Progression of the faecal microbiome in preweaning dairy calves that develop cryptosporidiosis

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Abstract

**Background** Cryptosporidiosis is a diarrheal disease that commonly affects calves under 6-weeks-old. The causative agent, *Cryptosporidium parvum*, has been associated with the abundance of specific taxa in the faecal microbiome during active infection. However, the long-term impact of these microbiome shifts, and potential effects on calf growth and health have not yet been explored.

**Methods** 346 calves from three dairy farms had one faecal swab collected during the first week of life (W1). Thereafter, sampled calves were monitored for diarrhoeal disease and those that suffered a diarrhoea event were tested for *C. parvum* by lateral flow test (LFT). Calves that experienced diarrhoea and tested positive for *C. parvum* by LFT were assigned to the *Cryptosporidium*-positive (*Cp*+) group (n=32). Matched healthy (H) controls with no history of diarrhoea were selected from the remaining cohort (n=33). The selected subset of calves (n=65) were observed until weaning, collecting a faecal swab, at approximately Week 5 (W5) and Week 10 (W10) after birth, resulting in a total of 191 samples (W1; n=65, W5; n=64, W10; n=62). 16S rRNA gene amplicon sequencing was performed on all extracted samples.

**Results** Analysis of the longitudinal microbiome showed significant changes in the microbial diversity and composition across all three time points. Whilst *Firmicutes* were elevated in the *Cp*+ group at W5 compared to the H group, no other significant differences were detected between H and *Cp*+ groups. Whilst the core microbiota showed some taxa were exclusive to each group, the role of these taxa in health and disease has yet to be determined. Antibiotics were also found to have an impact on the relative abundance of some taxa. Though there was a difference in daily live weight gain between H and *Cp*+ groups, it did not reach significance at either W5 and W10, suggesting that *Cp*+ calves can catch up to their healthy counterparts once the infection has resolved.

**Conclusions** The findings of this study illustrate the changes in the microbial diversity and composition during the preweaning period in dairy calves. The results also indicate that the faecal microbiome is not predictive of cryptosporidiosis and imply that cryptosporidiosis doesn't cause long-term gut dysbiosis. This study furthers our understanding of the parasite-microbiome relationship and its impact on the bovine host.

**Introduction**

*Cryptosporidium parvum* is an apicomplexan protozoan and is the leading cause of calf diarrhoea, accounting for approximately 70% of all *Cryptosporidium* infections in cattle [1]. The parasite invades the small intestinal epithelium of cattle, usually within the first six weeks of life, and can cause clinical signs such as watery diarrhoea, weight loss, dehydration, and in especially severe cases, can be fatal. These signs not only compromise cattle health and welfare but also place a financial burden on the farmer. A recent study found that dairy calves infected with *C. parvum* between 3 days and 3 weeks of age incurred an average cost of €40 (~£35) per calf in losses through mortality, labour costs, and veterinary treatment.
Currently, no vaccine exists to tackle the challenge of bovine cryptosporidiosis, with the main treatment options limited to halofuginone, a coccidiostat with variable efficacy, and high toxicity [3].

Given the economic and welfare implications in conjunction with a lack of efficacious therapies, it is of paramount importance to explore alternative approaches for preventing and treating bovine cryptosporidiosis. The infection takes place specifically in the ileum of the small intestine and therefore it is likely that the parasite interacts with, manipulates, and is influenced by the commensal microbes that inhabit the gut. There are a large number of studies that show how the calf gut microbiome develops from birth to weaning in healthy and diarrhoeic calves [4–44]. A handful of studies investigate the progression of the intestinal microbiota in healthy calves [30, 31]. These studies highlight the microbial variation that occurs along the gastrointestinal tract (GIT). For example, they reported that Lactobacillus and Clostridium were the most prevalent genera in the ileum, whilst Prevotella and Faecalibacterium had the highest prevalence in the colon compared to other regions of the GIT [30]. While intestinal microbiome studies show a more accurate picture of the microorganisms that may directly interact with pathogens in the gut, the collection of tissue and digesta samples requires the culling of animals, and prevents follow-up sampling to study the microbiome development within the same animal. Faecal microbiome studies, however, provide the means for longitudinal study designs, and non-invasive sample collection. However, it can only be assumed that the faecal microbiome is a proxy for the microbial diversity and composition of the intestinal microbiome, as they are not directly comparable [45].

Existing research that explicitly studies the associations between the calf microbiome and C. parvum infection is limited. A recent study of calves experimentally infected with C. parvum showed that infection was associated with a high prevalence of Clostridium spp. as well as other potentially pathogenic bacteria such as Escherichia/Shigella, Listeria, and Campylobacter spp. [17]. Other studies have demonstrated that the faecal microorganisms of calves with cryptosporidiosis have a significantly higher abundance of Fusobacterium compared to healthy calves [13, 23]. Whilst these studies focus on the microbiome during C. parvum infection, there is little research on the diversity and composition of the microbiome prior to and post-infection leaving a gap in our knowledge of whether a particular microbiome may be predictive of cryptosporidiosis or whether C. parvum infection causes long-term gut dysbiosis if calves overcome infection. Using whole-genome shotgun sequencing, we previously found that whilst the diversity and composition of the microbiome did not predict infection, specific functional pathways of the microbiome were found to be significantly associated with a predisposition to C. parvum infection [46]. The aim of this study was to determine how the bacterial diversity and composition of the faecal microbiome in healthy and Cryptosporidium-positive calves changed between birth and weaning. The objective was to ascertain taxa which are associated with the pre-/post-infection or healthy faecal microbiome, that could further our understanding of the host-parasite-microbiome relationship, and therefore assist in the development of novel therapeutics.

**Materials and Methods**

**Ethics**
The study was conducted following ethical approval by the University of Liverpool Research Ethics Committee (VREC927) and procedures regulated by the Animals (Scientific Procedures) Act were conducted under a UK Home Office License (P191F589B).

**Animals**

346 female Holstein dairy calves from three commercial dairy farms, based in North Wales and Cheshire, UK (Farm 1, 2, and 3), were enrolled onto the study during the first week of life. Calves had rectal swabs collected at three time points; preweaning pre-\textit{C. parvum} infection (Week 1; \(n = 65\)), then at preweaning post-\textit{C. parvum} infection (Week 5 ± 2; \(n = 65\)), and during the weaning stage post-infection (Week 10 ± 2; \(n = 64\)); classified hereafter as W1, W5, W10, respectively. A subset of the Week 1 samples were also used in a shotgun sequencing study to determine the taxonomic and functional aspects of the faecal microbiome that may be associated with a predisposition to cryptosporidiosis [46]. Calves were weaned at approximately 10-weeks-old. Trimethoprim and sulfadiazine (2.5 mg trimethoprim/12.5 mg sulfadiazine/kg, Diatrim®, Dechra, UK), amoxicillin and clavulanic acid (7.0 mg amoxicillin/1.75 mg clavulanic acid/kg, Synulox™ RTU, Zoetis, UK), and tulathromycin (2.5 mg/kg, Draxxin®, Zoetis, UK) were routine antibiotics prescribed on Farm 2. Halofuginone lactate (100 µg/kg, Halocur®, MSD Animal Health, UK) was administered prophylactically (Farm 1 and 3) or sometimes therapeutically (Farm 2), and therefore treated calves were included in the study. The date of administration and type of treatment were recorded for all subjects.

All calves received a similar diet; cow colostrum was delivered within 24-hours of birth, followed by milk replacer. The calves were then weaned onto a typical cereal and hay-based diet. The veterinary team deemed the breed and farm management of the sample population of calves on all farms to be typical of the UK dairy calf population. Calf health was monitored throughout the study by body condition score (BCS), body weight in kilograms (measured by scales or weigh tape), faecal consistency scoring (0 = Normal, 1 = Semi-formed, pasty, 2 = Loose, but stays on top of bedding, 3 = Watery, sifts through bedding) and the Wisconsin (WI) scoring system to assess respiratory disease, to determine the overall health status of the calves [47, 48]. The serum total protein was quantified within the first week of life to confirm adequate passive transfer of maternal antibodies. Thoracic ultrasonography was employed to detect the severity of historic respiratory damage during the weaning stage (W10) and calves were assigned a lung score of 0–5 (0–1 = Normal, 2 = Lobular/patchy pneumonia, 3–5 = Severe lobar pneumonia − 1, 2 or ≥ 3 lobes affected). Calves did not display clinical signs of cryptosporidiosis at any of the sampling time points. Diarrhoea events usually occurred between the W1 and W5 sampling, though one calf did experience an additional diarrhoea event between the W5 and W10 sampling. The study design is presented in Additional file 1: Fig. S1.

**Sample collection**

Prior to the development of clinical signs of cryptosporidiosis, one faecal swab sample (Sterilin Regular Nylon Flocked Swabs 552C, Scientific Laboratory Supplies, UK) was collected from each of the 346 calves during the first week of life (W1), and snap frozen on dry ice directly after the sampling. Shortly
after the collection, samples were stored at –80°C until DNA extraction. Following W1 sampling, the calves were monitored by experienced veterinary clinicians using a faecal score (0–3) to determine the occurrence of diarrhoea events [48]. Calves that experienced a diarrhoea event after W1 sampling (faecal score of ≥ 2) were assessed for infectious agents using a lateral flow test (LFT) (MSD Rainbow Calf Scour Diagnostic Test, Farmacy, UK), used to detect *Rotavirus, Coronavirus, E. coli F5* (K99) and *C. parvum*. During the study period, 32 calves developed diarrhoea and tested positive for *C. parvum* on the LFT, two of which also tested positive for *Rotavirus*. 33 healthy matched controls were selected from the remaining sampled cohort. The healthy calves were selected on the basis that they showed no clinical signs of diarrhoeal disease during the whole sampling period, though calves with respiratory disease signs were included in the study. The control calves were matched to the *Cp*+ group by age, sex, farm and breed, and as closely matched for administration date and type of prophylactic treatment as possible. Selected calves were sampled again using available swabs at preweaning once diarrhoea events had resolved (W5; n = 65), and again during the weaning stage (W10; n = 64; one calf did not have a sample collected) (Sterilin Regular Nylon Flocked Swabs 552C, Scientic Laboratory Supplies; COPAN FLOQSwabs COPA961C, VWR, UK; Oropharyngeal Specimen Collection Swab MD300235, Medline Scientific, UK). The selected calves that did not experience a diarrhoea event and remained healthy throughout the study were referred to as the Healthy (H) group (n = 33). Calves that experienced a diarrhoea event (faecal score of ≥ 2) and received a positive test result for *C. parvum* were referred to as the *Cryptosporidium*-positive (*Cp*+) group (n = 32). All samples were categorised according to health status and sample time point (H1, H5, and H10; *Cp*+ 1, *Cp*+ 5, and *Cp*+ 10). Some W5 (n = 1) and W10 (n = 2) samples went missing in transit, resulting in a total of 191 samples (W1; n = 65, W5; n = 64, W10; n = 62) that were carried forward for extraction, sequencing, and analysis.

**DNA extraction**

DNA extraction of faecal swab samples (n = 191) was carried out using the DNeasy PowerLyzer Powersoil Kit (QIAGEN, UK) as per the manufacturer’s instructions with the following exceptions. Faecal swabs were transferred into Powerbead tubes, and sterilised scissors (submerged in 100% ethanol and flamed in a Bunsen burner between samples) were used to remove excess swab applicator. 500 µL of Powerbead solution was combined with C1 solution in the Powerbead tubes, which were arranged securely in a 24-tube adaptor on a Vortex Genie 2 for 15 minutes on speed 7.5. Solutions C2 and C3 were mixed 1:1 and 300 µL per sample was mixed with the sample supernatant. This mixture was incubated at 4°C for 5 minutes. 50 µL of Solution C6 was added directly to the centre of the white filter membrane to elute DNA. DNA was quantified by NanoDrop One (ThermoFisher Scientific, UK), and Qubit dsDNA Quantitation, Broad Range Kit and Qubit 3.0 (ThermoFisher Scientific, UK) and the optimum qPCR cycle length of 30 was determined by qPCR. All negative extraction and negative swab controls contained negligible quantities of DNA and so only one negative extraction control was selected to be carried forward for sequencing. DNA samples were stored at –80°C until sequencing.

**Amplicon library preparation**
191 gDNA samples and controls (One negative extraction control, three negative PCR plate controls, and three ZYMO community positive PCR plate controls) underwent PCR using previously described primers to amplify the V4 hypervariable region of the 16S rRNA gene [49]:

F: 5'ACACTCTTCTACACGACGCTCTTCCGATCTNNNNNGTGCCAGCMGCCGCGGTAA3'

R: 5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGAATACHVGGGTWTCTAAAT3'

5 µl of DNA entered a first round PCR with cycling conditions that consisted of 20 seconds at 95°C, 15 seconds at 65°C, 30 seconds at 70°C for 10 cycles then a 5 minutes extension at 72°C.

The primer design incorporated a recognition sequence to allow a secondary nested PCR process. Samples were first purified with AMPure XP Bead-Based Reagent (Beckman Coulter Ltd., UK) before entering the second PCR. The second PCR was performed to incorporate Illumina adapter sequences for sequencing of samples on the Illumina sequencing platforms. Barcodes for sample identification were also included at this point. Eight forward primers (i5) and twelve reverse primers (i7) each contained a separate barcode creating up to 96 different combinations. The barcode sequences were the same as those described in the Illumina Nextera protocol.

The general sequences of the forward and reverse primers are illustrated below. The 10 bp barcode is underlined.

N501 F: 5'AATGATACGGCGACCACCGAGATCTACATAGATCGCATACACTCTTTCCCTACACGACGCTC3'

N701 R: 5'CAAGCAGAAGACGGCATACGAGATTCGCCTTACTGTGACTGGAGTTCAGACGCTC3'

15 cycles of second round PCR were performed using the same conditions as above for a total of 25 cycles. Samples were purified using AMPure XP Bead-Based Reagent before being quantified using the Qubit dsDNA Quantitation High Sensitivity Kit (ThermoFisher Scientific, UK) and assessed using the Fragment Analyzer High Sensitivity NGS Fragment Kit (1-6000 bp) (Agilent Technologies LDA UK Limited, UK). Successfully generated amplicon libraries were taken forward. These final libraries were pooled in equimolar amounts using the Qubit and Fragment Analyzer data and cleaned up with AMPure XP Bead-Based Reagent at a 1:1 ratio. The quantity and quality of the pool was assessed by the Bioanalyzer High Sensitivity DNA Kit (Agilent, USA) and subsequently by qPCR using the KAPA Illumina Library Quantification Kit (Roche Diagnostics Ltd, UK) on a LightCycler LC480II (Roche Diagnostics Ltd, UK) according to manufacturer's instructions. Briefly, a 10 µl PCR reaction (performed in triplicate for each pooled library) was prepared on ice with 8 µl SYBR Green I Master Mix (Roche Diagnostics Ltd, UK) and 2 µl diluted pooled DNA (1:1000 to 1:100,000 depending on the initial concentration determined by the Qubit® dsDNA HS Assay Kit). PCR thermal cycling conditions consisted of 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds and 60°C for 45 seconds, melt curve analysis to 95°C and cooling at 37°C.

**Sequencing**
Following calculation of the molarity using qPCR data, template DNA was diluted to 12 pM and denatured for 5 minutes at room temperature using freshly diluted 0.2 N sodium hydroxide (NaOH) (Merck Life Science UK Limited, UK) and the reaction was subsequently terminated by the addition of HT1 buffer (Illumina®, San Diego, USA). To improve sequencing quality control, 15% PhiX was spiked-in. The libraries were sequenced on the MiSeq platform (Illumina®, San Diego, USA), generating 2x 250 bp and 2x 300 bp paired-end reads. See BioProject PRJEB70717 to access raw sequence data.

**Sequence processing**

The raw fastq files generated by the 16S rRNA gene sequencing were trimmed for the presence of Illumina adapter sequences using Cutadapt version 1.2.1 [50]. The option -O 3 was used, so the 3’ end of any reads which matched the adapter sequence for three or more base pairs were trimmed. The reads were further trimmed using Sickle version 1.2 with a minimum window quality score of 20 [51]. Reads shorter than 15 base pairs after trimming were removed. If only a single read pair passed this filter, it was included in the R0 file. Statistics showing the number of reads and read length per sample were generated using fastq-stats from EAUtils [52] (Additional file 1: Fig. S2-3).

After trimming, R1 and R2 compressed fastq files (.gz) were processed in mothur 1.47.0 using a custom pipeline based on the MiSeq SOP protocol [53, 54]. Contigs were generated and primers removed before aligning the sequences with the SILVA version 132 reference alignment. Sequences were screened and filtered, and unique sequences identified. These unique sequences were pre-clustered, and chimeras were removed before assignment to their taxonomy using the RDP (PDS) training set version 18 reference sequences. Any sequences from chloroplast, mitochondria, Archaea, Eukaryota or unknown lineages were removed and remaining sequences were assigned to operational taxonomic units (OTUs). OTU and consensus taxonomy tables were constructed as well as a tree file of representative OTU sequences using the get.oturep command. The full breakdown of the mothur pipeline is presented in Additional file 2.

**Statistical analysis**

All analysis of the resulting OTU and taxonomy tables was carried out in mothur 1.47.0 and RStudio with R version 4.3.1, using the following R packages: ggplot2 3.4.3, vegan 2.6-4, plyr 1.8.8, dplyr 1.1.3, scales 1.2.1, grid 4.2.2, reshape2 1.4.4, cowplot 1.1.1, phyloseq 1.44.0, ape 5.7-1, tidyverse 2.0.0, ggtext 0.1.2, RColorBrewer 1.1-3, egg 0.4.5, ggrepel 0.6.0, glue 1.6.2, pairwiseAdonis 0.4.1, ggmisc 0.5.4-1, markdown 1.12, ggprism 1.0.4, xlsx 0.6.5, readxl 1.4.3, gridExtra 2.3, euler 7.0.0, microbiome 1.22.0, microbiomeutilities 1.00.17, VennDiagram 1.7.3, glmmTMB 1.1.8, DHARMA 0.4.6, emmeans 1.8.9, car 3.1-2, moments 0.14.1, MaAsLin2 1.15.1, and hilldiv 1.5.1 [53–89]. The distribution of sample sequencing depth was determined (Additional file 1: Fig. S4). All sample sequences were rarefied to 19000 reads per sample before commencing further analysis to account for variation in read depth either in mothur for the diversity analysis or in phyloseq for the compositional analysis (Additional file 1: Fig. S5). 19000 reads
Alpha diversity was quantified by means of observed OTUs and the Shannon index in mothur. The resulting files were plotted in R as bar plots and scatterplots with local regression curves. Significant differences between subject parameters were determined using a pairwise Wilcoxon test with Benjamini-Hochberg correction for the bar plots and loess smoothing was implemented for the scatter plots to highlight trends in the data. The estimated marginal means were calculated to determine if there was a significant difference in alpha diversity between the H and Cp+ groups across the days of sampling.

Beta diversity was calculated by Bray-Curtis and Jaccard distance matrices in mothur using the dist.shared command. PCoA coordinates were produced by the pcoa commands in mothur and plotted in an ordination plot using vegan and ggplot2. Significant differences in beta diversity between H and Cp+ groups and sample time points were determined by a pairwise PERMANOVA test with Benjamini-Hochberg correction using the pairwise.adonis2 function.

Taxonomic relative abundances at phylum and genus level were determined in R using phyloseq. The OTU table was rarefied to 19000 reads per sample and parsed by taxonomic rank. Relative abundances of phyla and genera were plotted in stacked bar charts and the top 30 genera were plotted in individual boxplots. Significant differential abundances of phyla and genera were determined using MaAsLin2. Confounding variables such as farm, and routine treatments were included as fixed effects in addition to the parameters of interest; disease status and sample time point. Sample time point was included as a continuous variable (day of sampling) to account for intra-time point variation, and so the model would consider the three time points longitudinally (Additional files 3 and 5). Study ID was used as a random effect to account for repeated measures from the same animal at different time points. Pairwise multivariate comparisons were conducted using the Benjamini-Hochberg correction to recalculate q-values to correct for multiple testing to determine any significant differences in taxa between H and Cp+ groups within each sample time point. Q-values that were \( \leq 0.25 \) were considered significant (Additional files 4 and 6).

Daily live weight gain (DLWG; kg day\(^{-1}\)) for Farm 2, and total WI score and lung scores for all farms were plotted using ggplot2. A pairwise t-test or Wilcoxon test with Benjamini-Hochberg correction was performed to ascertain any significant differences between the H and Cp+ groups for each of these variables. All statistical analysis is presented in Additional file 2.

**Results**

**C. parvum infection may prolong the time taken for stabilisation of the faecal microbiome**

16S rRNA gene amplicon sequencing was performed in order to ascertain how the diversity of the calf faecal microbiome changed between birth and weaning in calves that developed cryptosporidiosis compared to healthy controls. There was a significant increase across time points in both observed OTUs.
(Median ± SE: W1; 199 ± 7, W5; 445 ± 12, W10; 619 ± 19, W1 vs W5; \(p < 2.22\times10^{-16}\), W1 vs W10; \(p < 2.22\times10^{-16}\), W5 vs W10; \(p = 1.40\times10^{-09}\)) and Shannon diversity (Median ± SE: W1; 2.49 ± 0.07, W5; 3.66 ± 0.05, W10; 4.27 ± 0.06, W1 vs W5; \(p < 2.22\times10^{-16}\), W1 vs W10; \(p < 2.22\times10^{-16}\), W5 vs W10; \(p = 7.90\times10^{-11}\)). However, there was no significant difference in the mean observed OTUs or Shannon diversity between H and \(Cp+\) groups within any of the time points (Fig. 1A-B; H1 vs \(Cp+\) 1; \(p = 0.45\), H5 vs \(Cp+\) 5; \(p = 0.96\), H10 vs \(Cp+\) 10; \(p = 0.56\)). The H and \(Cp+\) groups were stratified by day of sampling to consider intra-group variation and plotted as a local regression (Fig. 1C-D). The H group showed a higher count of observed OTUs by the weaning stage than the \(Cp+\) group but overall there was no significant difference in the estimated marginal means of observed OTUs (\(p = 0.34\), Fig. 1C). In addition, the Shannon diversity became stable during weaning in the H group, as opposed to the \(Cp+\) group where the diversity was still increasing during the weaning stage (Fig. 1D). These differences did not reach statistical significance in the estimated marginal means analysis of the Shannon index (\(p = 0.64\); Additional file 2).

Bray-Curtis PCoA showed that microbiome profiles grouped more closely over time (Fig. 2A), implying that there was a reduction in variance within each community. No statistically significant difference in variance was found between H and \(Cp+\) groups within any time point in either the Bray-Curtis or Jaccard metrics (Fig. 2A-B; \(p > 0.05\)). However, there was a statistically significant difference in both measures of beta diversity across the three time points (Fig. 2A-B; \(p = 0.001\)). This shows that \(C. parvum\) infection had little impact on the pre- or post-infection community diversity, whilst the similarity between community time points showed the greatest variance of all the variables. The reduction in variance over time is consistent with other studies that have investigated the development of the calf faecal microbiome.

**C. parvum has minimal impact on the calf pre- and post-infection faecal microbiome composition**

The faecal microbiome of calves that developed cryptosporidiosis or remained healthy was investigated to determine the impact of infection on the bacterial composition at three time points between birth and weaning. While the most influential variable contributing to the differences in composition of the faecal microbiome was the day of sampling, the relative abundance of the phylum, Firmicutes, was found to be significantly higher in the \(Cp+\) group compared to the H group at W5 (64.2% vs 59.1%; \(q = 9.41\times10^{-02}\), Additional file 4). However, the levels of **Firmicutes** between H and \(Cp+\) groups were no longer significantly different by W10 (\(q = 9.40\times10^{-01}\), Additional file 4). The two most abundant phyla in W1 were **Firmicutes** (50.9%) and **Proteobacteria** (18.0%) and the two most abundant phyla in W5 and W10 were **Firmicutes** (W5; 61.6%, W10; 55.2%) and **Bacteroidetes** (W5; 28.6%, W10; 36.4%). At the phyla level, **Firmicutes** (\(q = 1.61\times10^{-01}\)) and **Bacteroidetes** (\(q = 2.33\times10^{-15}\)) significantly increased in relative abundance over time (Fig. 3A, Additional file 3). Whilst **Proteobacteria** (\(q = 2.85\times10^{-08}\)), **Fusobacteria** (\(q = 5.49\times10^{-02}\)), and **Actinobacteria** (\(q = 2.15\times10^{-07}\)) significantly declined in relative abundance over time (Fig. 3A, Additional file 3).

At the genus level, the relative abundances of **Escherichia/Shigella** (\(q = 3.40\times10^{-08}\)), **Veillonella** (\(q = 1.40\times10^{-08}\)), **Bacteroides** (\(q = 1.42\times10^{-08}\)), **Clostridium sensu stricto** (\(q = 1.32\times10^{-03}\)), **Megamonas** (\(q = 1.40\times10^{-01}\)), **Collinsella** (\(q = 4.58\times10^{-06}\)), **Bifidobacterium** (\(q = 2.58\times10^{-06}\)) significantly decreased over the three time points
(Fig. 3B and 4). Genera that significantly increased over the three time points included *Phascolarctobacterium* (*q* = 1.59E-11), *Prevotella* (*q* = 1.80E-11), *Blautia* (*q* = 1.82E-05), unclassified *Lachnospiraceae* (*q* = 4.95E-12), unclassified *Ruminococcaceae* (*q* = 1.50E-22), and unclassified *Clostridiales* (*q* = 6.54E-17; Fig. 3B; Additional file 5). However, there were no significantly different genera between H and *Cp*+ groups within any of the time points (Additional file 6).

The results indicated that there were no statistically significant differences in any genera between H and *Cp*+ groups within W1, W5 and W10 time points (*q* ≥ 0.25; Additional file 6). This would suggest that the pre-scour relative abundance of genera in the faecal microbiome does not predispose calves to *C. parvum* infection and that *C. parvum* infection does not significantly impact the long-term development of the faecal microbiome post-infection in a farm setting. A core microbiome was established for H and *Cp*+ groups at each time point with an abundance of ≥ 0.01% in ≥ 80% of the samples (Fig. 3C, Additional file 7). H and *Cp*+ shared the majority of their genera, but had some taxa that were found exclusively within each group at the stated abundance and prevalence (Fig. 3C, Additional file 7). The genus found exclusively in the H1 group was *Enterococaceae* unclassified, then *Anaerobutyricum*, *Holdemanella*, *Succinlasticum*, *Escherichia/Shigella*, and *Gallibacterium* were found in H5 and *Ihubacter*, *Anaerobutyricum*, *Dorea*, and *Roseburia* in the H10. The genera found exclusively in *Cp*+1 group were *Enterococcus* and *Lactobacillales* unclassified, then *Alloprevotella*, *Limosilactobacillus*, *Clostridiales incertae sedis XIII* unclassified, *Ruminococcus* 2, and *Erysipelotrichaceae* unclassified were found in *Cp*+5, and there were no taxa exclusive to *Cp*+10. The unique taxa detected in each group were present at very low abundance (< 1%) and we do not yet understand the impact of rare taxa on host health or in the context of disease.

**Antibiotic and anti-cryptosporidial treatment impacts the relative abundance of some taxa in the calf faecal microbiome**

Antibiotic treatment was administered as part of routine farm management practices, and influenced the relative abundance of certain taxa in the microbiome irrespective of *C. parvum* infection. Trimethoprim/sulfadiazine was administered at birth to the majority of Farm 2 calves and significantly reduced the relative abundance of *Terrisporobacter* (*q* = 2.26E-02), *Streptococcus* (*q* = 9.87E-02), *Clostridium sensu stricto* (*q* = 1.19E-01), and *Streptococcaceae* unclassified (*q* = 1.54E-01) within the first week of life (Additional file 6). Trimethoprim/sulfadiazine was associated with a statistically significant reduction in *Enterococcus* (*q* = 8.29E-03), *Lactococcus* (*q* = 4.08E-02), and *Atopobiaceae* unclassified (*q* = 2.05E-01) in W5 samples (Additional file 6). The antibiotic, amoxicillin/clavulanic acid was also prescribed to some calves and significantly increased the abundance of *Bacteroides* (*q* = 4.08E-02), *Paraeggerthella* (*q* = 8.94E-02), and *Tyzzerella* (*q* = 2.37E-01) and significantly reduced the abundance of *Terrisporobacter* (*q* = 5.01E-02) with in the first week of life (Additional file 6). Tulathromycin was only administered after W1 sampling, and it significantly reduced the relative abundance of *Agathobaculum* (*q* = 1.15E-01), and *Enterococcus* (*q* = 1.54E-01) in the W5 samples (Additional file 6). In W10 samples, tulathromycin raised the relative abundance of *Clostridia* unclassified (*q* = 1.13E-01), and *Oscillibacter* (*q* = 2.05E-01), whilst a reduction in the relative abundance of *Coprococcus* (*q* = 8.94E-02), *Peptacetobacter*
(q = 2.05E-01), *Agathobaculum* (q = 2.05E-01), *Faecalibacterium* (q = 2.42E-01), and *Allisonella* (q = 2.46E-01) was observed (Additional file 6). Halofuginone lactate was prescribed either prophylactically or therapeutically and was significantly associated with an increase in the relative abundance of *Porphyromonas* (q = 8.94E-02), *Clostridiales incertae sedis* XIII unclassified (q = 2.05E-01), and *Eggerthellaceae* unclassified (q = 2.37E-01) in W5 samples (Additional file 6). Though the taxa associated with treatment may be present at a low abundance in the microbiome, a change in their proportion due to antibiotic usage could still impact overall calf health and recovery from infection.

**C. parvum infection does not significantly impact long-term calf weight gain or respiratory health**

As *C. parvum* infection did not significantly impact upon the development of the microbiome, and the composition of the microbiome has previously been linked to growth, we wanted to determine if the results of this analysis were reflected in other indicators of calf health including daily live weight gain (DLWG; kg day$^{-1}$), total Wisconsin (WI) scores and lung scores. As part of the health monitoring that was conducted throughout the study, calves on Farm 2 were weighed at each time point. The data showed that the DLWG was not significantly impacted by *C. parvum* infection though it did approach statistical significance (Fig. 5A, H5 vs Cp + 5; p = 0.069, H10 vs Cp + 10; p = 0.091). This would suggest that *C. parvum* infection may reduce the calf DLWG marginally, however, the infected dairy calves appeared to have sufficient resilience to maintain an adequate DLWG post-infection.

Calves also received a WI score (0–3) for four signs of respiratory disease including a cough, nasal discharge, eye, and ear score. The sum of these scores reflects the severity of respiratory disease. Calves with two or more clinical parameters scoring 2–3 or a total score of ≥ 5 are considered to have respiratory disease. In W10, calves underwent thoracic ultrasonography to detect historic lung damage and were given a lung score (0–5). Comparison of total WI scores and lung scores between H and Cp + groups showed that there was no statistically significant difference between the groups (Fig. 5B-C). In spite of this, Cp + 10 calves had a tendency towards higher WI scores than H10 calves (Fig. 5B; p = 0.14), suggesting that *C. parvum* may have some influence on the severity of respiratory infection, which could be attributed to the weakening of the immune system as a result of infection.

**Discussion**

The main aim of this study was to describe differences between the faecal microbiomes of pre- and post-infection calves that developed cryptosporidiosis or remained healthy, to determine if *C. parvum* impacts the overall progression of the microbiome during the preweaning stage of life. Based on previous studies of the calf microbiome that investigated during active *C. parvum* infection, we predicted that there would be significant differences in certain bacterial taxa in the Cp + group compared to the H group prior to and following infection. Despite some studies showing statistically significant taxa differences between the microbiomes of diarrhoeic Cp + calves and the microbiomes of healthy control calves, it appears that the pre-infection faecal microbiome does not show any features that may predispose calves to cryptosporidiosis. This result is in line with the previously cited study we conducted that examined the
microbiome of the same calves prior to *C. parvum* infection using shotgun sequencing, and found no statistically significant differences in diversity or composition between H and *Cp +* groups in the W1 time point [46]. While the relative abundance of the phylum, *Firmicutes*, was found to be significantly higher in the *Cp +* group compared to the H5 group, this difference did not remain significant in the W10 time point, suggesting that *C. parvum* infection may not have a long-lasting impact on the faecal microbiome. A higher abundance of *Firmicutes* has previously been observed in *C. parvum* infected mice, however another study reported a lower relative abundance of *Firmicutes* in *C. parvum* infected mice compared to uninfected controls, which only emphasises the considerable variability between the microbiomes of *C. parvum* infected hosts [90, 91]. Interestingly, *Faecalibacterium* was present at a higher median relative abundance in the H group compared to the *Cp +* group at every time point. Whilst this difference was not statistically significant, another study has shown a positive association between *Faecalibacterium* and better health, improved growth, and reduced incidence of diarrhoea in dairy calves, implying the probiotic potential of this bacterium [36]. Other studies have observed a specific increase in *Fusobacterium* during infection, however, there was no statistically significant difference in this bacterium between H and *Cp +* groups before or after infection in this study [13, 23]. Our findings give us an indication of the causality of the changes to the microbiome observed in other studies, demonstrating further that they must be caused by *C. parvum*. In addition, the study outcome indicates that in a farm setting, there is no particular microbial composition or shared pre-disposing factor that could be targeted therapeutically/prophylactically. This is noteworthy, as in an experimental setting, certain taxa may be shown to predispose animals to infection, yet targeting these taxa in a farm setting may not be beneficial for the majority of animals. The results of this study also imply that the dysbiosis observed in the faecal microbiome of calves with an active infection in other studies were caused by direct parasite interactions with the microbiota. The outcome of our study paints a positive picture of the impact of *C. parvum* infection on the microbiome in dairy calves, as there appear to be no statistically significant long-term effects on the development of the calf microbiome which is essential for good health and the development of a strong immune system.

Whilst the impact of *C. parvum* on the pre- and post-infection calf faecal microbiome was minimal, the changes in diversity and the relative abundance of several taxa over time were substantial. There are various studies that have examined the development of the calf faecal microbiome, particularly during the first week of life, as this is the critical stage when calves are most vulnerable to infection [4, 6–9, 11, 12, 20–22, 24–27, 32, 33, 35–37, 40, 41, 44, 92]. The majority of studies correspond with our results showing an increase in alpha diversity and a reduction in beta diversity with age, and that the most abundant phyla prior to weaning are *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*. Several studies with comparable results showed that during the preweaning period, *Faecalibacterium* relative abundance increased and then declined again, *Bacteroides* and *Escherichia/ Shigella* are highly abundant in the first week of life and then drop off over time, whilst *Ruminococceae*, *Lachnospiraceae* and *Prevotella* became more highly abundant later in the pre-weaning period [8, 21, 22, 24, 26, 27]. Other studies also exhibited trends not observed in our data, for example one study showed relatively high levels of *Bacteroides-Prevotella* (33.0-60.6%) and *Clostridium-Eubacterium* (9.90–19.0%) groups between
1–12 weeks of age [40]. However, in our study, these genera made up a much smaller proportion of the overall composition and were far more varied in abundance between time points. There is a lot of variation in the taxa that may characterise different age groups between calf faecal microbiome studies largely due to differences in study design, diet, breed, location, and farm management practices. The variation during the preweaning stage may create a challenge in the development of efficacious pre-/pro-/post-biotics for the maintenance of healthy calves or to mitigate disease.

The only step farmers took to mitigate cryptosporidiosis on the participating farms was the administration of halofuginone lactate either prophylactically (Farm 1 and 3) or therapeutically (Farm 2), though this was not always consistent. Therefore, a starker difference between healthy and infected calf microbiomes may have been observed pre- and post-infection had the calves not received anti-cryptosporidial treatment. We found that halofuginone lactate had a positive association with *Eggerthellaceae* unclassified, *Clostridiales incertae sedis* XIII unclassified, and *Porphyromonas*. *Porphyromonadaceae* bacterium DJF B175 has been associated with high daily weight gain in calves, so perhaps halofuginone lactate could improve weight gain in calves if it also promotes this type of bacteria [27]. One study shows that calves that received halofuginone lactate had a higher mean relative abundance of *Actinobacteria* and higher microbial diversity during a cryptosporidiosis outbreak compared to untreated calves [13]. While this trend was not observed in our study, the current literature is not sufficient to explain the associations that were identified.

The administration of various antibiotics had an effect on the relative abundance of certain taxa in the faecal microbiome of the calves. This was an expected outcome as it is well-documented that antibiotics can alter the calf gut microbiome [93–103]. Most of these studies were not specific to the particular antibiotics used on the farms in this study, and often studied the effect of a mixture of antibiotics or waste milk containing antibiotic residues. In our study, tulathromycin seemed to have the most impact on taxa in the order *Clostridiales*, having caused a statistically significant change in the relative abundance of genera within this taxon. A study looking at the impact of antibiotics on the faecal microbiome also found that treatment had the most influence on the order of *Clostridiales* [41]. One study showed that tulathromycin reduced the relative abundance of *Bifidobacterium* within the first week of life and increased the abundance of *Escherichia/Shigella* at around 3 weeks [104]. In two other studies that investigated the effect of tulathromycin on the faecal microbiome in cattle, they found that this antibiotic had no statistically significant impact on the microbiome [105, 106]. Differences in the study design, diet, breed, location, and farm management practices likely account for the inconsistencies observed between studies.

Our findings suggest that *C. parvum* infection does not significantly affect daily live weight gain in dairy calves. This could be attributed to the treatment of *Cp*+ calves with halofuginone lactate following the diagnosis of cryptosporidiosis on Farm 2, reducing the severity of infection, leading to the mitigation of weight loss. One study showed that correct treatment with halofuginone lactate given to calves with cryptosporidiosis was negatively associated with weight gain compared to untreated infected calves at 3 months of age which the authors explain may be due to the delay and potential expansion of the lifecycle.
of *C. parvum* induced by halofuginone lactate [107]. In contrast, a study conducted on beef calves showed a statistically significant difference in weight gain at 6 months between healthy calves and calves that developed severe cryptosporidiosis and were treated with halofuginone lactate, though this could be as a result of high disease severity, breed differences or differing farm management practices [108].

A previous study has shown an association between diarrhoea incidence and respiratory disease, possibly linked to a weakened immune system or inconsistent colostrum supplementation [109]. Though there were some small differences in total WI scores and lung scores between the H and Cp+ groups, and the Cp+10 calves had a tendency towards higher WI scores than the H10 calves, these differences did not reach significance. Moreover, the administration of prophylactic antibiotics may have contributed to the overall low severity of respiratory signs observed in the calves. Whilst the study was designed to prioritise the collection of faecal samples for microbiome analysis, the recording of weight and respiratory scores was not our primary objective and therefore the data may not provide enough power to detect significant differences.

**Limitations**

The study had limitations that may have had an impact on the final results and therefore, we will reflect on them here.

The main limitation of the study design was the use of LFT to diagnose the calves with cryptosporidiosis. Whilst this method of detection doesn't have the highest sensitivity/specificity of all the available tests, it does have the advantage of being easy-to-use, cost-effective, and fast especially for a study where great numbers of large animals were required to be tested on a farm environment. The positive LFT coupled with the confirmation of diarrhoea by experienced veterinary clinicians was regarded as adequate criteria for the diagnosis of cryptosporidiosis.

It is challenging to draw robust conclusions on the effect of specific antibiotics from this data as they were only prescribed on one of the farms and therefore significant differences could also be attributed to the farm location or other environmental factors. In addition, all of the taxa were present at an abundance of less than 1% and therefore can be classified as rare or low abundance. While the antibiotics may have affected these taxa, we do not yet understand the role that low abundance taxa play in the microbiome and whether they have a substantial impact on calf health and resistance to infection.

Some samples went missing in transit, which may marginally reduce the power of the study. However, these samples are missing completely at random (MCAR) and therefore do not introduce bias to the study design.

**Conclusion**
To summarise, *C. parvum* infection had minimal impact on the microbial diversity and composition of the calf faecal microbiome pre- or post-infection when compared with healthy calves, with age having the greatest impact on the development of the microbiome. This leads us to speculate that the changes observed in other studies are a result of direct interactions with the parasite during infection and not a consequence of pre-existing microbiome differences. In addition, *C. parvum* does not affect the overall long-term progression of the microbiome. Though this is surprising it is not an unwelcome outcome as it is indicative of the calf’s ability to restore the gut microbiome post-infection, which is important for overall health and protecting cattle against further gastrointestinal disease.

**Abbreviations**

*C. parvum*

*Cryptosporidium parvum*

*Cp+

*Cryptosporidium*-positive group

H

Healthy control group

GIT

Gastrointestinal Tract

FMT

Faecal Microbiota Transplantation

LFT

Lateral Flow Test

BCS

Body Condition Score

WI

Wisconsin

CGR

Centre of Genomic Research, University of Liverpool

DLWG

Daily Live Weight Gain

**Declarations**

**Ethics approval**

The study was conducted following ethical approval by the University of Liverpool Research Ethics Committee (VREC927), and procedures regulated by the Animals (Scientific Procedures) Act were conducted under a United Kingdom (UK) Home Office Licence (P191F589B).

**Consent for publication**
Not applicable

Availability of data and material

All raw sequence data was deposited in the European Nucleotide Archive under BioProject: PRJEB70717.

Competing interests

Not applicable

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Authors' contributions

MFH was involved with the study design and managed the sample processing and QC, as well as sequence processing in mothur and microbiome analysis in mothur and R. MFH also prepared Fig. 1-5 and Additional file 1: Fig S1, S4-5, and wrote the main manuscript. BEG conducted the faecal sample collection, storage, and transport and provided all relevant metadata. LB generated the sequencing libraries. EEV sequenced the libraries on the MiSeq platform (Illumina®, San Diego, USA). RG conducted the initial trimming of the raw fastq data and provided Additional file 1: Fig. S2-3. CJS provided guidance/supervision for the sample DNA extractions and sequence processing in mothur and the statistical analysis in R. CJS, JSD, JLC, and GO all contributed to the funding, and study design, and provided guidance/supervision on the sample processing, data analysis, and writing of the manuscript. All authors read and approved the final manuscript.

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Figure 1

**Alpha diversity increased with age but was not influenced by Cryptosporidium parvum.**

**A** Observed OTUs between healthy control and Cryptosporidium-positive groups at Week 1, 5 and 10. A pairwise Wilcoxon test shows differences in observed OTUs between time points. The red dots indicate the mean.

**B** Shannon diversity between healthy control and Cryptosporidium-positive groups at Week 1, 5 and 10. A pairwise Wilcoxon test shows differences in Shannon indices between time points. The red dots indicate
the mean. C Local regression (LOESS) of observed OTUs between healthy control and Cryptosporidium-positive groups stratified by day of sampling shows within time point variation. D Local regression (LOESS) of Shannon diversity between healthy control and Cryptosporidium-positive groups stratified by day of sampling shows within time point variation.

Figure 2
Beta diversity altered with age but was not impacted by *Cryptosporidium parvum*. **A** Weighted Bray-Curtis PCoA plot shows similarity between healthy control and *Cryptosporidium*-positive groups at Week 1, 5 and 10, and dissimilarity between sample time points (H1 vs Cp+1, $p = 0.49$; H5 vs Cp+5, $p = 0.40$; H10 vs Cp+10, $p = 0.89$; W1 vs W5 vs W10, $p = 0.001$). **B** Unweighted Jaccard PCoA plot displays similarity between healthy control and *Cryptosporidium*-positive groups at Week 1, 5 and 10, and dissimilarity between sample time points (H1 vs Cp+1; $p = 0.20$, H5 vs Cp+5; $p = 0.69$, H10 vs Cp+10; $p = 0.90$; W1 vs W5 vs W10, $p = 0.001$).
Figure 3

The pre-/post-infection taxonomic composition and core microbiome were minimally impacted by *Cryptosporidium parvum*. A Top 5 most abundant phyla at Week 1, 5 and 10 in healthy control and *Cryptosporidium*-positive groups. B Top 15 most abundant genera at Week 1, 5 and 10 in healthy control and *Cryptosporidium*-positive groups. C Venn diagrams of the core microbiota in healthy control and *Cryptosporidium*-positive groups at Week 1, 5 and 10 time points. Genera were detected at 0.01% abundance in at least 80% of the samples.
Figure 4

The most abundant genera experienced changes in relative abundance over time. Relative abundance of the top 30 most abundant genera for healthy control and Cryptosporidium-positive groups at Week 1, 5, and 10. The red dot shows the mean relative abundance.
Figure 5

Daily live weight gain and respiratory disease scoring was not impacted by *Cryptosporidium parvum*. The red dots indicate the mean. A Daily live weight gain in kilograms per day of healthy control and *Cryptosporidium*-positive groups at Week 5 and 10 on Farm 2. B Total Wisconsin scores for healthy control and *Cryptosporidium*-positive groups at Week 1, 5, and 10. C Lung scores for healthy control and *Cryptosporidium*-positive groups at Week 10.
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFile1SupplementaryFigures.pptx
- AdditionalFile2AnalysisPipeline.pdf
- AdditionalFile3MaAsLin2PhylumLongitudinalResults.txt
- AdditionalFile4MaAsLin2PhylumPairwiseResults.txt
- AdditionalFile5MaAsLin2GenusLongitudinalResults.txt
- AdditionalFile6MaAsLin2GenusPairwiseResults.txt
- AdditionalFile7CoreMicrobiome.xlsx
- AdditionalFile8Metadata.txt