

Dynamic Changes of Intestinal Microbiota and Metabolite Composition in Pre-Weaned Beef Calves

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Research

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Abstract

Background: Gut microbes and their metabolites are essential for maintaining host health, but few studies have elucidated the combined effects of microbial and metabolite interactions on the growth and development of pre-weaned calves over time. Therefore, the aim of this study was to explore dynamic changes of intestinal microbiota and metabolites among newborn calves classified as healthy, sub-healthy, and those that died early during their growth and development.

Results: 16S rRNA gene sequencing and metabolomics analysis was employed to track the dynamic changes in faecal microflora abundance and metabolite levels (fatty acids and amino acids) in calves before weaning. The results demonstrated that the alpha diversity of the faecal microbiota increased with calf growth and development. Specifically, the abundances of *Porphyromonadaceae bacterium* DJF B175 and *Alistipes shahii* gradually increased in healthy calves over time. Inversely, the abundance of *Enterobacteriaceae* was higher in the sub-healthy group than in the healthy group. Meanwhile, the faeces of calves in the early death group had significantly higher medium-long-chain fatty acid concentration than those in the healthy group. The faecal amino acid concentration decreased significantly with weekly age in the healthy and sub-healthy groups.

Conclusions: The study findings provide a new understanding of calf growth and development prior to weaning. Even under the same management conditions, microorganisms and their metabolites interact to play different dynamic regulatory roles in the growth and development of newborn calves. Further studies are warranted to determine the mechanisms involved.

Background

The calf stage is the most critical period for cattle growth and development, with the survival and growth rates of calves directly impacting economic benefits. The gut microbiota is established during the first 7 weeks of life and is associated with calf health, diarrhoea, and weight gain [1]. In humans, the gut microbiota undergoes dynamic changes in childhood, acquiring several bacterial taxa that are beneficial to human health [2]. On the one hand, the immature gut microbiota is thought to influence the growth and energy metabolism of infants [3, 4]. On the other hand, the microbiota is particularly vulnerable to antibiotic damage in infancy, and its disruption may impact growth in early life [5]. In ruminants, the calf intestine also undergoes rapid microbial structural changes that are closely linked to gut health and disorders, while supporting nutrient absorption and metabolism to promote calf growth [6, 7].

Intestinal microbiota imbalance in newborn animals causes diarrhoea, along with the discharge of large amounts of harmful intestinal bacteria, which can further cause intestinal infections and immune disorders [8]. Faecal microbiota transplantation (FMT) and microbiological intervention can improve growth performance and regulate calf health [9]. Furthermore, studies have shown that probiotic intake can improve the growth performance of calves. For example, oral administration of *Feacalibacterium prausnitzii* reportedly improved the gastrointestinal health and weight gain of pre-weaned calves [10].

Further, calves fed *Saccharomyces cerevisiae boulardii* and *Lactobacillus acidophilus* exhibited reduced abundance of pathogenic genera *Streptococcus* and *Tyzzarella 4*, while the abundances of beneficial genera *Ruminococcus*, *UCG-005*, *Roseburia*, and *Olsenella* were increased [11]. Increased proportions of these beneficial bacteria improve the gastrointestinal tract (GIT) environment of ruminants, promote calf growth and development, and reduce deaths due to diarrhoea.

Metabolites such as fatty acids (FAs) and amino acids (AAs) produced by gut microbes can regulate the health, growth, and development of the host [12–14]. A previous study of cow calf intestinal microbiota and metabolites during growth and development revealed that the abundance of microorganisms related to AA, carbohydrate, and energy metabolism was increased, and the short-chain fatty acid (SCFA) concentration was directly related to carbohydrate utilisation [15]. Moreover, medium-chain fatty acids (MCFAs) are known to be absorbed directly via the portal vein and exert antibacterial effects that may prevent the overgrowth of pathogenic bacteria in the GIT [16].

In order to determine which strains and their metabolites in the intestinal tract may promote healthy calf growth, and which factors may be responsible for growth retardation and even death during the pre-weaning period. We tracked dynamic changes of faecal microbiome composition and metabolite levels in calves classified as healthy, sub-healthy, and those that died within 2-3 weeks of birth using 16S rRNA gene sequencing and metabolomics analysis, and explored their correlation with the growth and development of calves.

Methods

Animals management and grouping

The animal experiments were approved by the Ethics Committee of Yanbian University (SYXK2020-0009). Eighteen newborn Yanbian yellow cattle calves were selected from a specialised breeding farm in Yanji City (Jilin Province China) and their growth performance was tracked under strict management. Mothers and calves were separated from the herd, and calves were fed only their mothers' milk for the first three days of life. Subsequently, all calves were free to ingest calf starter and water. From birth to the end of the experimental period, each calf resided with its mother. All calves had access to a physical space where they could eat feed and interact with other calves. All groups were provided the same calf starter in a feeding shed to minimise interindividual differences caused by feeding or diet. Throughout the study, the floor of the feeding shed was cleaned frequently to keep it dry. Buckets and feed pails were cleaned daily. Calves were not treated with any medications during the study. Five of the 18 calves died between 2 and 3 weeks after birth and were classified as the dead group (D). The remaining 13 calves were divided into healthy (H) and sub-healthy (SH) groups based on whether their net weight gain over the 8-week period was above average.

Faecal sample collection and body size measurement

Faecal samples were collected from calves by rectal enema while wearing sterile, disposable, laboratory latex gloves. No faecal samples were collected on the day of birth, considering that the calves were weak and might not defaecate that day. Therefore, only physical data were measured on the day of birth. Faecal samples and body data were collected weekly for 8 weeks. The obtained faeces were immediately loaded into clean, sterilized 1.5 mL centrifuge tubes, transported to the laboratory in dry ice, and stored at -80°C for subsequent processing. Parameters of calf body size including body weight, body length, and body height and so on, were measured when the calf was upright and stable [17].

Faecal microbial profiling

The collected calf faeces were analysed by 16S rRNA gene amplicon sequencing of the bacterial V3-V4 region. Genomic DNA was extracted from the samples using CTAB or SDS methods. The purity and concentration of DNA were determined by agarose gel electrophoresis. After detection, sample DNA was diluted to 1 ng/ μL with sterile water in a centrifuge tube. Using diluted genomic DNA as a template, amplification of the V3-V4 region was conducted using specific primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') with barcodes, along with Phusion® High-Fidelity PCR Master Mix with GC buffer (New England Biolabs, Ipswich, MA, USA), according to the manufacturer's instructions. The PCR products were purified using magnetic beads and detected by 2% agarose gel electrophoresis after fully mixing. Finally, the PCR products were recovered from the target strip using a gel recovery kit (Qiagen, Hilden, Germany). The TruSeq® DNA PCR-free Sample Preparation Kit (Illumina Inc., San Diego, CA, USA) was used for library construction. The constructed library was quantified using Qubit (Thermo Fisher Scientific, Waltham, MA, USA) and qPCR. Sequencing was conducted using the NovaSeq 6000 in PE250 mode (Illumina Inc.) by Novogene Co., Ltd. (Beijing, China).

Sample data were separated according to barcode and PCR amplification primer sequences. After removal of barcode and primer sequences, FLASH version 1.2.7 software (<http://ccb.jhu.edu/software/FLASH/>) was used to splice the reads of each sample, expressed as raw tags. The raw tags underwent strict filtering to obtain high-quality clean tag data, which were compared with the species annotation database to detect and remove chimeric sequences, yielding the final effective tags (<https://github.com/torognes/vsearch/>). Uparse algorithm version 7.0.1001 software (<http://www.drive5.com/uparse/>) was used to determine the effective clustering tags [18]. Sequences were clustered into operational taxonomic units (OTUs) with 97% identity, and representative OTU sequences were selected. Species annotation was performed on representative OTU sequences using the Mothur method and SSUrRNA database of SILVA138 (<http://www.arb-silva.de/>) (threshold set at 0.8~1) to obtain taxonomic information at kingdom, phylum, class, order, family, genus, and species levels [19, 20]. MUSCLE version 3.8.31 software (<http://www.drive5.com/muscle/>) was used to conduct multiple sequence alignment [21]. Finally, data from all samples were homogenized using the sample with the least amount of data as the standard. Subsequent alpha and beta diversity analyses were based on the homogenised data. QIIME version 1.9.1 software was used to calculate Chao1, ACE, Shannon, and Simpson indices.

Fatty acid analysis

SCFAs were extracted from calf faeces using an acetonitrile-water mixture (1:1 v/v) and derivatized using 3-nitrophenylhydrazinehydrochloride. SCFAs were analysed using a Jasper HPLC system coupled to a 4500MD mass spectrometer (SCIEX, Framingham, MA, USA). Briefly, individual SCFAs were separated on a Kinetex C18 column (100 x 2.1 mm, 2.6 μ m; Phenomenex, Torrance, CA, USA) using 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). Octanoic acid-1- 13 C (Sigma-Aldrich, St. Louis, MO, USA) and butyric-2,2-d $_2$ (CDN Isotopes, Pointe-Claire, QC, Canada) were used as internal standards for quantitation [22].

Long chain fatty acids (LCFAs) were extracted from calf faeces using a modified version of Bligh and Dyer's method, as previously described [23]. Briefly, tissues were homogenised in 750 μ L chloroform-methanol mixture (1:2 v/v) with 10% deionised water, followed by incubation at 4°C for 30 min. Subsequently, 350 μ L deionised water and 250 μ L chloroform were added to the mixture. The lower lipid-containing organic phase of samples was extracted into a clean tube. Lipid extraction was performed twice, and the lipid extracts were pooled into a single tube and dried under vacuum. Samples were stored at -80°C until further analysis.

LCFAs were analysed using a 1290 Infinity UHPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled with a QTRAP 6500+ mass spectrometer (SCIEX), as previously described [24, 25]. Lipids were separated by normal-phase (NP)-HPLC using a Luna silica column (150 x 2.0 mm, 3 μ m; Phenomenex) with chloroform-methanol-ammonium hydroxide (89.5:10:0.5 v/v/v; mobile phase A) and chloroform-methanol-ammonium hydroxide-water (55:39:0.5:5.5 v/v/v/v; mobile phase B). Internal standards 16:0-d $_3$ 1 (Sigma-Aldrich) and 20:4-d $_8$ (Cayman Chemical Company, Ann Arbor, MI, USA) were used for quantitation.

Amino acid analysis

Faecal samples were extracted with a methanol-water mixture (8:2 v/v), vortexed, incubated at 4°C for 30 min, and then centrifuged at 4°C for 5 min at 12,000 rpm. Subsequently, the supernatant was dried under vacuum with no heating, and the sample was reconstituted in a 2% acetonitrile aqueous solution containing a mixture of isotope standards. Following vortex mixing and centrifugation, the supernatant was analysed by LC-MS/MS, as previously described [24].

Statistical analysis

All statistical analyses were performed using GraphPad Prism software (San Diego, CA, USA) and IBM SPSS version 17.0 software (IBM Corp., Armonk, NY, USA). All data are expressed as the mean \pm standard error of the mean (SEM). Student's *t*-test or repeated measure analysis of variance (ANOVA) was used to compare groups. Statistical significance was set at $P < 0.05$.

Results

Experimental design and sample collection

To date, most studies investigating the growth and development of dairy calves have reported comparisons between healthy and diarrhoeal calves [26, 27]. However, we believe that the increase degree in body weight better reflects healthy growth and development. To this end, body parameters were measured along with faecal sample collection for 8 weeks after the birth of each calf (Fig. 1a). A total of 18 calves were included in the study, from which 708 faecal samples were collected. After grouping (Fig. 1b), the body weights, body lengths, and rump widths of calves in groups H and SH differed significantly (Fig. 1c-e and Additional file 1: Figure S1a). However, the body heights, hip heights, and chest, abdominal, and cannon circumferences did not differ among the groups (Additional file 1: Figure S1b, c).

Dynamic changes in faecal microbiota of healthy calves

The growth and development of calves are accompanied by transformation of the faecal microbiota [28, 29]. The results of non-metric multidimensional scaling (NMDS) analysis revealed that the faecal flora of healthy calves was independently distributed each week and could be clearly distinguished between weeks 1 and 8 (Fig. 2a, b). A previous study reported that the composition of the microbial community in dairy calf faeces changed rapidly, and Chao1 and Shannon indices increased significantly over time [28]. In the current study, the alpha diversity of the microbial community in group H increased with weekly age (Fig. 2c and Additional file 2: Fig. S2a, b). In the early stage of life, the faecal microbes belonged mainly to the phyla *Firmicutes*, *Bacteroidota* and *Proteobacteria* (Fig. 2d). The abundances of *Firmicutes* and *Proteobacteria* declined slightly, while that of *Bacteroidota* gradually increased with weekly age. Meanwhile, the relative abundance of the family *Lactobacillaceae* declined over time, while those of *Rikenellaceae* and *Prevotellaceae* gradually increased (Fig. 2e). Several strains at the species level displayed significant differences between weeks 1 and 8 (Fig. 2f). The results of the LEfSe analysis indicated that the abundances of *Lactobacillus johnsonii*, *Lactobacillus amylovorus*, *Escherichia coli*, *Serratia marcescens*, *Lactobacillus reuteri*, *Nitrospira* sp., *Aquihabitans daechungensis*, and *Enterococcus faecalis* were downregulated with the growth and development of calves (Fig. 2g). Interestingly, the relative abundances of *Porphyromonadaceae* bacterium DJF B175 and *Alistipes shahii* gradually increased with weekly age (Fig. 2h, i), while those of *L. johnsonii* and *L. reuteri* decreased (Fig. 2j). A heatmap was used to illustrate the changes in relative abundance of intestinal flora at the genus level (Additional file 2: Figure S2c). The abundances of *Bacteroides*, *Lactobacillus*, *Faecalibacterium*, *Subdoligranulum*, *Ruminococcus gnavus* group, *Collinsella*, *Acinetobacter*, *Pseudomonas*, and *Stenotrophomonas* were increased in the early stage of life, but decreased gradually with calf growth. Conversely, the abundances of *Alloprevotella*, *Prevotella*, *Rikenellaceae* RC9 gut group, *UCG-005*, *Treponema*, *Odoribacter*, *Agathobacter*, *Lachnospiraceae* NK3A20 group, and *Lachnoclostridium* were increased in the later stage of calf life. These results confirmed that the structure of the intestinal microbiome changed in healthy calves, and microbiota diversity increased with calf growth. In particular,

the relative abundances of *P. bacterium* DJF B175 and *A. shahii* increased with weekly age in the intestinal microbiome of healthy calves, while those of *L. johnsonii* and *L. reuteri* decreased.

Comparative analysis of microbial dynamics in sub-healthy calves

Newborn calves are highly susceptible to intestinal bacterial imbalance, which can lead to indigestion and gastrointestinal infections, resulting in stunted growth and even high mortality [30]. The developmental dynamics of faecal microbes in group SH and differences between groups H and SH are noteworthy. Similar to that of group H, the Shannon and Simpson indices of the microbiota of group SH increased significantly with weekly age. The Chao1 and ACE indices also showed an upward trend over time (Additional file 3: Figure S3a-d). Furthermore, the NMDS results indicated that the faecal flora of group SH was also independently distributed each week and differed clearly between weeks 1 and 8 (Fig. 3a and Additional file 3: Figure S3e). Additionally, the intestinal flora of group SH was also dominated by the phylum *Firmicutes*, *Proteobacteria*, and *Bacteroidota*, but was also enriched with *Actinobacteria* and other bacteria (Fig. 3b). At the family level, the abundances of *Lachnospiraceae* and *Muribaculaceae* increased with calf growth and development, while those of *Lactobacillaceae* and *Ruminococcaceae* gradually decreased (Fig. 3c). Specifically, *L. amylovorus*, *L. johnsonii*, and *L. reuteri* were downregulated at the species level (Fig. 3d). The abundance of *F. prausnitzii* decreased similarly, while that of *E. coli* gradually increased.

Comparison of the faecal analysis of groups H and SH at week 8 revealed that 1338 OTUs were shared between the microbiota of the two groups, accounting for 67.20% of the total OTUs in group SH (1338/1991) (Fig. 3e). Meanwhile, compared with that of group H, the abundance of *Bacteroidota* was lower in the microbiota of group SH, while the abundances of *Firmicutes* and *Proteobacteria* were relatively higher (Fig. 3f). At the strain level, the abundances of *E. coli* was higher in the microbiota of group SH than in that of group H (Fig. 3g). And the LEfSe analysis also demonstrated that the microbiota of group SH was enriched with *Bacteroides thetaiotaomicron*, *L. reuteri*, *L. amylovorus*, and *Intestinimonas timonensis* (Fig. 3h). Furthermore, the relative abundances of *L. amylovorus*, *Bifidobacterium pseudolongum*, *Lactobacillus mucosae*, *S. marcescens*, and *I. timonensis* were significantly higher in the microbiota of group SH than in that of group H, whereas those of *P. bacterium* DJF B175 and *Parabacteroides goldsteinii* were significantly lower (Additional file 3: Figure S3f). The results described above indicated that sub-healthy calves possessed similar faecal microbiological dynamics as those of healthy calves. However, the abundance of *Lactobacillaceae* and the *Firmicutes* to *Bacteroidetes* ratio were greater in the microbiota of group SH than in that of group H.

Microflora analysis of factors that result in calf death

In the current study, five calves died in the second to third week after birth. We further compared the intestinal flora of groups H, SH, and D at week 1. Venn diagram revealed that group H had more independent OTUs than those shared with the other groups (Fig. 4a). In terms of alpha diversity, the

intestinal microbiota of group H possessed higher diversity and richness than that of groups SH and D at week 1 (Additional file 4: Figure S4a-d). The relative abundance histogram revealed that the microbiota of group D was relatively enriched with *Proteobacteria* compared to that of groups H and SH, while less enriched with *Bacteroidetes* and *Actinobacteria* (Fig. 4b). *Bacteroidaceae* at the family level and *Bacteroides coprophilus* at the species level also demonstrated similar trends (Fig. 4c, d). In addition, the microbiota of group D was more enriched with *Enterobacteriaceae*, *Lachnospiraceae*, *Prevotellaceae*, and *E. coli* than that of groups H and SH. *Ruminococcaceae* were present in the lowest abundance in the microbiota of group D among the three groups. The histogram and heatmap further demonstrated that the microbiota of group H was significantly enriched with some strains at week 1, including *Bacteroides massiliensis*, *Ruminococcus* sp. HUN007, *Butyrivibrio crossotus*, *Lachnospiraceae* bacterium, and *B. coprophilus*, compared to that of groups SH and D (Fig. 4e and Additional file 4: Figure S4e). However, the abundances of *E. coli*, *Enterococcus cecorum*, *Bacteroides clarus*, and *Lactobacillus salivarius* were significantly increased in the microbiota of group D compared with that of groups H or SH, and were accompanied by increased abundance of *Lactobacillus murinus*. Interestingly, *P. bacterium* DJF B175 was identified again at week 1, and was more abundant in the microbiota of group H compared to that of groups SH and D. The heatmap illustrates relative abundance of intestinal microflora at the genus level for all three groups (Fig. 4f). The microbiota of group D was enriched with *Acinetobacter*, *Clostridium sensu stricto 1*, *Blautia*, *Alloprevotella*, *Dorea*, *Escherichia shigella*, *Ruminococcus torques* group, *Lysinibacillus*, *Pseudomonas*, *Stenotrophomonas*, *Delftia*, *Bifidobacterium*, *Ruminococcus gnavus* group, *Ruminococcus gauvreauii* group, and *Lachnoclostridium* compared to that of groups H and SH. Meanwhile, the microbiota of group H was enriched with *Bacteroides*, *UCG-005*, *Collinella*, *Streptococcus*, *Parabacteroides*, *Ruminococcus*, *Rikenellaceae RC9 gut* group, and *Akkermansia* compared to that of groups SH and D.

Faecal FAs are associated with calf health

FAs are important metabolites of the gut microbiome that regulate the development of muscle and adipose tissue [31, 32]. The results of principal component analysis (PCA) indicated that faecal FAs in groups H and SH were independently distributed from those in group D at week 1 (Fig. 5a, b). In terms of total faecal FA concentration, group H had lower proportions of MCFAs and LCFAs at week 1 than groups SH and D, but not SCFAs (Fig. 5c-e). Specifically, the faecal SCFAs in group D contained a relatively higher concentration of iso-capric acid (iso-C6) than those in groups H and SH, while the faecal MCFAs in group D contained significantly higher proportions of octanoic acid (C8), decanoic acid (C10), and lauric acid (C12) (Fig. 5f, g). In addition, some faecal LCFAs in group D, including C22:5, C22:4, C20:3, C18:4, C18:3, C18:2, C17:1, C16:1, C15:0, C14:0, and C13:0, were upregulated to different degrees compared to those in groups H and SH (Fig. 5h). These results were also supported by a heatmap of faecal FA analysis results (Fig. 5i). However, the faecal SCFA, MCFA, and LCFA levels in groups H and SH at week 8 did not show the same differences observed at week 1 (Additional file 5: Figure S5a-f).

Levels of faecal AAs change with weekly age

As important metabolites of intestinal flora, AAs play an important role in maintaining host health [33, 34]. The faecal AA concentration in groups H and SH could be distinguished between weeks 1 and 8 (Fig. 6a and Additional file 6: Figure S6a), demonstrating significant downregulation with the growth and development of calves (Fig. 6b). The levels of most faecal AAs in group H decreased significantly over time, including glycine (Gly), L-serine (L-Ser), L-threonine (L-Thr), L-proline (L-Pro), 4-hydroxyproline (Hyp), L-asparagine (L-Asn), L-aspartic acid (L-Asp), L-lysine (L-Lys), L-glutamine (L-Gln), L-glutamic acid (L-Glu), pyroglutamic acid, L-arginine (L-Arg), and taurine. These changes are also illustrated in a heatmap of the AA analysis results (Fig. 6c). However, faecal levels of L-isoleucine (L-Ile), L-leucine (L-Leu), L-Gln, L-methionine (L-Met), L-phenylalanine (L-Phe), pyroglutamic acid, L-citrulline, L-tyrosine (L-Tyr), and N-benzoylglycine were confirmed to be varying degrees of higher in group D at week 1 compared to groups H and SH (Fig. 6d). In particular, the faecal L-Met concentration in group D differed significantly from that in groups H and SH. Similar to group H, faecal levels of L-Ser, L-Pro, 4-Hydroxyproline, L-Asp, L-Lys, L-Gln, pyroglutamic acid and total faecal AA concentration were significantly downregulated in group SH with weekly age (Additional file 6: Figure S6b). The heatmap also serves as evidence of faecal AA downregulation (Additional file 6: Figure S6c). Taken together, these results indicate that the average AA concentration in faeces was downregulated with the growth and development of calves.

Discussion

Due to the incomplete rumen system of calves prior to weaning, undigested food components reach the intestine where microbial metabolism produces many compounds to regulate calf growth and development, including FAs and AAs [35, 36]. Increased intestinal permeability and disturbance of the intestinal microbiota are key factors leading to disease and growth retardation in calves. Various studies have described the development of the gastrointestinal microbiome in calves [37–40], but limited information is available regarding the specific changes that occur in the faecal flora and metabolome of calves. In the current study, we found significant differences in the faecal microbiota and FA and AA metabolism between calves classified as healthy and sub-healthy, as well as those that died within two to three weeks of birth. This early establishment of cross-communication between microbes and metabolites may have profound implications for the health of cattle later in life.

As the newborn grows and is introduced to solid foods, microbiota diversity increases, and the intestinal microbial community converges toward an adult-like state [41]. These changes in the composition and maturity of gut microbes are reportedly related to growth status [42]. Our data confirmed that the microbiota composition changed gradually from weeks 1 to 8 and alpha diversity increased, reflecting maturation of the gut microbiota in healthy calves (Fig. 2a-c and Additional file 2: Figure S2a, b). In group H, the relative abundances of *Rikenellaceae* and *Prevotellaceae* in the microbiota gradually increased over time (Fig. 2e, g). Both decreases of these bacterial families have been previously linked to gut-related diseases, such as inflammation and oxidative stress [43, 44]. In contrast, the abundance of *S. marcescens*, a pathogenic organism that can cause a variety of inflammatory responses and has immunosuppressive effects [45–47], gradually decreased with weekly age and differed significantly between weeks 1 and 8 (Fig. 2f, g). Moreover, the abundances of *A. shahii* and *P. bacterium* DJF B175

gradually increased with the growth and development of calves (Fig. 2f, h, i). These strains and the genus to which they belong reportedly utilise some polysaccharides to regulate the intestinal barrier, improve its permeability, and reduce harmful metabolites, all of which are beneficial to host health [48–51]. However, additional studies are needed to determine whether these bacteria can promote healthy calf growth and their mechanisms of action.

Intestinal dysbiosis refers to an imbalance between beneficial and pathogenic bacteria, resulting in inhibition of beneficial flora and overproliferation of pathogenic bacteria [52–54]. Intestinal inflammation and other typical metabolic diseases have been reportedly associated with intestinal flora imbalance [55, 56]. Comparing the microbiota of calves in groups H and SH at week 8 revealed that the abundance of *Firmicutes* was higher in group SH, while that of *Bacteroidota* was lower (Fig. 3f). The *Firmicutes* to *Bacteroidota* ratio in the gut microbiome has been linked to a variety of diseases, such as inflammatory bowel disease and metabolic syndrome [57]. The higher *Firmicutes* to *Bacteroidetes* ratio in the microbiota of group SH reflected the "unhealthy" state of the intestinal flora. Further, the abundances of *E. shigella* and *Fusobacterium*, typical pathogens closely associated with intestinal inflammation and even colorectal cancer, were higher at week 8 in the microbiota of group SH (Fig. 3g, h) [58, 59]. Notably, the abundance of *P. bacterium* DJF B175 was gradually upregulated over time in the microbiota of group H, but was lower in the microbiota of group SH at week 8 compared with group H (Additional file 3: Figure 3f). The same downregulation occurred with *P. goldsteinii*, a strain that is associated with enhanced intestinal integrity and promotes resistance against intestinal and respiratory inflammation [60, 61].

In a follow-up analysis, we conducted a comparison of the three groups at the first week after birth. *Enterobacteriaceae*, *E. coli*, and *E. cecorum* were more enriched in the microbiota of group D than in that of groups H and SH (Fig. 4c-e). Several of these bacterial genera and strains have been shown to disseminate genes encoding antimicrobial resistance and are associated with intestinal inflammation. Their high proliferation seems to reduce intestinal resistance to other intestinal pathogens, thereby aggravating several diseases related to inflammation, such as necrotising enteritis, sepsis, and bone infection [62–64]. As mentioned above, the abundance of *P. bacterium* DJF B175 in the microbiota decreased with decreasing calf health in a comparison of the three groups (Fig. 4e). In addition, *UCG-005*, *Rikenellaceae RC9 gut group*, and *Akkermansia* were enriched in the microbiota of group H in contrast to that of groups SH and D (Fig. 4f). Some of these strains have been verified to maintain the balance of intestinal flora, promote average daily weight gain, and exert positive effects on growth and development [65–67]. Overall, the balance of the gut flora is important for nutrient intake and normal physiological activities. Suppression or infringement of beneficial microbiota by harmful bacteria results in a serious imbalance that may lead to retardation of calf growth and development or even death. The study findings suggest that early death of calves may be related to intestinal microbiome dysregulation, particularly, severe upregulation of strains negatively associated with growth, such as *E. coli*, and relatively low abundance of beneficial strains. In contrast, enriched *P. bacterium* DJF B175, *Akkermansia*, and *Bacteroides* populations in the intestinal microbiome may promote the healthy growth of calves.

In addition to changes in microbiota composition, the faecal FA composition differed significantly between groups D and H at one week of age, with higher faecal FA levels in group D, especially MCFAs (Fig. 5). Microbes can metabolise FAs, regulate the absorption of FAs by intestinal cells, and affect host energy metabolism, all of which are closely related to the pathogenesis of some diseases [68, 69]. MCFAs are important substrates in mammalian energy metabolism and synthesis, and can also improve immune and inflammatory responses in intestinal cell lines [70, 71]. For example, lauric acid inhibits a variety of pathogens, including *E. coli*, can significantly reduce inflammatory responses, and improves serum levels of inflammatory cytokines IL-6, TNF- α , IL-4, and IL-10 [72, 73]. When the intestinal flora is in a healthy state, the body maintains an environment suitable for nutrient metabolite absorption. However, disordered intestinal flora impairs the function of the intestinal barrier, which weakens the absorption of FAs by intestinal epithelial cells and leads to loss of large amounts of FAs [74]. Therefore, high FA concentrations in the intestine lead to an imbalance in host metabolic homeostasis and weakened disease resistance. Indeed, caprylic, nonanoic, and decanoic acid levels were shown to be higher in the watery faeces of patients with diarrhoea [75]. A similar study reported higher levels of branched-chain fatty acids (BCFAs) in the faeces of diarrhoeal calves compared to those of healthy calves [76]. Moreover, levels of faecal LCFAs in patients with colorectal cancer were higher than those in healthy subjects [77]. These studies support that intestinal microbiome disorder may lead to intestinal FA imbalance, which may disrupt the metabolic homeostasis of calves and damage their health.

Changes in the AA metabolism calves in the current study further confirmed the faecal microbiota results. Levels of some faecal AAs were higher in one-week-old calves in group D than in groups H and SH (Fig. 6d). Intestinal bacteria are known to alter the distribution of free AAs (FAAs) in the GIT and affect the bioavailability of host AAs [78]. For example, *Clostridium sporosporum* can reportedly degrade tryptophan and secrete indole propionic acid through the metabolic pathway of tryptophan [79]. Therefore, higher AA concentrations in the intestine are thought to be the result of incomplete fermentation. Studies have reported that faecal FAA concentrations were significantly increased in patients with inflammatory bowel disease [80, 81]. Further, the remission of diarrhoea in calves receiving FMT was accompanied by varying reductions in faecal AA concentrations [9]. Comparing the faecal AA results with the bacterial results, the abundance of *Clostridium* sp., the main AA-fermenting bacteria, was significantly decreased in the microbiota of group D, whereas the abundance of *E. coli*, the main bacteria responsible for methionine synthesis and transformation, was significantly increased (Fig. 4e) [82–84]. These findings may explain the abnormal increase in intestinal AA concentrations at one week of age and the intestinal bacteria imbalance in group D. Moreover, faecal AA concentrations were lower at week 8 than week 1 in groups H and SH (Fig. 6b-c) likely because the intestinal flora structure of calves tended to be stable and AA use increased at eight weeks of age. Combining the faecal FA and AA results, we speculate that differences and disorder of intestinal microbiota distribution in calves might lead to abnormal intestinal function, absorption of MCFAs and LCFAs, and influence AA catabolism, resulting in an increased risk of early death. However, due to the lack of detection of serum metabolites, we could not judge the absorption of FAs and AAs in calves.

Conclusions

Our study tracked the dynamics of faecal microbes and their metabolites in beef calves during the pre-weaning period. We compared differences and associations between the gut microbiome environments of calves classified as healthy, sub-healthy, and those that died within 2-3 weeks of birth. The abundances of some microbes displayed significant changes with the growth and development of calves, changes that were particularly pronounced between healthy calves and those that died early in life. At the same time, levels of metabolites derived from microorganisms also varied with weekly age, reflecting the effects of intestinal microflora and their metabolites on the health and growth of calves. The combined application of microbiome analysis and metabonomics provides a reference for subsequent research on improving the growth and development of calves via changes in their intestinal flora.

Abbreviations

FA, fatty acid; AA, amino acid; SCFA, short-chain fatty acid; MCFA, medium-chain fatty acid; LCFA, long-chain fatty acid; BCFA, branched-chain fatty acid; NMDS, non-metric multidimensional scaling; PCA, principal component analysis; OTU, operational taxonomic unit; ANOVA, analysis of variance; FAA, free amino acid; L-Ala, L-Alanine; Gly, Glycine; L-Ser, L-Serine; L-Thr, L-Threonine; L-Cys, L-Cysteine; L-Pro, L-Proline; L-Val, L-Valine; L-Ile, L-Isoleucine; L-Leu, L-Leucine; L-Asn, Asparagine; L-Asp, L-Aspartic acid; L-Lys, L-Lysine; L-Gln, Glutamine; L-Glu, L-Glutamic acid; L-Met, L-Methionine; L-His, L-Histidine; L-Phe, L-Phenylalanine; L-Arg, L-Arginine; L-Tyr, L-Tyrosine; L-Trp, L-Tryptophan

Declarations

Ethics approval and consent to participate

All animal studies were approved by the institutional animal care and Use Committee of the College of Medicine (Yanbian University).

Consent for publication

Not applicable

Availability of data and material

The raw sequences of the 16S rRNA genes obtained from the faecal samples have been deposited into NCBI Sequence Read Archive (SRA) under the accession number PRJNA783159 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA783159>). All data relevant to the study are included in the article or uploaded as supplementary materials (<https://pan.baidu.com/s/1tKL2p6wuyIWXAqhyAPt0nA>).

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

L-H Q and J-D K conceived the project and contributed to experimental design; L-H Q, J-D K, and H L performed experiments, interpreted the results, prepared the figures and wrote the manuscript; C Y, Z-B L and S H contributed to wrote the manuscript; C H, D W, Y L, J W, D L, J Z, S C, L Y, X L and C Y performed animal studies; all authors discussed the results and approved the manuscript.

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Figures

Fig.1

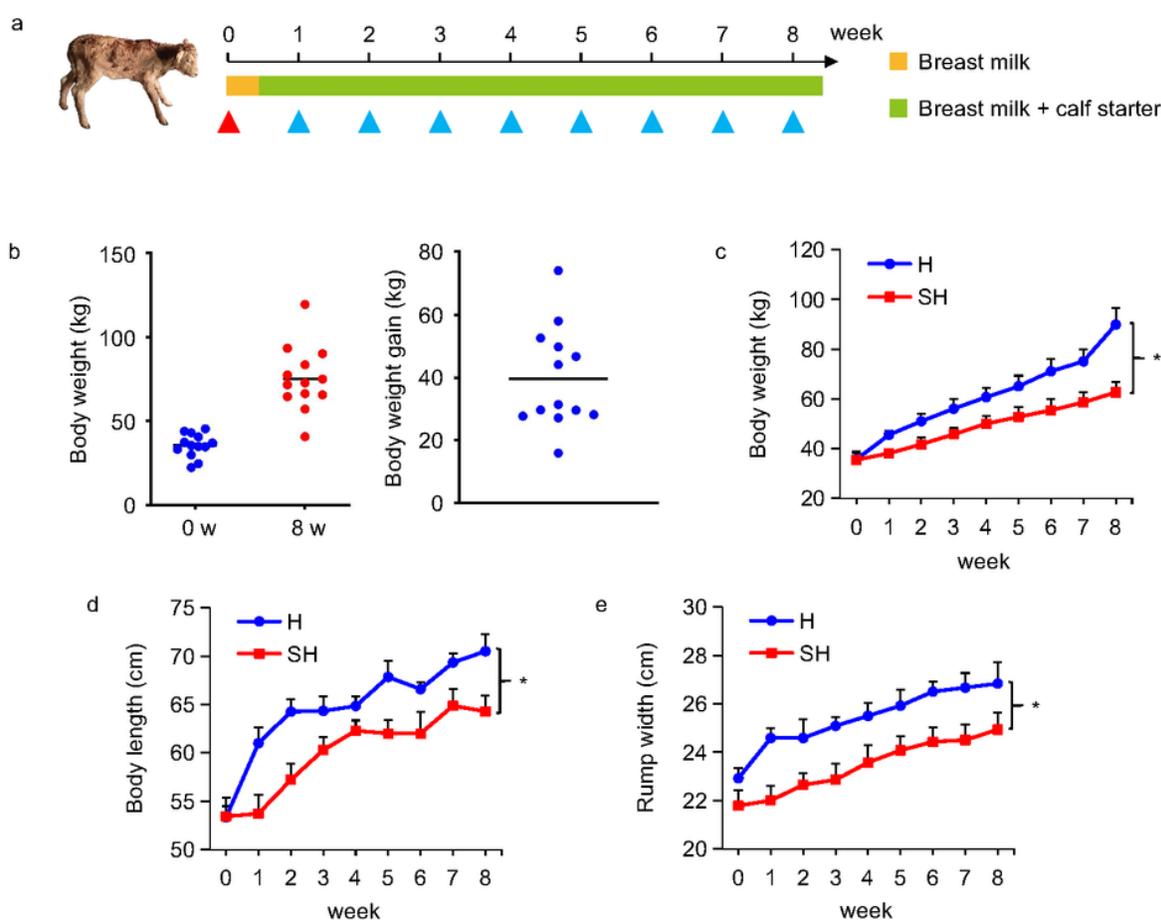


Figure 1

Experimental design and grouping. (a) Experimental design in calves including feeding management and sampling arrangements. Detailed descriptions are provided in the Methods section. (b) Body weight of the calves (n=13) at 0 and 8 weeks as well as the body weight gain at 8 weeks after birth. (c-e) After

grouping, body weight (c), body length (d) and hip circumference (e) of calves in the healthy group (n=6) and sub-healthy group (n=7). Data are shown as mean \pm SEM. The P values were determined using an analysis of variance (ANOVA). *p<0.05. Source data are provided as a Source Data file.

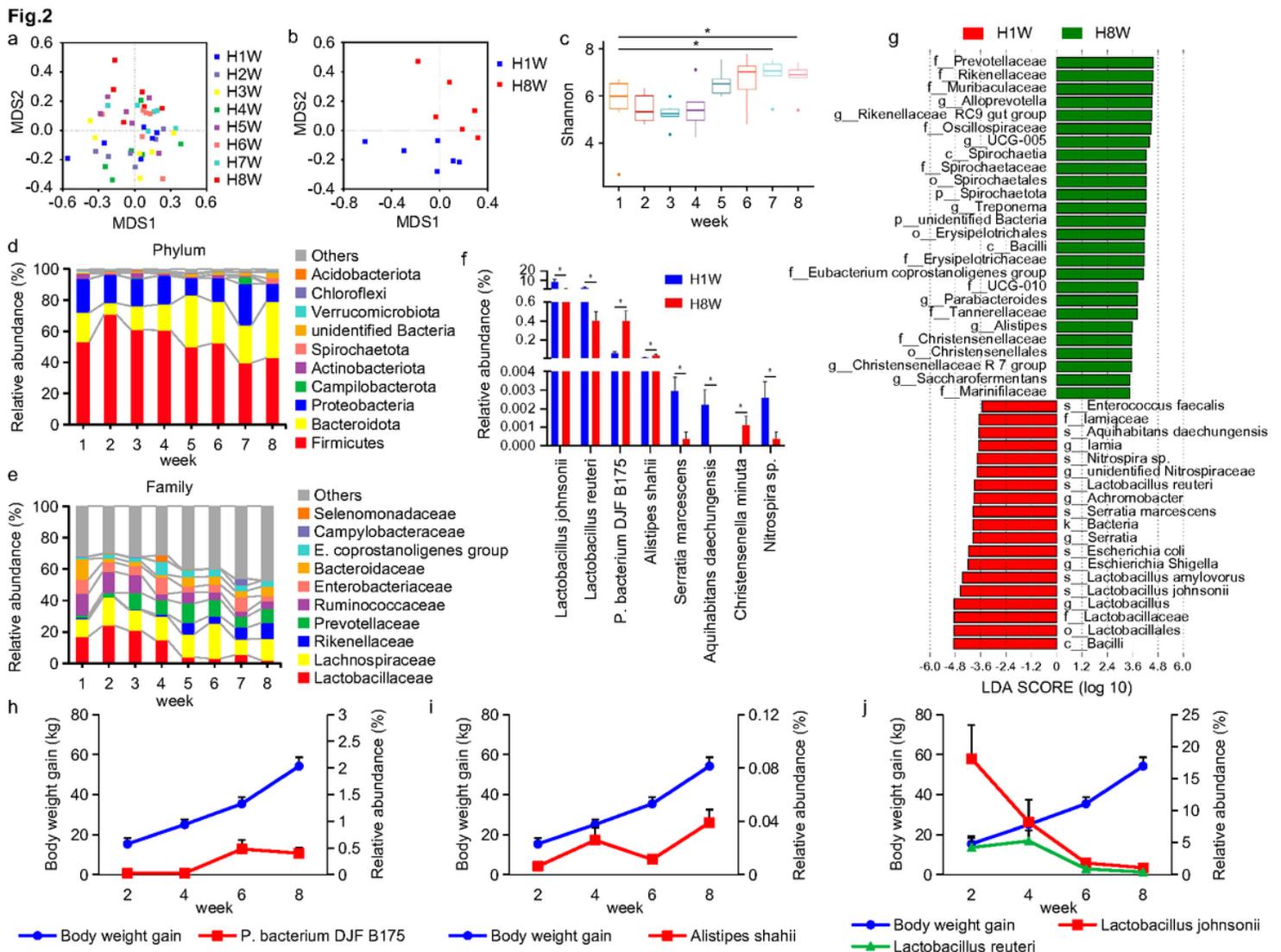


Figure 2

Intestinal microbiota of healthy calves changes with weekly age. (a-b) Based on Bray-Curtis Dissimilarity, NMDS of bacterial 16S rRNA gene sequence data from faecal samples of healthy group calves (n=6) 0-8 weeks after birth were shown. The NMDS scatter plot shows different bacterial communities for each week. (c) The alpha diversity boxplot was measured using the Shannon diversity index. The median is represented by a thick horizontal line in the middle of the box. The bottom and top lines correspond to the 25th and 75th percentile, respectively. Outliers are shown as small circles. (d-e) The relative abundance of bacteria at phylum (d) and family (e) levels over time. (f) The strains with significant differences between the first week and the eighth week after birth. (g) The LDA scores computed for OTUs differentially abundant between two time-point groups (log₁₀ LDA>3.4). (h-j) The relative abundances of the species *Porphyromonadaceae bacterium DJF B175* (h), *Alistipes shahii* (i), *Lactobacillus johnsonii* and

Lactobacillus reuteri (j) as well as the bodyweight gain over time. Data are shown as mean \pm SEM. The P values were determined using the Mann-Whitney *U* test (two-tailed) and Student's *t*-test. * $p < 0.05$.

Fig.3

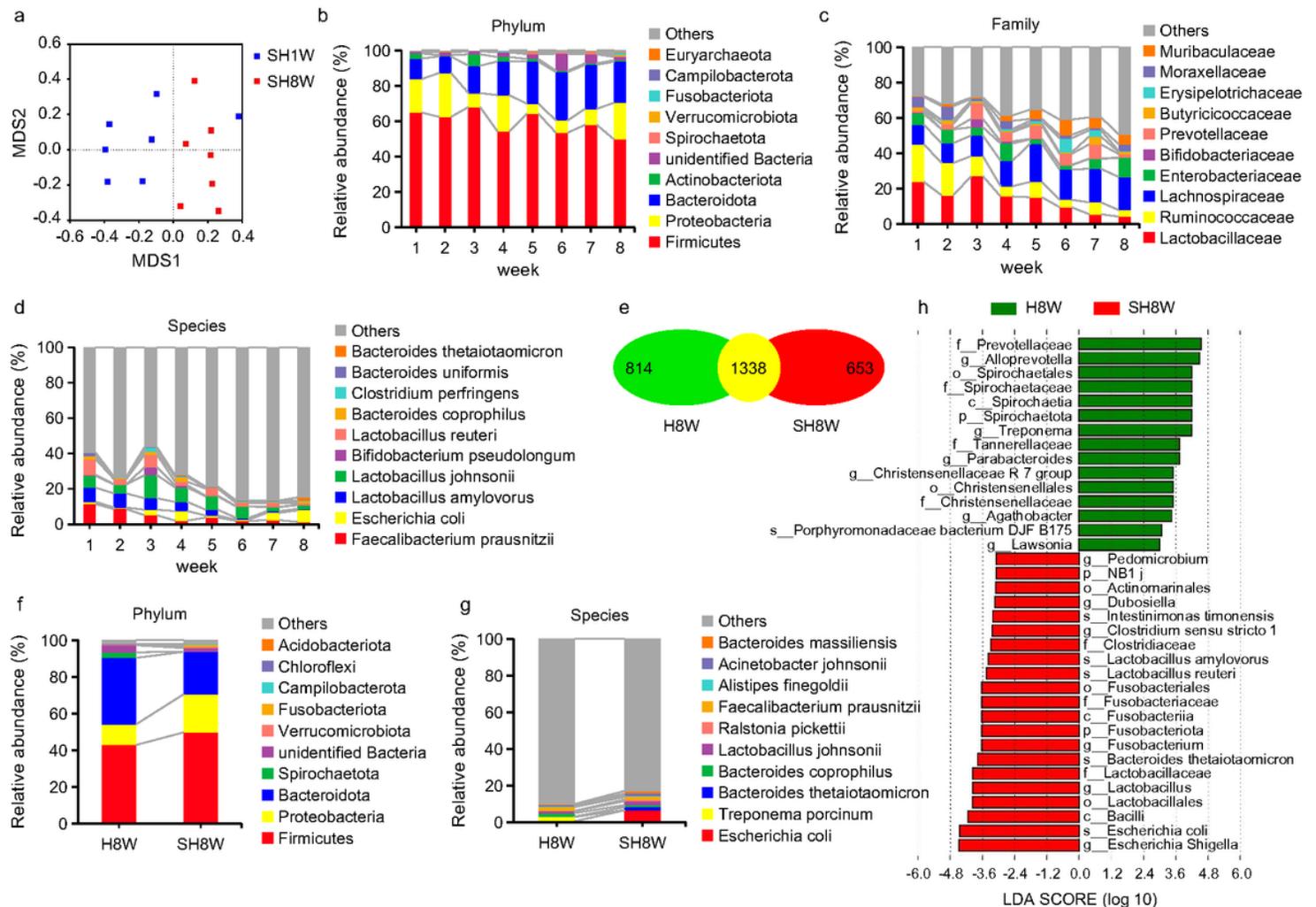


Figure 3

Comparative analysis of microbe dynamics in the sub-healthy group. (a) Based on Bray-Curtis dissimilarity, NMDS of bacterial 16S rRNA gene sequence data from faecal samples of weeks 1 and 8 in sub-healthy group calves (n=7) were shown. (b-d) Bar plot depicting the relative abundance of bacteria at phylum (b), family (c) and species (d) levels over time in the sub-healthy group. (e) Venn diagram showing shared OTUs between H (n=6) and SH (n=7) group at week 8. (f-g) Bar plot depicting the relative abundance of bacteria at phylum (f) and family (g) levels between H and SH group at week 8. (h) Histogram shows the LDA scores computed for OTUs differentially abundant between two groups (log₁₀ LDA>3.0).

Fig.4

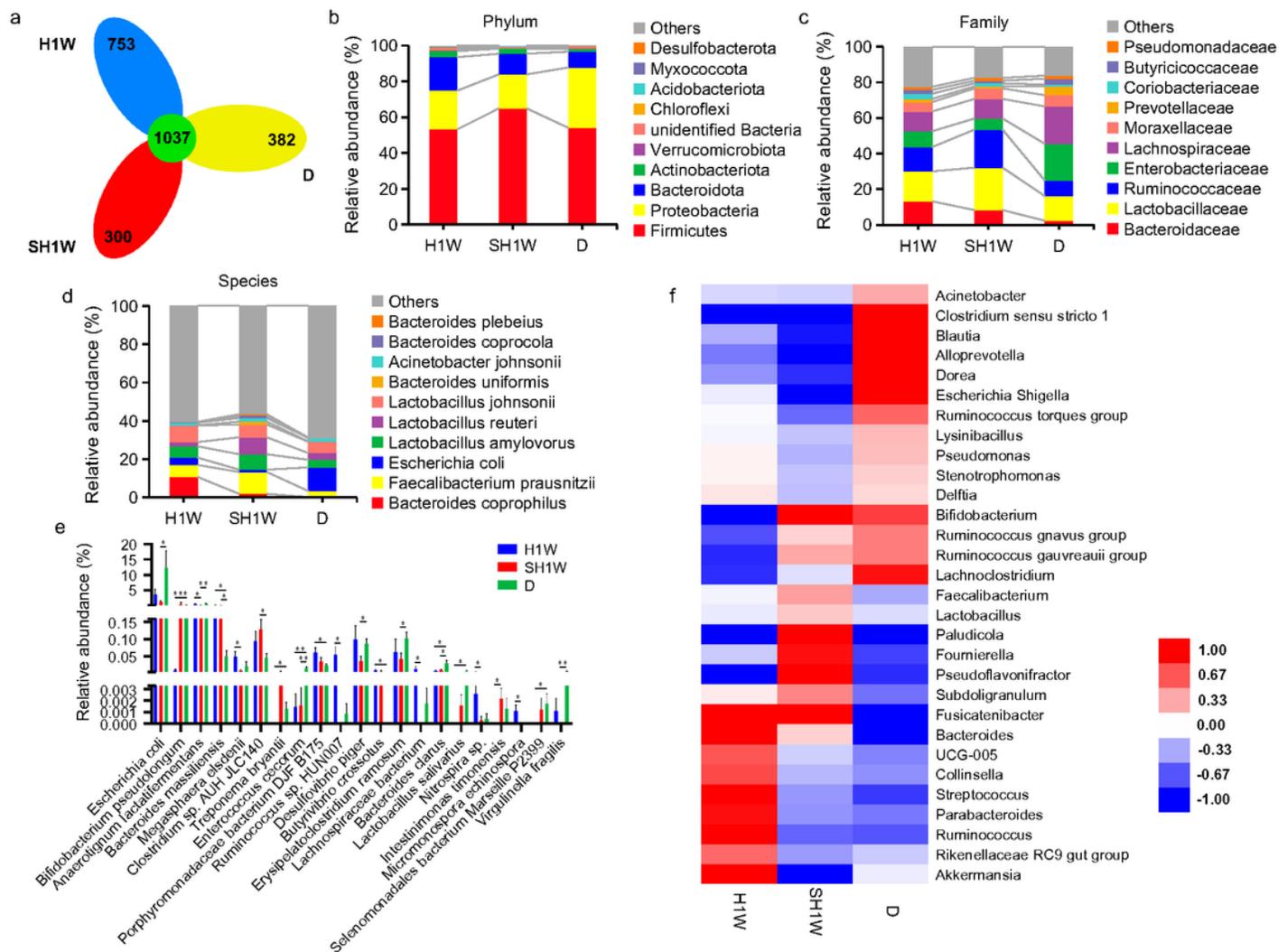


Figure 4

Comparisons of the intestinal flora among groups H, SH, and D at week 1. (a) Venn diagram showing shared OTUs among H, SH, and D groups (n=6, 7, and 5) at week 1. (b-d) Bar plot depicting the relative abundance of bacteria at phylum (b), family (c) and species (d) levels in three groups. (e) The strains with the significant difference among the three groups at week 1. (f) The relative abundance of bacteria at the genus level among the three groups was clustered and represented in a heatmap. Data are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

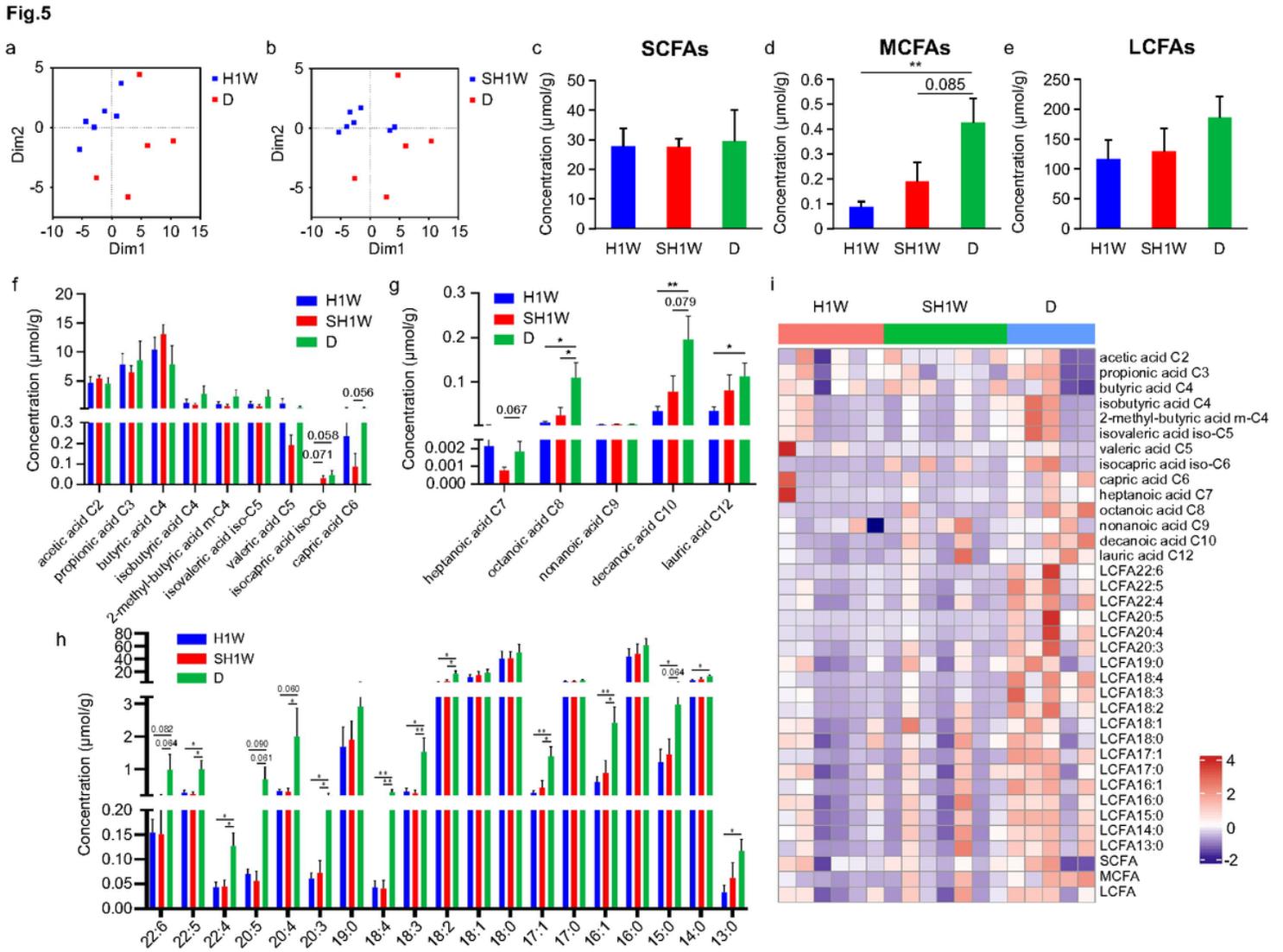


Figure 5

Difference in faecal fatty acid concentration of 1 week-old calves. (a-b) PCA of fatty acid concentration analysis data from faecal samples of H, SH, and D groups (n=6, 7, and 5) at week 1. (c-e) The total fatty acid concentration of faecal samples at SCFA (c), MCFA (d) and LCFA (e) levels in three groups at week 1. (f-h) Specific concentrations of fatty acids of different lengths in feces of three groups at week 1. (i) Detailed concentrations of fatty acids among three groups at week 1 were clustered and represented in a heatmap. Data are shown as mean ± SEM. *p<0.05, **p<0.01.

Fig.6

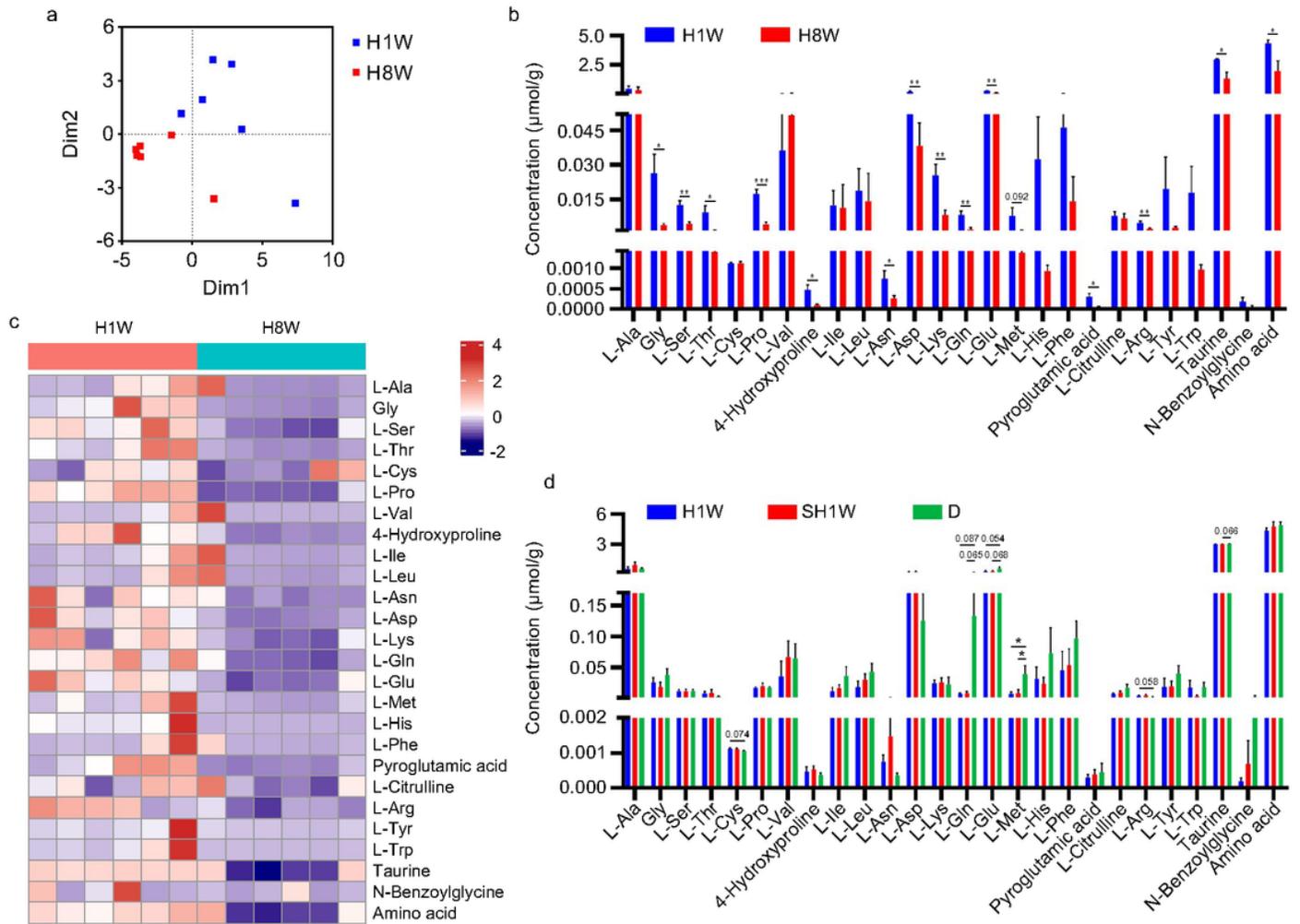


Figure 6

Amino acid concentration decreased with the growth and development of calves. (a) PCA of amino acid concentration analysis data from faecal samples of the H group (n=6) at weeks 1 and 8 was shown. (b) Bar plot depicting the concentration of different amino acids of faecal samples between week 1 and 8. (c) Detailed concentrations of amino acids of H group at week 1 and 8 were clustered and represented in a heatmap. (d) Bar plot depicting the concentration of different amino acids of faecal samples among H, SH, and D groups (n=6, 7, and 5) at week 1. Data are shown as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Supplementary Files

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

- [SupplementaryMaterial.pdf](#)