-Lassila et al., 2018). The development of the neonate's immune system depends on microbial stimulation, as well as parasitic and viral stimulation (Chase et al., 2008; Ianiro et al., 2022; Kelly and Coutts, 2000). The gastrointestinal and respiratory tracts are predominant sites of microbial contact (Renz et al., 2012). To the authors' knowledge, the development of intestinal microbiota in neonatal calves has not been thoroughly studied. In a recent study carried out in Finland, small amounts of microbial DNA were observed in the rectums of newborn calves, and the rectal microbiota changed rapidly during the first week of life (Alipour et al., 2018). It has been found that in beef cattle, taxonomic groups belonging to the phyla *Firmicutes* and *Bacteroidetes* are dominant in the gastrointestinal tract in general, as well as in the colon (Jami et al., 2013; Myer et al., 2017). The highest species

* Corresponding author.
E-mail address: elisabeth.dorbek-kolin@emu.ee (E. Dorbek-Kolin).

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richness and diversity have been detected at weaning (Klein-Jöbstl et al., 2014). One genus, *Faecalibacterium* spp., has been found to be positively associated with weight gain and negatively associated with diarrhoea in dairy calves during the first week of life (Oikonomou et al., 2013). This indicates that *Faecalibacterium* may be beneficial for neonatal calves.

Cryptosporidiosis, caused by the zoonotic protozoan parasite *Cryptosporidium* spp., is a common clinical problem in calves. It has been demonstrated that calves in their second week of life have the highest number of *Cryptosporidium* spp. oocysts (Santín et al., 2008). *Cryptosporidium parvum* (*C. parvum*) is associated with clinical diseases, although other *Cryptosporidium* species are also found in cattle (Thomson et al., 2017). The worldwide prevalence of *C. parvum* varies from 3.4% to 96.6% (Thomson et al., 2017), whereas in Estonia, 66% of the investigated farms had at least one calf shedding *Cryptosporidium* spp. oocysts (Santoro et al., 2018). It has been suggested that the ability of *Cryptosporidium* spp. to infect the gut may be affected by the age-related changes in the gut microbiota or changes in the diet of calves (Thomson et al., 2017). As *Cryptosporidium* spp. infects intestinal epithelial cells (McDonald et al., 2000), I-FABP (intestinal fatty acid binding protein) can be a good biomarker for evaluating the intestinal epithelial damage and permeability in calves (Ok et al., 2020).

In Europe, halofuginone lactate (HL) has been approved as a prophylactic and therapeutic treatment for calf cryptosporidiosis. HL is used to prevent and reduce diarrhoea caused by *C. parvum,* and in both indications, the oocyst excretion has been demonstrated to be reduced (European Medicines Agency, 2019).

Tissue damage (*e. g.* trauma or infection) initiates a host inflammatory response called the acute phase response (APR) (Baumann and Gauldie, 1994). This response is a vital part of innate immunity and is an important protective mechanism in neonatal infections. Acute phase proteins (APPs) are produced by the liver, induced by proinflammatory cytokines such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumour necrosis factor-alpha (TNF-α) (Baumann and Gauldie, 1994). In cattle, the two major APPs are haptoglobin (Hp) and serum amyloid A (SAA) (Eckersall and Bell, 2010). The concentration and kinetics of systemic APPs during the inflammatory response are related to the severity of tissue damage and the time-course of the inflammation process (Baumann and Gauldie, 1994; Kent, 1992; Petersen et al., 2004). Thus, measuring the circulating levels of APPs provides valuable information about the ongoing APR and can be used as non-specific disease markers (Thompson et al., 1992; Van Leeuwen and Van Rijswijk, 1994; Petersen et al., 2004). In general, changes in APPs after birth reflect the physiological adaptation of the newborn to extrauterine life (Orro et al., 2008).

In humans, it has been shown that the immunity effects of early-life microbial exposure are durable and persist into later life (von Mutius, 2007; Ege et al., 2011). Immunological maturation may have a long-term impact on an animal's success as a production animal (Chester-Jones et al., 2017). High SAA concentrations in studies with 2–3-week-old beef calves (Seppä-Lassila et al., 2017), rearing calves (Seppä-Lassila et al., 2018), reindeer calves (Orro et al., 2006), and lambs (Peetsalu et al., 2019) predict lower long-term weight gain up to the age of slaughter. These observations suggest that early factors activating an APR can have a long-term negative impact on an animal's life, including lower weight gain. To the authors' knowledge, the relationships of neonatal infections with APR and the development of the immune system have not yet been thoroughly addressed.

The main aim of this study was to investigate the relationship between faecal microbiota and systemic innate immunity response in 2-week-old calves, as measured in terms of the APP and pro-inflammatory cytokine concentrations in the serum. Genera associated with the systemic innate immunity response were further analysed to explore the potential relationship between microbiota and growth rate during the first 9 months of life. In addition, we investigated the relationship between acute cryptosporidiosis and the intestinal microbiota and measured I-FABP concentration in blood as it is a marker of intestinal permeability.

2. Materials and methods

The present study is a part of a large-scale study, the previous parts of which have been published by Niine et al. (2018a) – APR of calves up to 6 weeks of age during an acute cryptosporidiosis outbreak; and Peetsalu et al. (2022) – association of colostrum cytokine and APP concentrations with APR of calves during the first 3 weeks of life.

2.1. Animals

The calves included in this study were all from the same large dairy farm located in Järva County in Central Estonia. At the time of the study, there were approximately 1800 Estonian Holstein dairy cows on the farm. All the included calves ($n = 112$) were females born between 21 January and 16 March 2015. Twins and male calves were excluded from the study. For this study, we analysed faecal and serum samples taken during the second week of the calves' lives.

The calves were separated from their mothers immediately after birth and weighed on a digital scale. They received three litres of colostrum from their dams once within 2 h after birth. Colostrum quality was determined by means of visual examination and a Kruuse colostrum densimeter (Jørgen Kruuse A/S, Langeskov, Denmark). If the specific gravity of the colostrum was <1035 or total protein was <50 g/l, deep frozen colostrum from another dam was provided to the calf. At 9 months of age, the calves were weighed again. A more detailed description of the management of farm and veterinary services provided to calves is presented in Niine et al. (2018a). Briefly, after the onset of an acute cryptosporidiosis outbreak, treatment with HL (Halocur, Intervet International B.V., Boxmeer, Netherlands) was initiated. The calves were retrospectively allocated into three groups, based on the HL treatment regimen they received: 1) not treated, 2) treated incorrectly (treatment started >48 h after birth and lasted <7 d), and 3) treated correctly (treated according to manufacturer's instructions; started <48 h after birth and lasted ≥7 d).

2.2. Feeding

Twice a day, the calves were fed 2–3 kg of warmed unpasteurised milk, in addition to free access to hay and prestarter feed (Prestarter, Agrovarustus OÜ, Estonia). This feeding regimen was maintained until the age of 15–17 d, after which the calves received milk powder (Josera GoldenSpezial, Josera GmbH & Co. KG, Germany) solution (140 g of milk powder diluted in 1 l of warm water) twice a day (3 l/day) and continued free access to prestarter feed.

2.3. Sample collection

Serum and faecal samples were ethically collected from calves during their second week of life. Serum samples ($n = 112$) were taken from the jugular vein using an 18-G needle into sterile evacuated tubes (VACUETTE® TUBE 9 ml CAT Serum Clot Activator). The samples were then transported to the laboratory and centrifuged (1800 ×*g* for 10 min). Serum samples were stored at −20 °C until analysis. Faecal samples (n = 112) were collected directly from the rectum using disposable gloves. The collected faecal samples were stored at 4 °C for up to 48 h until further analysis (detection of *Cryptosporidium* spp. oocysts and DNA extraction).

2.4. Laboratory analysis

The SAA concentration was measured using a commercial ELISA kit (Phase BE kit, Tridelta Development Ltd., Dublin, Ireland). The concentration of Hp was measured using a method defined by Makimura and Suzuki (1982), with alterations of using tetramethylbenzine (0.06

mg/ml) as a substrate and using microtitration plates (Alsemgeest et al., 1994). The detection limits were 0.3 mg/l for SAA and 60 mg/l for Hp. The concentrations of interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumour necrosis factor-alpha (TNF-α) were measured using commercial ELISA kits from Cusabio Biotech (Wuhan, Hubei, China). The detection limits for IL-1β, IL-6, and TNF-α were 15.6 ng/l, 2.5 ng/l, and 50.0 ng/l, respectively. The concentration of intestinal fatty acid binding protein (I-FABP), a marker of intestinal permeability, was measured using a commercial ELISA kit (bovine I-FABP ELISA kit (MBS035016); MyBiosource Inc., San Diego, CA, USA). The results were read using a spectrophotometer (Magellan Sunrise™, Tecan Group Ltd., Männedorf, Switzerland). The inter- and intra-assay coefficients of variability for all protein detection methods were < 15%.

Faecal samples were prepared for the detection of *Cryptosporidium* spp. using an immunofluorescence staining method, as described by Niine et al. (2018a). The results were measured as the approximate number of oocysts per gram of faecal matter (opg). Faecal DNA was extracted from approximately 200 μg of faeces using the PSP® Spin Stool DNA Kit (Stratec Biomedical AG, Birkenfeld, Germany) and the bead beating process, according to the manufacturer's instructions.

2.5. Microbiota analysis

The hypervariable regions V3-V4 of the 16S ribosomal RNA (rRNA) genes were sequenced using the Illumina MiSeq platform in the DNA core facility of the University of Helsinki, as described previously (Alipour et al., 2018; Husso et al., 2020). Samples and controls were first amplified using 1× Phusion Hot Start II High-Fidelity PCR Master Mix (Thermo Scientific), 2.5% DMSO (Thermo Scientific), 500 nM of 16S rRNA V3 and V4 gene primers (341F and 785R; Metabion), and 1.25 μl of DNA extract, in 25 μl total volume. The thermal cycling conditions included an initial denaturation step at 98 °C for 30 s, and 12 cycles of denaturing at 98 °C for 10 s, annealing at 56 °C for 30 s and extension at 72 °C for 20 s. The final extension step was at 72 °C for 5 min. T100™ Thermal Cycler (Bio-Rad Laboratories) was used.

The 2nd round PCR amplifications were performed using an Illumina forward and reverse primer set, Phusion Hot-Start II polymerase (Finnzymes/Thermo Scientific), High Fidelity buffer and 2.5% DMSO. The following thermal cycling conditions were applied with an Arktik thermal cycler (Finnzymes/Thermo Scientific): initial denaturation at 98 °C for 30 s, 17 cycles at 98 °C for 10s, 65 °C for 30s, 72 °C for 10s, and a final extension at 72 °C for 5 min. The final 16S rRNA gene amplicons were sequenced on an Illumina MiSeq sequencer using the v2 600 cycle kit paired-end (325 bp + 285 bp).

In addition to calf faecal samples, we sequenced no template controls, a ZymoBIOMICS Microbial Community Standard (Zymo Research, USA), and an in-house adult cow faecal standard. The observed composition of the commercial standard matched the expected composition provided by the manufacturer (data not shown).

The detailed bioinformatics pipeline is described in the Supplementary Materials. Briefly, the read quality was first inspected using FastQC and MultiQC (Andrews et al., 2015; Ewels et al., 2016). Leftover primes and spacers were then trimmed using Cutadapt version 1.10 (Martin, 2011). A mapping file was created for QIIME2 and validated using Keemei (Rideout et al., 2016). The FASTQ- files were imported into QIIME2 version 2018.4, where the DADA2 plugin was used to denoise and filter the reads, call amplicon sequence variants (ASVs), and generate a feature table (Callahan et al., 2016; Bolyen et al., 2019). A naïve Bayes classifier was trained in QIIME2 against the SILVA v132 97% database, extracted to only include the V3–V4 reference region, and used to assign taxonomy to ASVs (Quast et al., 2013; Bokulich et al., 2018). Singleton sequences and sequences derived from chloroplasts or mitochondria were removed.

2.6. Statistical analysis

We used variance partitioning analysis to investigate how the systemic innate immunity response markers, *Cryptosporidium* spp. infection, and age explain faecal microbiota variability on the genus level. This analysis partitions the variation in faecal microbiota data into components with respect to different tables of exploratory variables and their combinations using adjusted R-squares in redundancy analysis ordination (Borcard et al., 1992). Continuous variables were used for variance partitioning (except HL treatment).

Random forest analysis was used to investigate how well faecal microbiota explain our chosen parameters – cytokines, APPs, *Cryptosporidium* spp. infection, HL treatment, and I-FABP. For random forest analysis, continuous variables (except IL-1β) were divided into low, moderate, and high concentration groups of similar size, based on the concentration levels from lowest to highest in serum. Based on the concentration of IL-1β, the calves were divided into two groups: low (below the detection level of the assay) and high (above the detection level of the assay). Calves were also divided into groups based on the amount of *Cryptosporidium* spp. oocysts shed in their faeces (opg); these groups were: no oocysts found, low oocyst count, and high oocyst count. The division into low- and high oocyst groups was based on the median value of oocysts found. For treatment of HL, the calves were divided into three groups: not treated, treated incorrectly, and treated correctly. All the categorical variables and their values are presented in Table 1. Genus-level microbiota data were used for the statistical analysis. All genera with a prevalence of ≥10% in 112 calves were included (n = 102).

Table 1
Categorical variables used in the statistical analysis.

Variable	Group	Value	n
SAA (mg/l)	Low	34.0–101.2	38
	Moderate	102.1–153.2	37
	High	154.3–487.9	37
Hp (mg/l)	Low	95–218	38
	Moderate	221–884	37
	High	891–2830	37
IL-1β (ng/l)	Low	<15.6*	99
	High	16.9–207.4	13
IL-6 (ng/l)	Low	<2.5*–4.2	38
	Moderate	4.3–8.6	37
	High	9.3–130.7	37
TNF-α (ng/l)	Low	<50*–221	38
	Moderate	230–396	37
	High	429–4205	37
I-FABP (ng/l)	Low	63–234	38
	Moderate	236–303	37
	High	308–1797	37
ADWG (g/d)	Low	406–646	34
	Moderate	648–741	33
	High	750–982	33
Cryptosporidium spp. (opg)	No oocysts found	0	54
	Low oocyst count	208–399,910	29
	High oocyst count	475,293–5,764,653	29
HL treatment group	Not treated	n/a	21
	Treated incorrectly	n/a	39
	Treated correctly	n/a	52

Continuous variables from calves (n = 112) were categorised into three similar sized groups based on the concentrations, from lowest to highest (low, moderate, and high). Average daily weight gain data from 100 calves were available. The median value of oocysts found in *Cryptosporidium*-positive samples was used to categorise them into low and high oocyst groups. Calves treated with halofuginone lactate (HL) were divided into groups treated incorrectly (treatment started >48 h after birth and lasted <7 d), and correctly (treatment started <48 h after birth and lasted ≥7 d).
SAA – serum amyloid A, Hp – haptoglobin, IL-1β – interleukin 1-beta, IL-6 – interleukin 6, TNF-α - tumour necrosis factor alpha, I-FABP – intestinal fatty acid binding protein, HL – halofuginone lactate, n/a – not applicable, ADWG – average daily weight gain. * Under the detection limit of the ELISA kit.

The 30 most influential genera in the random forest analysis, as measured by mean decrease in model accuracy, were further analysed using negative binomial regression, to investigate the associations of microbial genera with *Cryptosporidium* spp. and marker variables. Genus was always included as a response variable. Age at sampling and *Cryptosporidium* spp. oocyst count were included as categorical explanatory variables in all models. The abundance of all the bacteria in the sample was included as an exposure variable. Wald test *p*-values were calculated for categorical variables, and Holm-Šidák adjusted p-values were calculated to account for multiple comparisons (out of 30 Wald test *p*-values). For pairwise comparisons between 3-level categorical variables after a significant Holm-Šidák corrected Wald test, Bonferroni correction was used. Graphs of the observed proportions of negative binomial probabilities along with the Poisson model were used to evaluate how negative binomial distribution suited to the models used. Association with average daily weight gain (ADWG) was investigated for all genera ($n = 5$) that were significantly associated with I-FABP or any systemic innate immunity response marker. ADWG at 9-months of age (low, moderate, and high; $n = 34$, 32, and 34, respectively) was used as an explanatory categorical variable. To control for possible confounding factors, age at sampling and *Cryptosporidium* spp. oocyst counts in faeces were included as categorical explanatory variables, and calf birth weight was included as a continuous explanatory variable. Bonferroni correction was used for pairwise comparison of p-values for categorical variables.

Linear regression models were used to evaluate the associations between microbial diversity (Shannon index) and age, innate immunity response marker variables, *Cryptosporidium* spp. infection, and HL treatment. The Shannon index was a response variable; age (continuous variable), protein groups, *Cryptosporidium* spp. infection groups, and HL treatment groups were explanatory variables. Stepwise backward elimination procedure was used to select the final models. The linearity assumption between the response and continuous explanatory variables was evaluated using Lowess smoothing curve graphs. Interactions and confounders (change in coefficient of over 10% after variable elimination) were controlled for. Residual plots were used to evaluate the overall model assumptions.

For variance partitioning analysis package 'vegan' (Oksanen et al., 2019) and for random forest analysis package 'randomForest' (Liaw and Wiener, 2002) with R version 4.0.1 (http://www.R-project.org/) were used. For negative binomial regression models and linear regression model, STATA/IC 14.2 for Windows (StataCorp LP, College Station, TX, USA) was used. Excel 2016 (Microsoft, Redmond, WA, USA) was used for the initial data management.

3. Results

3.1. Microbiota composition on phylum level

The most prevalent phylum in all 2-week-old calves in our study ($n = 112$) was *Firmicutes*, with a mean relative abundance (±SD) 50.9 ± 26.8%. This was followed by *Bacteroidetes* (28.6 ± 25.8%), *Proteobacteria* (8.4 ± 12.4%), *Fusobacteria* (7.9 ± 15.9%), and *Actinobacteria* (3.9 ± 9.0%) (Fig. 1A). These five phyla contributed to 99.7% of all bacterial taxa found in the faecal samples. Age-related changes were observed in the composition of the microbiota, as shown in Fig. 1A. The overall trend of *Bacteroidetes* increased, whereas the relative abundances of *Firmicutes*, *Actinobacteria*, and *Fusobacteria* declined. Bacterial diversity, as measured using the Shannon index, increased with age ($p = 0.001$) (Fig. 2A).

As seen in Fig. 1B, the mean relative abundance of *Firmicutes* was the highest in the group with high *Cryptosporidium* spp. oocyst counts. The relative abundances of *Bacteroidetes* and *Actinobacteria* were, on average, lower in the high oocyst count group. A high count of *Cryptosporidium* spp. oocysts was negatively associated with Shannon's diversity index ($p = 0.007$) (Fig. 2B).

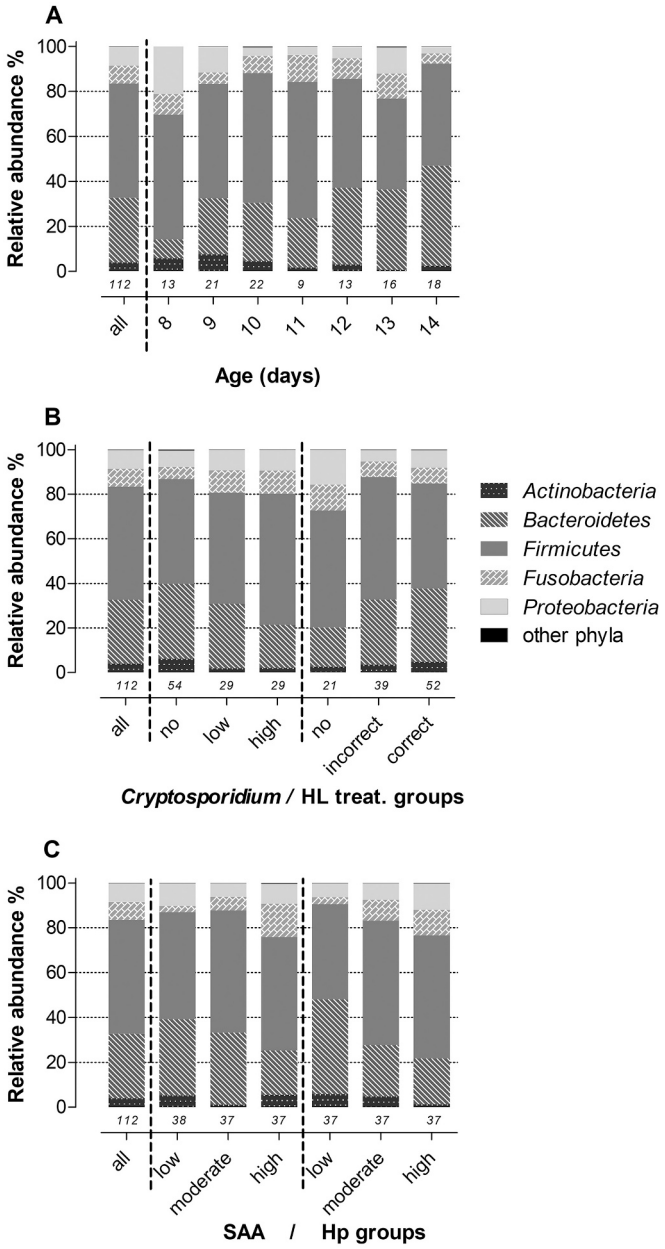

Fig. 1. Microbiota composition at phylum level in calves based on days of age (A), *Cryptosporidium* spp. infection status and halofuginone lactate (HL) treatment groups (B), and serum amyloid A (SAA) and haptoglobin (Hp) concentrations groups (C). The small numbers given in italics under the columns indicate the number of calves in that group. Column 'all' shows the composition of microbiota in all calves included in this study. The three groups of *Cryptosporidium* spp. infection status were: no – no oocysts found, low – low oocyst count (below the median of the same week), and high – high oocyst count (above the median of the same week). HL treatment groups were: no – no treatment received, incorrect – treatment started >48 h after birth and lasted <7 d, correct – treatment started <48 h after birth and lasted ≥7 d.

The mean relative abundances of *Actinobacteria* were the highest in the HL treatment groups that did receive treatment (incorrect or correct), and *Fusobacterium* was the highest in the not treated group (Fig. 1B). *Cryptosporidium* spp. infection and HL treatment groups correlated with each other: correct HL treatment and *Cryptosporidium* spp. negative results; incorrect HL treatment and low *Cryptosporidium* spp. oocyst counts; and no HL treatment and high *Cryptosporidium* spp. oocyst counts mirrored each other.

The mean relative abundance of the phyla was similar in all three

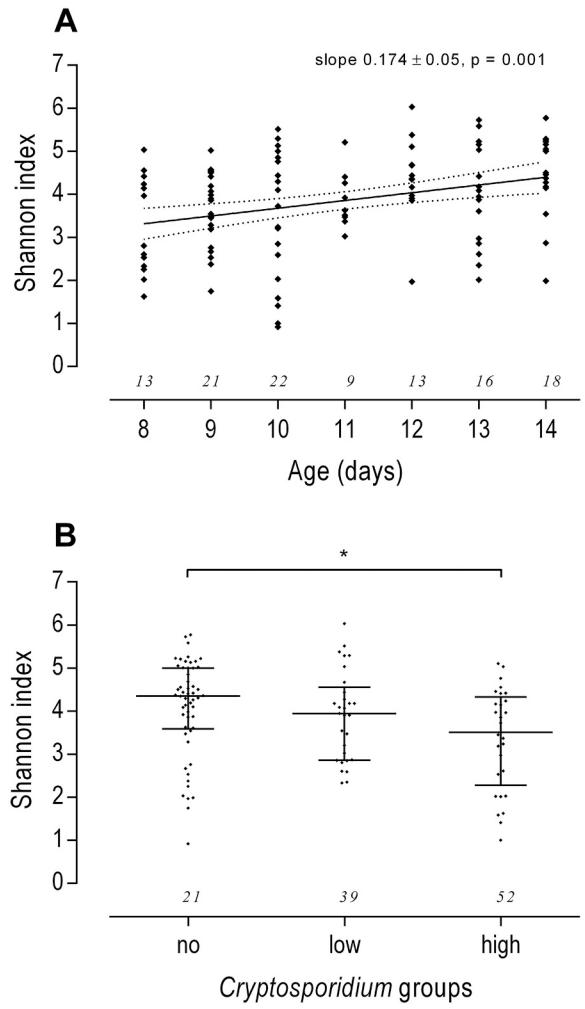

Fig. 2. Faecal microbiota diversity by age (days) (A) and *Cryptosporidium* spp. infection group (B), as evaluated using linear regression model. The three groups of *Cryptosporidium* spp. infection status were: no – no oocysts found, low – low oocyst count (below the median of the same week), and high – high oocyst count (above the median of the same week). The small numbers given in italics under the columns indicate the number of calves in that group.
* Significant difference, Bonferroni-corrected $p < 0.05$.

groups of SAA and Hp (Fig. 1C). The higher the SAA and Hp concentrations, the greater the relative abundance of *Fusobacteria* and lower the relative abundance of *Bacteroidetes*.

3.2. Variance-partitioning analysis

The variation in intestinal microbiota at the genus level explained by the study variables is shown in Fig. 3. As seen in this figure, most of the variation was explained by factors other than APPs, *Cryptosporidium* spp. infection, HL treatment, cytokines, or age; a total of 6.0% of microbial variation was explained by these variables. However, it should be noted that APPs (SAA and Hp) independently explain half (3.0%) of the explainable microbial variation, and cytokines (IL-1β, IL-6, TNF-α) were completely overlapped by the APP explanation. In other words, cytokines did not explain the variability in microbiota more than the APPs. The combined APP group and the combined *Cryptosporidium* spp. infection and HL treatment group overlapped with each other for 1.2% of the explained microbiota genus variation. Age was more independent and explained approximately 1.4% of the 6.0% microbial variation.

When *Cryptosporidium* spp. infection and HL treatment groups were added separately to the variance partitioning analysis, all microbial variation explained by HL treatment overlapped with the *Cryptosporidium* spp. infection group. Therefore, *Cryptosporidium* spp. infection and HL treatment groups were added as a combined explanatory variable. When SAA and Hp were included separately in the model, 1.0% (together 1.16%; Fig. 3) of the explained microbiota variations overlapped with each other.

3.3. Random forest analysis

Random forest analysis classified the calves' faecal samples (n = 112) into SAA, Hp, *Cryptosporidium* spp. infection, and HL treatment groups. The relative importance of the 30 most influential genera (out of 102) is shown in Fig. 4. The proportion of groups that were classified correctly by this random forest analysis based on bacterial genera was 42.9% for SAA, 43.7% for Hp, 45.5% for *Cryptosporidium* spp. infection, and 46.2% for HL treatment. The 30 most influential genera by group were further analysed using negative binomial models, to investigate associations with variable groups (Holm-Šidák corrected Wald test *p*-values). In these models, we also accounted for the possible confounding factors age and *Cryptosporidium* spp. infection in the APPs.

Fusobacterium was the most influential genus for SAA, and the fourth most influential genus for Hp. The most influential genus for Hp was [*Ruminococcus*] torques group, but it was not found to be associated with SAA. *Ruminiclostridium 9* was the most influential genus for *Cryptosporidium* spp. infection, and it was ranked 16th for HL treatment. *Erysipelotrichaceae* UCG-009 was the most influential genus for HL treatment, but it was not found to be associated with *Cryptosporidium* spp. infection.

3.4. Negative binomial models

The association between *Cryptosporidium* spp. infection and the presence of *Ruminiclostridium 9* was negative (Fig. 5A). The abundance of *Ruminiclostridium 9* was higher in the no *Cryptosporidium* spp. oocysts group than in the low and high oocyst count groups (Bonferroni corrected $p = 0.039$ and $p < 0.001$, respectively). This bacterial genus was found in 59% of the calves studied ($n = 112$). We also found other bacterial genera (*Alistipes*, *Parasutterella*, and *Faecalibacterium*) with a similar association, except that both the no and low *Cryptosporidium* spp. oocyst count groups had significantly higher abundances than the high oocyst count group (Fig. 5B–D). The 30 most influential genera did not show any statistically significant associations with the HL treatment groups in the negative binomial models.

The *Fusobacterium* genus was positively associated with SAA (Fig. 6A), and was found in 83% of the calves (n = 112). The moderate and high SAA groups had significantly higher *Fusobacterium* abundance than the low SAA group (Bonferroni corrected $p = 0.027$ and $p < 0.001$, respectively). The *Collinsella* genus was found to be associated with SAA and found in 99% of the faecal samples. The association was found to be non-linear, since in the moderate SAA group, abundance of *Collinsella* was lower than in the low or high SAA groups. However, the distribution of *Collinsella* abundance did not follow a negative binomial distribution; thus, the models (for SAA and *Cryptosporidium* spp. infection groups) were not validated (data not shown).

The high Hp concentration group had a positive association with genus [*Ruminococcus*] torques group abundance (Fig. 6B) and a negative association with *Flavonifractor* abundance (Fig. 6C).

Peptostreptococcus was found in 44% ($n = 49$) of the calves, and its abundance was positively associated with high concentrations of both SAA and Hp (Fig. 7A–B). All genera that were significantly associated with inflammatory markers were further analysed for possible associations with nine-month weight gain ($n = 100$), using similar negative binomial models. These five genera were *Fusobacterium*, *Peptostreptococcus, Collinsella*, [*Ruminococcus*] torques group, and *Flavonifractor*. The association between weight gain at the age of 9 months and *Peptostreptococcus* was negative (Fig. 7C). The high ADWG group had a lower abundance of *Peptostreptococcus* than the low and moderate

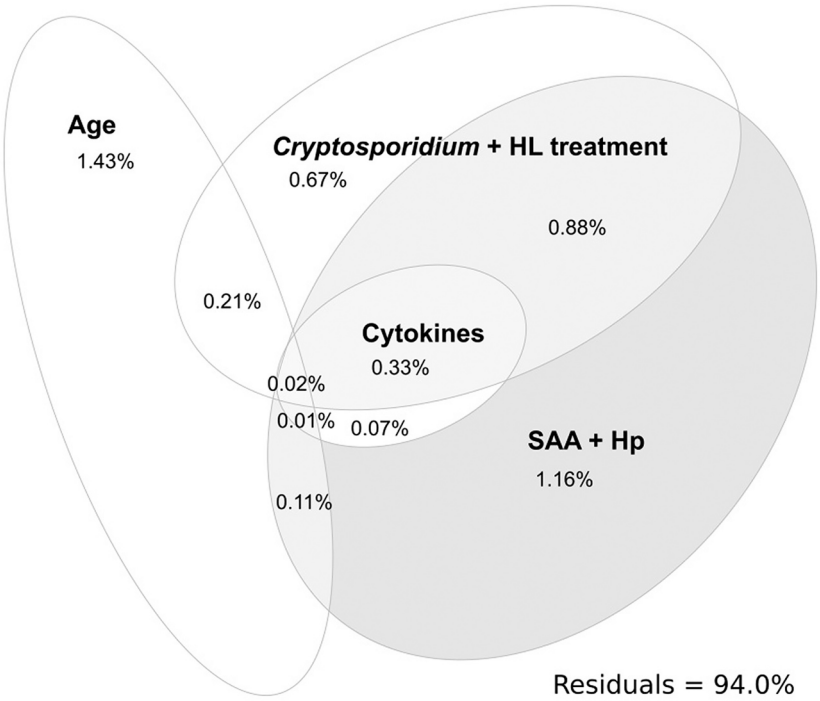

Fig. 3. Variance partitioning analysis of the calves' ($n = 112$) intestinal microbiota at the genus level ($n = 102$). Results are represented as percentiles of the 6.0% of microbial variance explained by these variables. The continuous variables included in the analysis were age at sampling in days ('Age'), cytokines in ng/l (including interleukin-1β, interleukin-6, tumour necrosis factor-α) ('Cytokines'), serum amyloid A (SAA) and haptoglobin (Hp) together in mg/l ('SAA + Hp'), and *Cryptosporidium* spp. infection (opg) and halofuginone lactate (HL) treatment groups together ('*Cryptosporidium* + HL treatment').

ADWG groups (Bonferroni corrected $p < 0.01$ and $p = 0.030$). No statistically significant associations were found between cytokines, I-FABP, HL treatment groups, and genera (data not shown).

4. Discussion

Infection with enteric pathogens can alter the composition of microbiota, and alterations in the composition of microbiota can confer or promote infection by pathogens (Laurent and Lacroix-Lamandé, 2017). The present study shows that there are some genera that have the potential to trigger the inflammatory response, but on a larger scale, microbiota seems to influence the innate immunity response only slightly. For example, *Peptostreptococcus* was positively associated with both APPs investigated (SAA and Hp) and negatively associated with long-term ADWG. Higher oocyst shedding of *Cryptosporidium* spp. is associated with decreased microbial diversity of faecal microbiota of neonatal calves, as was also reported by Rahman et al. (2022); age has the opposite effect.

The rectal microbial 16S rRNA gene profile of 2-week-old calves was dominated by *Firmicutes*, followed by *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and *Actinobacteria*, all of which constituted 99.7% of the microbiota. We found that microbial diversity increased with age, as demonstrated in a previous study (Dill-Mcfarland et al., 2017), and that a high *Cryptosporidium* spp. oocyst count was negatively associated with diversity (Fig. 2). In negative binomial models of *Cryptosporidium,* the four genera associated with *Cryptosporidium* spp. infection (*Ruminiclostridium 9*, *Alistipes*, *Parasutterella*, and *Faecalibacterium*) decreased in abundance, the more oocysts were found. Carey et al. (2021) found that a low abundance of *Megasphaera* was associated with diarrhoeal symptoms during cryptosporidiosis in human infants. They also found that the microbiome of these children was predictive of diarrhoea prior to and at the time of *Cryptosporidium* spp. infection. In mice, *Cryptosporidium* spp. infection has been associated with an increase in the relative abundance of *Bacteroidetes* and a decrease in that of *Firmicutes* (Mammeri et al., 2019). These findings indicate that the presence of *Cryptosporidium* spp. may steer the intestinal bacterial environment to a less favourable direction for some bacterial species. Alternatively, a high abundance of some bacterial species may inhibit or reduce *Cryptosporidium* spp. infection. As this was a cross-sectional study, we can only speculate about the causal relationships.

However, there have been studies suggesting that *Fusobacterium* and *C. parvum* have a synergistic effect (Ichikawa-Seki et al., 2019). Previous studies have hinted to the fact that some specific microbial phyla (indole producing bacteria) may modulate the immune response against *Cryptosporidium* spp. infections in humans (Laurent and Lacroix-Lamandé, 2017). The fact that *Cryptosporidium* spp. influences the microbial composition of calf faeces, or vice versa, needs to be considered when conducting field studies, so that the results of these studies can accurately represent the current situation in farms. *Cryptosporidium* spp. are widespread worldwide (O'Handley and Olson, 2006), and their influence on the host should not be ignored. This study is a field cohort study that reflects this situation.

HL is used as the standard care, as there are no alternatives in the European market. HL is used as a prophylactic treatment for *Cryptosporidium* spp. infection, which functions by reducing the excretion of oocysts (Silverlås et al., 2009), delaying shedding, and improving the survival of calves (Niine et al., 2018a). This treatment needs to be considered as a possible confounder of the objectives of this study. Associations between microbiota and innate immunity response in the present study were obtained from statistical analyses, where the influence of HL treatment and *Cryptosporidium* spp. infection have been accounted for, and should thus not be biased by them. In some countries, HL is so widely used that having a non-treatment control group in field studies is considered unethical (Seale et al., 2019); thus, HL treatment should always be considered when studying the effects of *Cryptosporidium* spp. and microbiota of calves in field studies.

We found that HL treatment was associated with increased faecal microbial diversity. It should be noted that the effects of *Cryptosporidium* spp. infection and HL treatment on faecal microbiota cannot always be differentiated, as they are 100% overlapping in variance partitioning analysis. However, in the negative binomial models, four genera were negatively associated with *Cryptosporidium* spp. infection, and no genera were associated with the HL treatment groups. This indicates that *Cryptosporidium* spp. infection may modulate the faecal microbiota more than HL treatment.

The *Fusobacterium* genus includes several species of gram-negative

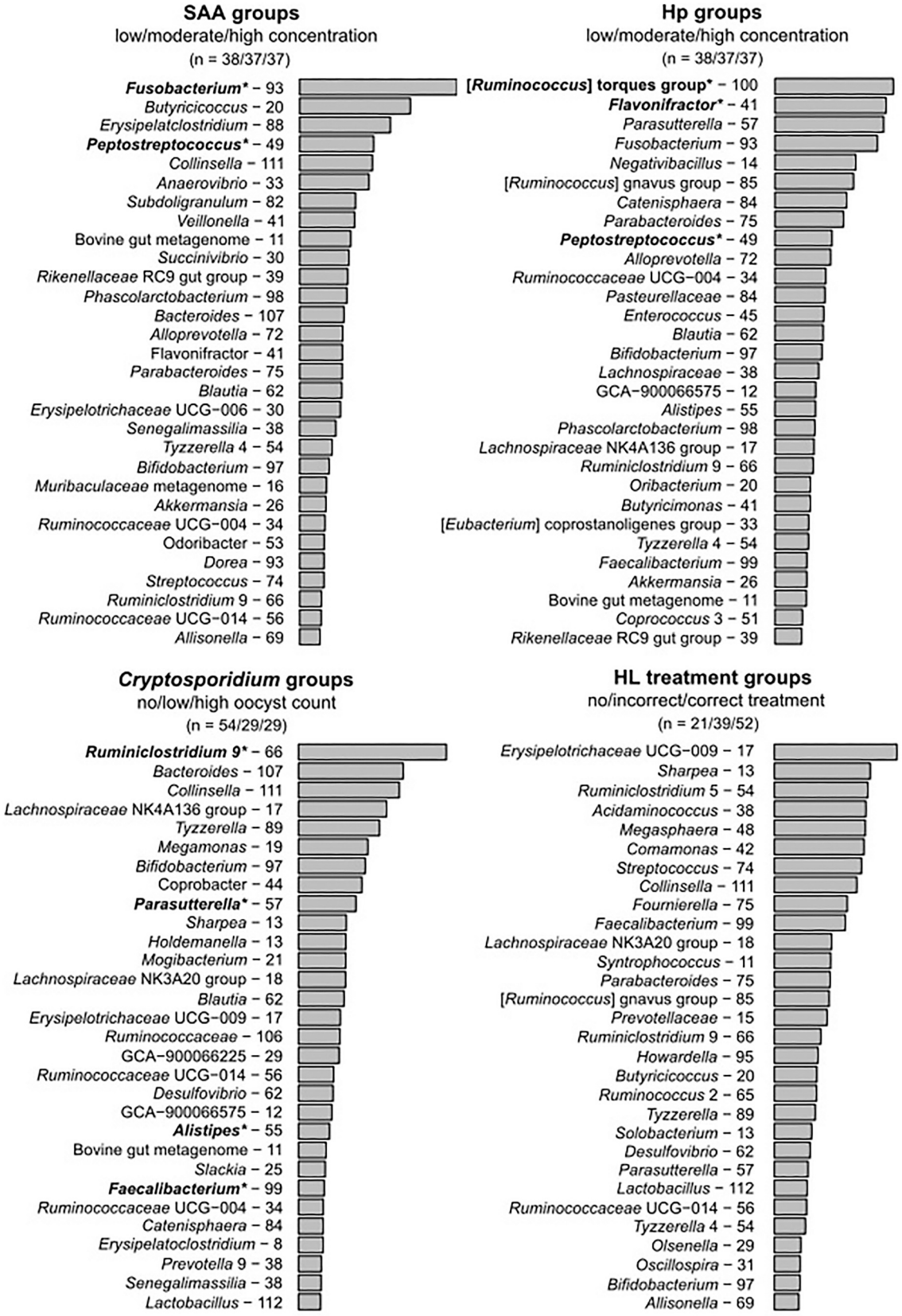

Fig. 4. Random forest analysis results of the 30 most influential genera (out of 102) by groups of calves ($n = 112$). Lengths of the bars (mean decrease in accuracy) show the relative importance of genera. Serum amyloid A (SAA) and haptoglobin (Hp) groups were defined according to concentrations, from lowest to highest. *Cryptosporidium* spp. infection groups were defined according to faecal oocyst count (no oocyst and below or above the median of the same week), while halofuginone lactate (HL) treatment groups were defined on the basis of the received treatment (incorrect – treatment started >48 h after birth and lasted <7 d, correct – treatment started <48 h after birth and lasted ≥7 d). The number after the genus name represents the number of calves in which this genus was found.
* Genera with significant association with variable group (Holm-Sidák corrected Wald test), as analysed using negative binomial models.

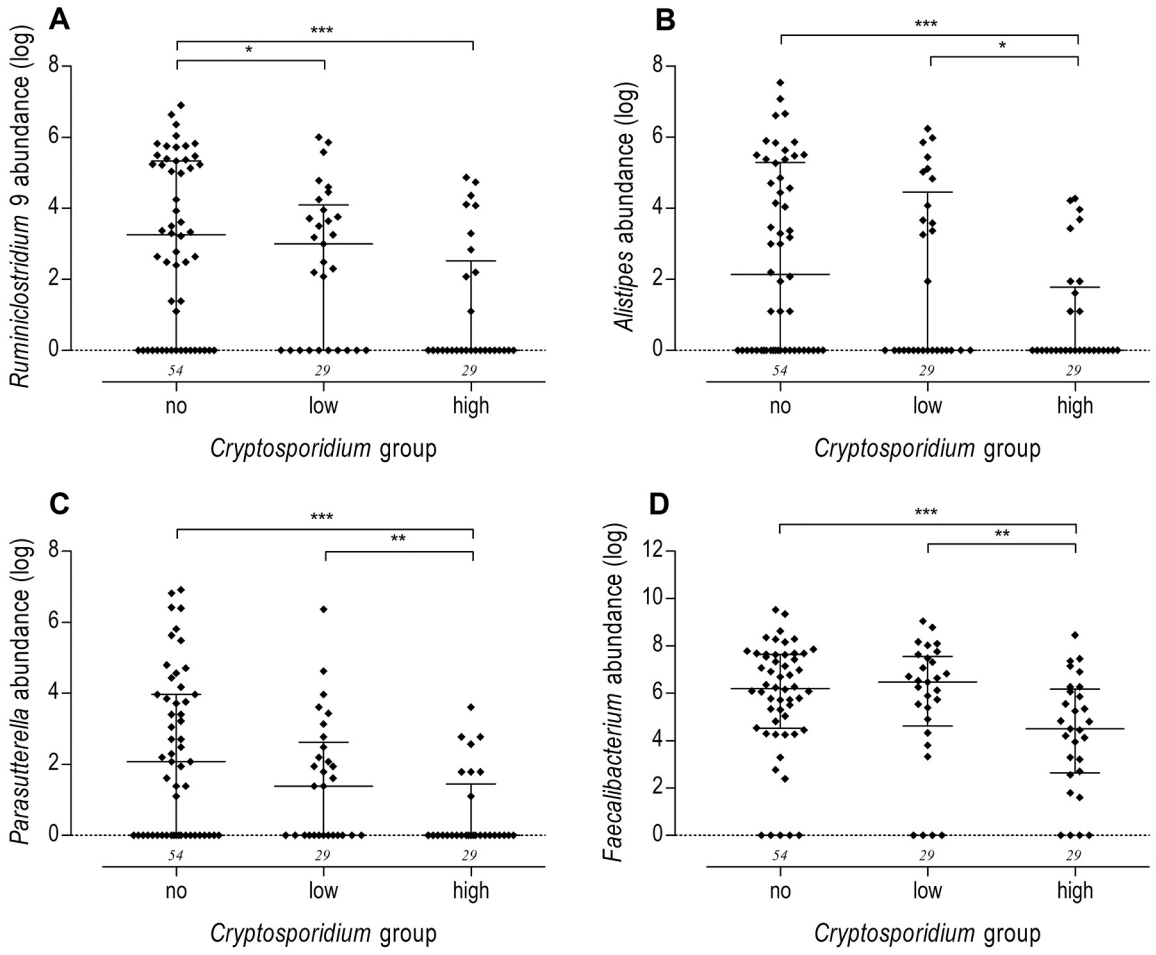

Fig. 5. Association of *Cryptosporidium* spp. groups with four genera, as assessed using negative binomial model. *Cryptosporidium* spp. infection groups (no oocysts found, low oocyst count, and high oocyst count) and *Ruminiclostridium* 9 (A), *Alistipes* (B), *Parasutterella* (C), and *Faecalibacterium* (D) abundances in samples are shown. Log-transformed abundance of these genera, with median and interquartile range, is presented by groups. The small numbers given in italics under the columns represent the number of calves in that group.
* Significant difference, Bonferroni-corrected $p < 0.05$.
** Significant difference, Bonferroni-corrected $p < 0.01$.
*** Significant difference, Bonferroni-corrected $p < 0.001$.

rods that are obligately anaerobic, non-spore forming, and can be motile or non-motile (Hofstad, 2006). *Fusobacterium* is an opportunistic bacterium with pro-inflammatory effects, among other properties (Wong and Yu, 2019). There are both pathogenic and non-pathogenic species of *Fusobacterium*, and all known species affect both animals and humans, with the two most common pathogenic species being opportunistic *F. necrophorum* and *F. nucleatum* (Nagaraja et al., 2005). In our study, the relative abundance of *Fusobacterium* (7.9%) was higher than reported previously. Previous studies have found abundances of 0.76%–5.67% in calves of up to 7 weeks of age (Oikonomou et al., 2013), and 2.0% from birth to 15 d of age in pathogen-free calves (Ichikawa-Seki et al., 2019). *Fusobacterium* abundance was positively associated with SAA concentration (Fig. 6). High *Cryptosporidium* spp. oocyst counts were also positively associated with SAA concentration in the second week of life in these calves (Niine et al., 2018a), but as we controlled the *Cryptosporidium* spp. infection groups in the *Fusobacterium* and SAA model, this means that *Fusobacterium* independently initiated the calves' APR. A study by Ichikawa-Seki et al. (2019) showed an increase in *Fusobacterium* in calf faecal matter to 14.1%, coinciding with infection with *C. parvum*. They also found more severe diarrhoea in calves with concurrent *Cryptosporidium* spp. and *Fusobacterium* infections. The authors of that study suggested that *Fusobacterium* can have an important aggravating effect on *Cryptosporidium* spp. infections. Our study did not show a direct association between *Cryptosporidium* spp. infection and *Fusobacterium,* but as SAA is directly related to the severity of clinical disease, an additional positive association between SAA and *Fusobacterium* supports the synergistic effect of these two infections with clinical disease in calves.

The gut microbiota is influenced by genetics (Fan et al., 2020), both paternal and maternal (Amin and Seifert, 2021). The bacterial composition varies within the gastrointestinal tract of calves, and is influenced by the colostrum and microbiota on the dam's skin, at both the udder and vagina (Yeoman et al., 2018). However, the differences in the overall microbial composition of calves in this study are probably influenced by the acute outbreak of cryptosporidiosis and HL treatment, or other factors leading to *Cryptosporidium* spp. infection, including *Fusobacterium* overgrowth. Since this was a cross-sectional study, causal relationships could not be proven.

Variance partitioning analysis (Fig. 3) showed that most of the microbial variation (residuals 94.0%) was not explained by the variables investigated in this study. The cytokine group completely overlapped with the larger APP group. We can, therefore, state that APPs reflect the activation of cytokines (which have a shorter half-life than APPs) as markers of inflammation. Cytokines are known to increase the levels of APPs. It should be noted that since no associations were found in the negative binomial model, the associations of cytokines with microbiota

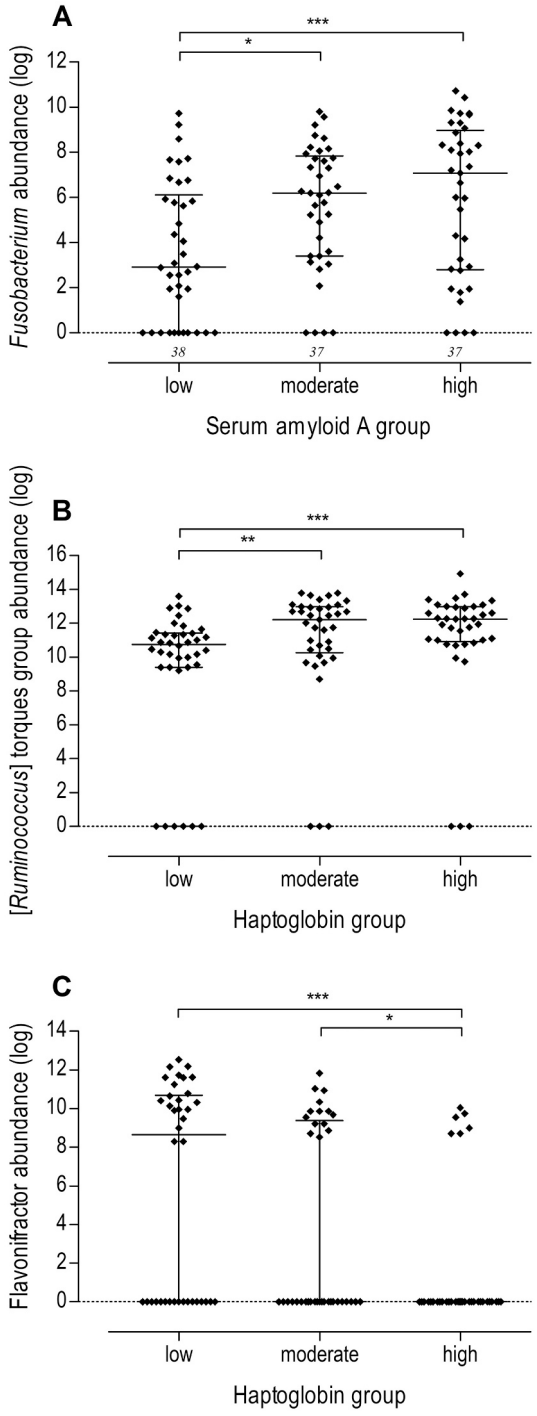

Fig. 6. Association of APPs with three genera, as assessed using negative binomial model. The results of negative binomial models of the associations of serum amyloid A (low, moderate, and high) and *Fusobacterium* abundance (A), as well as those of haptoglobin group (low, moderate, and high) and [*Ruminococcus*] torques group (B), and *Flavonifractor* abundance per sample (C). Log-transformed abundance of bacteria genera, with median and interquartile range, is presented by groups. The small numbers given in italics under the columns represent the number of calves in that group.
* Significant difference, Bonferroni-corrected $p < 0.05$.
** Significant difference, Bonferroni-corrected $p < 0.01$.
*** Significant difference, Bonferroni-corrected $p < 0.001$.

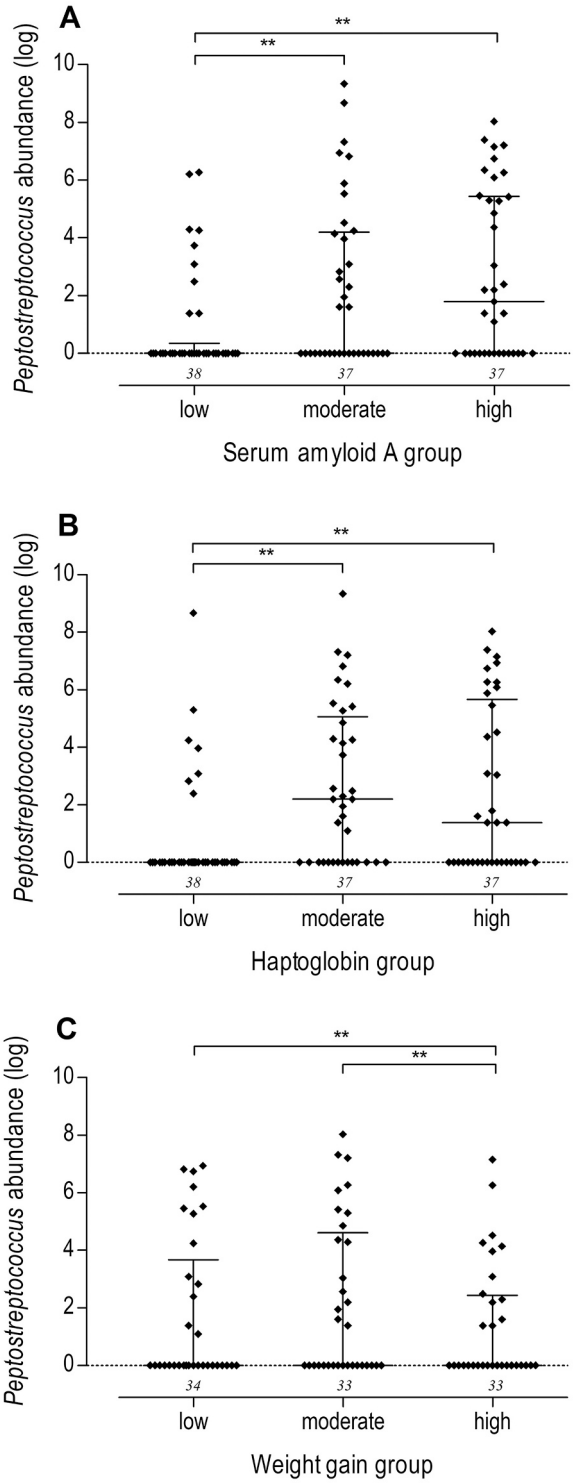

Fig. 7. Associations of APPs and weight gain with *Peptostreptococcus* using negative binomial model. The results of negative binomial models of the associations of serum amyloid A (A), haptoglobin (B) ($n = 112$) and weight gain (C) (n = 100) groups, and *Peptostreptococcus* abundance per sample. Log-transformed abundance of bacteria genera, with median and interquartile range, is presented by groups. The small numbers given in italics under the columns represent the number of calves in that group.
** Significant difference, Bonferroni-corrected $p < 0.01$.
*** Significant difference, Bonferroni-corrected $p < 0.001$.

seem to be relatively negligible compared to those of APPs.

Fusobacterium was the most influential genus explaining SAA, and the fourth most influential genus explaining Hp in the random forest analysis. The positive association between *Fusobacterium* and SAA was confirmed by a negative binomial model. However, *Fusobacterium* was not associated with the calves' ADWG at 9 months. The genus that was positively associated with SAA and Hp and negatively associated with ADWG was *Peptostreptococcus* (Fig. 6). *Peptostreptococcus* spp. are anaerobic, non-spore-forming, gram-positive cocci (Wanger et al., 2017). In humans, an increase in *P. anaerobius* abundance in the faecal and mucosal microbiota is associated with colorectal cancer (Cheng et al., 2020). However, little is known about the effects of this genus in ruminants or other animal species. SAA has been shown to increase in response to microbial colonisation (Murdoch et al., 2019). This process can trigger APR without manifesting as a clinical disease, but as a subclinical infection, and microbiota composition itself might have a long-lasting effect on an animal's weight gain and future immune response. SAA is not simply a marker for inflammation and infection; it can also be seen as a marker for the induction of an adaptive immune response, as it attracts Th2 T-cells (Xu et al., 1995), and thus provides an indication of the production of memory T-cells. The neonatal immune system is biased toward a Th2-dominant response with minimal Th1 activity (Zaghouani et al., 2009), and SAA and IL-6 have been shown to be related to this bias (Furuhashi et al., 2012; Schulte et al., 2008). Furthermore, SAA has been shown to be a soluble pattern recognition receptor related to Th2 immunity (Smole et al., 2020), and thus, SAA can also be a marker for the Th2-bias. These findings suggest that some factors initiating systemic APR affect overall health, and thus the future weight gain of the animal, probably by inducing biased priming of the immune system. We speculate that this is caused by subclinical rather than clinical disease, as our previous studies have shown a negative association between serum SAA concentrations during the second week of life and further gain in clinically healthy ruminants (Orro et al., 2006; Seppä-Lassila et al., 2017; Seppä-Lassila et al., 2018; Peetsalu et al., 2019). In the same calves as in the present study, SAA, Hp, and IL-6 serum concentrations in the second week of life were also negatively associated with ADWG at 9 months (Peetsalu et al., 2022 – submitted for publication). Multiple studies in different species, namely beef calves (Seppä-Lassila et al., 2017), dairy calves (Seppä-Lassila et al., 2018), reindeer calves (Orro et al., 2006), and lambs (Peetsalu et al., 2019) living under different conditions, have found this negative association, which cannot be explained by management factors such as climate, feeding, and colostrum intake. *Peptostreptococcus* may be one of the factors behind this phenomenon of the neonatal period's long-term influence on weight gain.

Serum Hp concentrations were positively associated with [*Ruminococcus*] torques group and negatively associated with *Flavonifractor* abundance (Fig. 5). Although controversial, these results support the possible stimulatory effect of microbiota on the calves' inflammatory responses. Studies in rearing calves (Seppä-Lassila et al., 2018) and lambs (Niine et al., 2018b) also found a positive association between Hp concentration in the second week and future weight gain. Mild inflammation can also be associated with lower systemic Hp concentrations in calves (Arthington et al., 2003; Seppä-Lassila et al., 2015). This can be explained by the binding of blood Hp to free haemoglobin (Eaton et al., 1982), resulting in a decrease in circulating Hp in the blood before the inflammatory response initiates Hp production in the liver. The associations found in these previous studies support our hypothesis that there are some early APR-activating factors that can have a negative long-term impact on an animal's life, including lower weight gain.

5. Conclusions

The results of our study show that there are associations between microbial composition and the systemic innate immune response, mainly with respect to SAA and Hp, but not with pro-inflammatory cytokines. This indicates that the bacterial composition has a systemic effect on neonatal calves. However, most of the variability in the intestinal microbiome does not affect the systemic innate immune response, as only a fraction of all faecal microbial variability can be accounted for by APR marker variation. The positive association of the genus *Peptostreptococcus* and APPs, and the negative association of this same genus and ADWG indicate that a high abundance of certain bacterial genera during early life may have long-term effects on the calf. *Cryptosporidium* spp. infection is negatively associated with microbial diversity. There is a need to further investigate the development and composition of intestinal microbiota to better understand how altering the microbial composition may benefit or harm the calf during the neonatal period and in its future life.

Ethics statement

Sample collection was conducted with permission issued by the Ethical Committee of Animal Experiments of the Estonian Ministry of Agriculture (no. 7.2–11/2).

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2022.07.008.

References

Alipour, M.J., Jalanka, J., Pessa-Morikawa, T., Kokkonen, T., Satokari, R., Hynönen, U., Iivanainen, A., Niku, M., 2018. The composition of the perinatal intestinal microbiota in cattle. Sci. Rep. 8, 1–14. https://doi.org/10.1038/s41598-018-28733-y.

Alsemgeest, S.P.M., Kalsbeek, H.C., Wensing, T., Koeman, J.P., van Ederen, A.M., Gruys, E., 1994. Concentrations of serum Amyloid – a (SAA) and haptoglobin (HP) as parameters of inflammatory diseases in cattle. Vet. Q. 16, 21–23. https://doi.org/10.1080/01652176.1994.9694410.

Amin, N., Seifert, J., 2021. Dynamic progression of the calf's microbiome and its influence on host health. Comput. Struct. Biotechnol. J. 19, 989–1001. https://doi.org/10.1016/j.csbj.2021.01.035.

Andrews, S., Krueger, F., Seconds-Pichon, A., Biggins, F., Wingett, S., 2015. FastQC. A Quality Control Tool for High Throughput Sequence Data. http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/.

Arthington, J.D., Eicher, S.D., Kunkle, W.E., Martin, F.G., 2003. Effect of transportation and commingling on the acute-phase protein response, growth, and feed intake of newly weaned beef calves. J. Anim. Sci. 81, 1120–1125. https://doi.org/10.2527/2003.8151120x.

Baumann, H., Gauldie, J., 1994. The acute phase response. Immunol. Today 15, 74–80. https://doi.org/10.1016/0167-5699(94)90137-6.

Bokulich, N.A., Kaehler, B.D., Rideout, J.R., Dillon, M., Bolyen, E., Knight, R., Huttley, G. A., Gregory Caporaso, J., 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6, 90. https://doi.org/10.1186/s40168-018-0470-z.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C.,

Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K. Bin, Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M.G. I., Lee, J., Ley, R., Liu, Y.-X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A.V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A., Robeson, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37, 852–857. https://doi.org/10.1038/s41587-019-0209-9.

Borcard, D., Legendre, P., Drapeau, P., 1992. Partialling out the spatial component of ecological variation. Ecology 73, 1045–1055. https://doi.org/10.2307/1940179.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.

Carey, M.A., Medlock, G.L., Alam, M., Kabir, M., Uddin, M.J., Nayak, U., Papin, J., Faruque, A.S.G., Haque, R., Petri, W.A., Gilchrist, C.A., 2021. Megasphaera in the stool microbiota is negatively associated with diarrheal cryptosporidiosis. Clin. Infect. Dis. 73, e1242–e1251. https://doi.org/10.1093/cid/ciab207.

Chase, C.C.L., Hurley, D.J., Reber, A.J., 2008. Neonatal immune development in the calf and its impact on vaccine response. Vet. Clin. North Am. - Food Anim. Pract. 24, 87–104. https://doi.org/10.1016/j.cvfa.2007.11.001.

Cheng, Y., Ling, Z., Li, L., 2020. The intestinal microbiota and colorectal cancer. Front. Immunol. 11, 3100. https://doi.org/10.3389/fimmu.2020.615056.

Chester-Jones, H., Heins, B.J., Ziegler, D., Schimek, D., Schuling, S., Ziegler, B., de Ondarza, M.B., Sniffen, C.J., Broadwater, N., 2017. Relationships between early-life growth, intake, and birth season with first-lactation performance of Holstein dairy cows. J. Dairy Sci. 100, 3697–3704. https://doi.org/10.3168/jds.2016-12229.

Dill-Mcfarland, K.A., Breaker, J.D., Suen, G., 2017. Microbial succession in the gastrointestinal tract of dairy cows from 2 weeks to first lactation. Sci. Rep. 7, 1–12. https://doi.org/10.1038/srep40864.

Eaton, J.W., Brandt, P., Mahoney, J.R., Lee, J.T., 1982. Haptoglobin: a natural bacteriostat. Science (80-.) 215, 691–693. https://doi.org/10.1126/science.7036344.

Eckersall, P.D., Bell, R., 2010. Acute phase proteins: biomarkers of infection and inflammation in veterinary medicine. Vet. J. https://doi.org/10.1016/j.tvjl.2010.04.009.

Ege, M.J., Mayer, M., Normand, A.-C., Genuneit, J., Cookson, W.O.C.M., Braun-Fahrländer, C., Heederik, D., Piarroux, R., von Mutius, E., 2011. Exposure to environmental microorganisms and childhood asthma. N. Engl. J. Med. 364, 701–709. https://doi.org/10.1056/NEJMoa1007302.

European Medicines Agency, 2019. EMEA/V/C/000040 - IB/0016 Halocur [product information]. https://www.ema.europa.eu/en/documents/product-information/halocur-epar-product-information_en.pdf. Accessed June 2022.

Ewels, P., Magnusson, M., Lundin, S., Käller, M., 2016. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics 32, 3047–3048. https://doi.org/10.1093/bioinformatics/btw354.

Fan, P., Bian, B., Teng, L., Nelson, C.D., Driver, J., Elzo, M.A., Jeong, K.C., 2020. Host genetic effects upon the early gut microbiota in a bovine model with graduated spectrum of genetic variation. ISME J. 14, 302–317. https://doi.org/10.1038/s41396-019-0529-2.

Furuhashi, K., Shirai, T., Suda, T., Chida, K., 2012. Inflammatory markers in active pulmonary tuberculosis: association with Th1/Th2 and Tc1/Tc2 balance. Kekkaku 87, 1–7.

Hansson, H., Lagerkvist, C.J., 2016. Dairy farmers' use and non-use values in animal welfare: determining the empirical content and structure with anchored best-worst scaling. J. Dairy Sci. 99, 579–592. https://doi.org/10.3168/jds.2015-9755.

Hofstad, T., 2006. The genus Fusobacterium. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E. (Eds.), The Prokaryotes, Proteobacteria: Delta, Epsilon Subclass, vol. 7. Springer New York, New York, NY, pp. 1016–1027. https://doi.org/10.1007/0-387-30747-8_51.

Husso, A., Jalanka, J., Alipour, M.J., Huhti, P., Kareskoski, M., Pessa-Morikawa, T., Iivanainen, A., Niku, M., 2020. The composition of the perinatal intestinal microbiota in horse. Sci. Rep. 10, 1–12. https://doi.org/10.1038/s41598-019-57003-8.

Ianiro, G., Iorio, A., Porcari, S., Masucci, L., Sanguinetti, M., Perno, C.F., Gasbarrini, A., Putignani, L., Cammarota, G., 2022. How the gut parasitome affects human health. Ther. Adv. Gastroenterol. 15, 10–12. https://doi.org/10.1177/17562848221091524.

Ichikawa-Seki, M., Motooka, D., Kinami, A., Murakoshi, F., Takahashi, Y., Aita, J., Hayashi, K., Tashibu, A., Nakamura, S., Iida, T., Horii, T., Nishikawa, Y., 2019. Specific increase of Fusobacterium in the faecal microbiota of neonatal calves infected with *Cryptosporidium parvum*. Sci. Rep. 9, 1–3. https://doi.org/10.1038/s41598-019-48969-6.

Jami, E., Israel, A., Kotser, A., Mizrahi, I., 2013. Exploring the bovine rumen bacterial community from birth to adulthood. ISME J. 7, 1069–1079. https://doi.org/10.1038/ismej.2013.2.

Kelly, D., Coutts, A.G.P., 2000. Early nutrition and the development of immune function in the neonate. Proc. Nutr. Soc. 59, 177–185. https://doi.org/10.1017/S0029665100000197.

Kent, J., 1992. Acute phase proteins: their use in veterinary diagnosis. Br. Vet. J. https://doi.org/10.1016/0007-1935(92)90081-B.

Klein-Jöbstl, D., Schornsteiner, E., Mann, E., Wagner, M., Drillich, M., Schmitz-Esser, S., 2014. Pyrosequencing reveals diverse fecal microbiota in Simmental calves during early development. Front. Microbiol. 5, 1–8. https://doi.org/10.3389/fmicb.2014.00622.

Laurent, F., Lacroix-Lamandé, S., 2017. Innate immune responses play a key role in controlling infection of the intestinal epithelium by Cryptosporidium. Int. J. Parasitol. 47, 711–721. https://doi.org/10.1016/j.ijpara.2017.08.001.

Liaw, A., Wiener, M., 2002. Classification and regression by randomForest. R News 2, 18–22.

Makimura, S., Suzuki, N., 1982. Quantitative determination of bovine serum haptoglobin and its elevation in some inflammatory diseases. Jpn. J. Vet. Sci. 44, 15–21. https://doi.org/10.1292/jvms1939.44.15.

Mammeri, M., Chevillot, A., Thomas, M., Julien, C., Auclair, E., Pollet, T., Polack, B., Vallée, I., Adjou, K.T., 2019. *Cryptosporidium parvum*-infected neonatal mice show gut microbiota Remodelling using high-throughput sequencing analysis: preliminary results. Acta Parasitol. 64, 268–275. https://doi.org/10.2478/s11686-019-00044-w.

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 17, 10–12. https://doi.org/10.14806/ej.17.1.200.

McDonald, V., Smith, R., Robinson, H., Bancroft, G., 2000. Host immune responses against Cryptosporidium. Contrib. Microbiol. 6, 75–91. https://doi.org/10.1159/000060371.

Murdoch, C.C., Espenschied, S.T., Matty, M.A., Mueller, O., Tobin, D.M., Rawls, J.F., 2019. Intestinal serum amyloid a suppresses systemic neutrophil activation and bactericidal activity in response to microbiota colonization. PLoS Pathog. 15, 1–30. https://doi.org/10.1371/journal.ppat.1007381.

Myer, P.R., Freetly, H.C., Wells, J.E., Smith, T.P.L., Kuehn, L.A., 2017. Analysis of the gut bacterial communities in beef cattle and their association with feed intake, growth, and efficiency. J. Anim. Sci. 95, 3215. https://doi.org/10.2527/jas2016.1059.

Nagaraja, T.G., Narayanan, S.K., Stewart, G.C., Chengappa, M.M., 2005. *Fusobacterium necrophorum* infections in animals: pathogenesis and pathogenic mechanisms. Anaerobe 11, 239–246. https://doi.org/10.1016/j.anaerobe.2005.01.007.

Niine, T., Dorbek-Kolin, E., Lassen, B., Orro, T., 2018a. Cryptosporidium outbreak in calves on a large dairy farm: effect of treatment and the association with the inflammatory response and short-term weight gain. Res. Vet. Sci. 117, 200–208. https://doi.org/10.1016/j.rvsc.2017.12.015.

Niine, T., Peetsalu, K., Tummeleht, L., Kuks, A., Orro, T., 2018b. Acute phase response in organic lambs associated with colostrum serum amyloid A, weight gain, and Cryptosporidium and Giardia infections. Res. Vet. Sci. 121, 117–123. https://doi.org/10.1016/j.rvsc.2018.10.013.

O'Handley, R.M., Olson, M.E., 2006. Giardiasis and cryptosporidiosis in ruminants. Vet. Clin. North Am. - Food Anim. Pract. 22, 623–643. https://doi.org/10.1016/j.cvfa.2006.07.002.

Oikonomou, G., Teixeira, A.G.V., Foditsch, C., Bicalho, M.L., Machado, V.S., Bicalho, R. C., 2013. Fecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA. Associations of faecalibacterium species with health and growth. PLoS One 8. https://doi.org/10.1371/journal.pone.0063157.

Ok, M., Yildiz, R., Hatipoglu, F., Baspinar, N., Ider, M., Üney, K., Ertürk, A., Durgut, M.K., Terzi, F., 2020. Use of intestine-related biomarkers for detecting intestinal epithelial damage in neonatal calves with diarrhea. Am. J. Vet. Res. 81, 139–146. https://doi.org/10.2460/ajvr.81.2.139.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H., Szoecs, E., Wagner, H., 2019. vegan: Community Ecology Package. R package version 2.5–6. https://cran.r-project.org/package=vegan.

Orro, T., Nieminen, M., Tamminen, T., Sukura, A., Sankari, S., Soveri, T., 2006. Temporal changes in concentrations of serum amyloid-A and haptoglobin and their associations with weight gain in neonatal reindeer calves. Comp. Immunol. Microbiol. Infect. Dis. 29, 79–88. https://doi.org/10.1016/j.cimid.2006.01.002.

Orro, T., Jacobsen, S., LePage, J.P., Niewold, T., Alasuutari, S., Soveri, T., 2008. Temporal changes in serum concentrations of acute phase proteins in newborn dairy calves. Vet. J. 176, 182–187. https://doi.org/10.1016/j.tvjl.2007.02.010.

Peetsalu, K., Tummeleht, L., Kuks, A., Orro, T., 2019. Serum amyloid A and haptoglobin concentrations in relation to growth and colostrum intake in neonatal lambs. Livest. Sci. 220, 217–220. https://doi.org/10.1016/j.livsci.2019.01.002.

Peetsalu, K., Niine, T., Loch, M., Dorbek-Kolin, E., Tummeleht, L., Orro, T., 2022. Effect of colostrum on the acute-phase response in neonatal dairy calves. J. Dairy Sci. https://doi.org/10.3168/jds.2021-21562.

Petersen, H.H., Nielsen, J.P., Heegaard, P.M.H., 2004. Application of acute phase protein measurements in veterinary clinical chemistry. Vet. Res. 35, 163–187. https://doi.org/10.1051/vetres:2004002.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596. https://doi.org/10.1093/nar/gks1219.

Rahman, S.U., Zhou, K., Zhou, S., Sun, T., Mi, R., Huang, Y., Han, X., Gong, H., Chen, Z., 2022. Curcumin mitigates Cryptosporidium parvum infection through modulation of gut microbiota and innate immune-related genes in immunosuppressed neonatal mice. Microb. Pathog. 164, 105424 https://doi.org/10.1016/j.micpath.2022.105424.

Renz, H., Brandtzaeg, P., Hornef, M., 2012. The impact of perinatal immune development on mucosal homeostasis and chronic inflammation. Nat. Rev. Immunol. 12, 9–23. https://doi.org/10.1038/nri3112.

Rideout, J.R., Chase, J.H., Bolyen, E., Ackermann, G., González, A., Knight, R., Caporaso, J.G., 2016. Keemei: cloud-based validation of tabular bioinformatics file formats in Google Sheets. Gigascience 5. https://doi.org/10.1186/s13742-016-0133-6.

Santín, M., Trout, J.M., Fayer, R., 2008. A longitudinal study of cryptosporidiosis in dairy cattle from birth to 2 years of age. Vet. Parasitol. 155, 15–23. https://doi.org/10.1016/j.vetpar.2008.04.018.

Santoro, A., Dorbek-Kolin, E., Jeremejeva, J., Tummeleht, L., Orro, T., Jokelainen, P., Lassen, B., 2018. Molecular epidemiology of Cryptosporidium spp. in calves in Estonia: high prevalence of Cryptosporidium parvum shedding and 10 subtypes identified. Parasitology 1–7. https://doi.org/10.1017/S0031182018001348.

Schulte, S., Sukhova, G.K., Libby, P., 2008. Genetically programmed biases in Th1 and Th2 immune responses modulate atherogenesis. Am. J. Pathol. 172, 1500–1508. https://doi.org/10.2353/ajpath.2008.070776.

Seale, J., Swain, J., Booth, R., Blake, D.P., 2019. A *Cryptosporidium parvum* genotype shift between week old and two week old calves following administration of a prophylactic antiprotozoal. Vet. Parasitol. 273, 32–35. https://doi.org/10.1016/j.vetpar.2019.07.009.

Seppä-Lassila, L., Orro, T., Lassen, B., Lasonen, R., Autio, T., Pelkonen, S., Soveri, T., 2015. Intestinal pathogens, diarrhoea and acute phase proteins in naturally infected dairy calves. Comp. Immunol. Microbiol. Infect. Dis. 41, 10–16. https://doi.org/10.1016/j.cimid.2015.05.004.

Seppä-Lassila, L., Eerola, U., Orro, T., Härtel, H., Simojoki, H., Autio, T., Pelkonen, S., Soveri, T., 2017. Health and growth of Finnish beef calves and the relation to acute phase response. Livest. Sci. 196, 7–13. https://doi.org/10.1016/j.livsci.2016.12.007.

Seppä-Lassila, L., Oksanen, J., Herva, T., Dorbek-Kolin, E., Kosunen, H., Parviainen, L., Soveri, T., Orro, T., 2018. Associations between group sizes, serum protein levels, calf morbidity and growth in dairy-beef calves in a Finnish calf rearing unit. Prev. Vet. Med. 161, 100–108. https://doi.org/10.1016/j.prevetmed.2018.10.020.

Silverlås, C., Björkman, C., Egenvall, A., 2009. Systematic review and meta-analyses of the effects of halofuginone against calf cryptosporidiosis. Prev. Vet. Med. https://doi.org/10.1016/j.prevetmed.2009.05.003.

Smole, U., Gour, N., Phelan, J., Hofer, G., Köhler, C., Kratzer, B., Tauber, P.A., Xiao, X., Yao, N., Dvorak, J., Caraballo, L., Puerta, L., Rosskopf, S., Chakir, J., Malle, E., Lane, A.P., Pickl, W.F., Lajoie, S., Wills-Karp, M., 2020. Serum amyloid A is a soluble pattern recognition receptor that drives type 2 immunity. Nat. Immunol. 21, 756–765. https://doi.org/10.1038/s41590-020-0698-1.

Thompson, D., Milford-Ward, A., Whicher, J.T., 1992. The value of acute phase protein measurements in clinical practice. Ann. Clin. Biochem. 29 (Pt 2), 123–131. https://doi.org/10.1177/000456329202900201.

Thomson, S., Hamilton, C.A., Hope, J.C., Katzer, F., Mabbott, N.A., Morrison, L.J., Innes, E.A., 2017. Bovine cryptosporidiosis: impact, host-parasite interaction and control strategies. Vet. Res. 48, 42. https://doi.org/10.1186/s13567-017-0447-0.

Van Leeuwen, M.A., Van Rijswijk, M.H., 1994. Acute phase proteins in the monitoring of inflammatory disorders. Baillieres Clin. Rheumatol. 8, 531–552. https://doi.org/10.1016/S0950-3579(05)80114-1.

von Mutius, E., 2007. Allergies, infections and the hygiene hypothesis – the epidemiological evidence. Immunobiology 212, 433–439. https://doi.org/10.1016/j.imbio.2007.03.002.

Wanger, A., Chavez, V., Huang, R.S.P., Wahed, A., Actor, J.K., Dasgupta, A., 2017. In: Wanger, A., Chavez, V., Huang, R.S.P., Wahed, A., Actor, J.K., Dasgupta, A.B.T.-M., M.D. in P (Eds.), Chapter 6 - Overview of Bacteria. Elsevier, pp. 75–117. https://doi.org/10.1016/B978-0-12-805351-5.00006-5.

Wong, S.H., Yu, J., 2019. Gut microbiota in colorectal cancer: mechanisms of action and clinical applications. Nat. Rev. Gastroenterol. Hepatol. 16, 690–704. https://doi.org/10.1038/s41575-019-0209-8.

Xu, L., Badolato, R., Murphy, W.J., Longo, D.L., Anver, M., Hale, S., Oppenheim, J.J., Wang, J.M., 1995. A novel biologic function of serum amyloid A. Induction of T lymphocyte migration and adhesion. J. Immunol. 155, 1184 LP – 1190.

Yeoman, C.J., Ishaq, S.L., Bichi, E., Olivo, S.K., Lowe, J., Aldridge, B.M., 2018. Biogeographical differences in the influence of maternal microbial sources on the early successional development of the bovine neonatal gastrointestinal tract. Sci. Rep. 8, 1–14. https://doi.org/10.1038/s41598-018-21440-8.

Zaghouani, H., Hoeman, C.M., Adkins, B., 2009. Neonatal immunity: faulty T-helpers and the shortcomings of dendritic cells. Trends Immunol. 30, 585–591. https://doi.org/10.1016/j.it.2009.09.002.