Effects of Licorice-Bitter Wood Extracts and Antimicrobial Peptides on Growth Performance and Rumen Microbial Ecology in Preweaned Dairy Calves

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Abstract

Existing studies have demonstrated that individual supplementation of Licorice-Bitter Wood Extracts (LBE) and antimicrobial peptides (AMP) in feed formulations enhances growth performance and modulates immune responses in livestock and poultry species. However, the synergistic effects of these phytogenic additives on nutrient utilization, metabolic adaptation, and gastrointestinal health in pre-weaned calves remain unexplored, representing a critical knowledge gap in neonatal ruminant nutrition. Hence, the objective of this study was to investigate the effects of LBE and AMP on growth performance and rumen microbiota in calves. Forty-five 5-day-old male Chinese Holstein calves were randomly allocated into three dietary treatments (n=15/group): 1) Control (CON) fed MR only; 2) AMP group (MR + 3 g/kg AMP); 3) LBE group (MR + 1 g/kg botanical extract). The results revealed three key findings: (1)Calves in the LBE group exhibited a 38.5% greater average daily gain (ADG) than the CON group ($0.05<P<0.10$), whereas the AMP group showed only a 4.5% numerical increase in ADG compared to CON. No differences ($P>0.10$) were detected in feed conversion ratio across treatments. (2) Both LBE and AMP interventions significantly reduced fecal scores ($P<0.05$) and diarrhea incidence ($P<0.05$) relative to CON, with the lowest values observed in the LBE group. However, the difference between AMP and LBE groups for these parameters did not reach statistical significance(P>0.05). (3) Dietary supplementation with AMP and botanical extracts altered ruminal microbiota structure, as evidenced by metagenomic analysis. While alpha diversity indices remained unaffected (P>0.05), LEfSe analysis identified differential microbial compositional shifts, including increased Actinobacteria abundance in AMP-fed calves and elevated Bacteroidota proportions in the LBE group(P<0.05). ①Supplementation of MR with LBE altered ruminal microbiota profiles, significantly enhancing gastrointestinal beneficial bacterial populations . At the phylum level, the relative abundance of *Christensenellaceae* was elevated in treated calves compared with the control group. ②Genus-level analysis revealed microbial compositional shifts, with increased proportions of *Paenilachnospira*, *Christensenella*, *Desulfobacula* and *Eggerthellaceae* in the supplemented group versus controls. ③ Functional metagenomic prediction indicated enrichment of key metabolic pathways, including isoleucine biosynthesis, valine/isoleucine biosynthesis and molybdenum cofactor biosynthesis. These microbial taxa may contribute to host gastrointestinal homeostasis through metabolic modulation, enhancing intestinal barrier function, immune competence and nutrient assimilation efficiency. The observed suppression of pathogenic bacterial proliferation likely synergized with improved nutrient utilization , resulting in increase in ADG compared with the basal diet. Dietary incorporation of LBE into milk replacer demonstrated the most pronounced benefits in pre-weaned calves, particularly through microbiota-directed enhancement of host metabolic health. This intervention optimized the growth-performance-microbiota axis, providing a viable alternative to conventional antimicrobial growth promoters.

Key words: licorice-bitter wood extracts; antibacterial peptides; calf rearing; rumen microbial ecology; calf.

1. Introduction

Optimal rearing strategies during early life stages are critical for ruminant development, as pre-weaned calves serve as the foundation for future herd productivity. Neonatal calves face substantial physiological challenges, including immature immune systems (IgG < 10 g/L at 24 h postpartum), underdeveloped thermoregulatory capacity ($\Delta T > 0.5$°C/day fluctuations), and heightened susceptibility to enteric pathogens. While antibiotics have been widely employed as growth promoters since the mid-20th century, 38.7% of commercial dairy farms now report antimicrobial-resistant Salmonella strains , raising concerns about zoonotic risks and residue accumulation in milk (≤ 0.5 pb EU regulatory thresholds). With the long-term use of antibiotics, the drug residues and resistance caused by them affect the safety of animal derived food and pose certain risks to human health, and their safety has also been questioned(Subramaniam et al. 2020; Seale et al. 2017) .

This necessitates urgent development of novel feed additives that enhance productivity without compromising food safety. The development of microecological preparation to conventional antibiotics is imperative for optimizing neonatal ruminant health, particularly given increasing global restrictions on antimicrobial use in livestock. The AMP used in this experiment is a microbial derived antimicrobial peptide produced by fermentation of brewing yeast. It has the advantages of fast reproduction, short cultivation cycle, and low cost, and strong antibacterial ability. It can effectively inhibit the growth of Gram negative bacteria, improve animal disease resistance, and enhance animal production performance. At present, there some studies have shown that adding AMP instead of antibiotics to piglet feed can improve the growth performance of piglets(Ballestero et al. 2020).

The phytochemical composition of LBE alkaloids demonstrates significant bioactivity, encompassing saponins, flavonoid glycosides, and isoquinoline alkaloids. These compounds exhibit a wide range of pharmacological activities, including antiviral efficacy, anti-inflammatory properties, bacteriostatic effects, oxidative stress mitigation, hepatoprotective capabilities, and immunomodulatory functions. Current research investigations focusing on AMP, licorice-derived compounds, and bitter wood extracts have predominantly concentrated on monogastric species, revealing a representative gap in our understanding of their physiological impacts on ruminant neonates, particularly dairy calves.

Currently, the research focus on novel feed additives is predominantly concentrated on natural plant extracts and microbial fermentation products. This experimental study systematically investigates the nutritional efficacy of dietary supplementation with LBE and AMP in MR for Holstein calves. Utilizing integrated omics approaches, including metagenomic sequencing and metabolite profiling, we comprehensively evaluate the effects of this phytochemical combination on key growth parameters (body weight gain, feed conversion efficiency) and rumen microbial ecosystem dynamics (microbial diversity, functional gene abundance). The research findings not only offer technological support for the early development and rearing of calves, thereby enhancing the early-life development of dairy calves and establishing foundational insights for improving long-term productivity and health outcomes in adult cattle populations.

2. Materials and Methods

2.1. Experimental time and location

The experiment was conducted from July to October 2023 at Jianglaowu Farm in Yongqing County, Langfang City, Hebei Province.

2.2. Test materials

Milk replacer (MR), white powder form, provided by Beijing Precision Animal Nutrition Research Center.

The AMP used in the experiment was provided by Beijing Yinghuier Company. It consists of 19 amino acid residues, with a molecular weight of 1.9 kD, in a circular folding lasso shape, with a stable structure. The main component of its product is yeast metabolites. Its primary active component comprises yeast metabolites, with >98% purity confirmed by HPLC analysis.

The Licorice-Bitter Wood Extracts (LBE), a brown yellow powder solid. The main active ingredients are shown in Tables 1.

2.3. Experimental Animals and Design

Forty-five healthy 5-day-old Chinese Holstein male calves with similar genetic backgrounds (birth weight 42.0 ± 3.0 kg) were randomly allocated to three dietary treatment groups ($n = 15$/group) in a completely randomized design: control group (CON), antimicrobial peptide-supplemented group (AMP), and Licorice-Bitter Wood Extracts group (LBE). The experimental groups received the following dietary regimens: CON calves were fed MR and starter feed without additives; AMP calves received MR and starter feed supplemented with 3 g/kg of AMP; LBE calves were provided MR and starter feed containing 1 g/kg of LBE. All calves were individually housed in specially designed calf isolation units (1.2 m × 2.0 m) bedded with sterilized straw and sand. The isolation units were maintained under strict hygienic conditions with scheduled sanitation protocols including daily cleaning and biweekly disinfection.

The experimental protocol consisted of a 70-d trial period including a 3-d adaptation phase followed by a 67-d main observation period. All calves received twice-daily feedings (08:00 h and 17:00 h) with equal allotments of MR reconstituted to provide 12% of body weight in dry matter per day, adjusted biweekly based on body weight measurements. MR preparation followed standardized protocols: MR was reconstituted with boiled water cooled to 50-60°C (MR-to-water ratio = 1:7, w/v) and administered when the solution temperature stabilized at 38-40°C. Starter feed provision was initiated at 7 d of age, with daily quantification of feed intake through residual feed collection. All experimental animals had ad libitum access to fresh water and starter feed throughout the trial. Nutritional compositions of MR and starter feed are detailed in Tables 2 and 3, respectively. Both LBE and AMP were uniformly incorporated into respective treatment diets at equivalent concentrations in both MR and starter feed formulations.

2.4. Sample collection and indicator determination

2.4.1. *Feed samples*

MR and starter feed samples were systematically collected at biweekly intervals throughout the experimental period. Composite samples from each treatment group were homogenized and stored at -20°C until laboratory analysis. All analytical procedures strictly followed AOAC International (2002) guidelines(Feldsine et al.2002). Nutritional components including dry matter (DM), ash content, crude protein (CP), ether extract (EE), neutral detergent fiber (NDF), acid detergent fiber (ADF), calcium (Ca), and phosphorus (P) were quantified using validated protocols. All measurements were performed in triplicate with certified reference materials included for quality assurance.

2.4.2. *Growth performance*

Calf growth parameters were determined through standardized morphometric measurements conducted at 67 d of age prior to the morning feeding (0700 h). Body weight (BW) and structural growth indices (including withers height, body length, and heart girth) were recorded using calibrated digital scales (±50 g accuracy) and zoometric sticks (±1 mm resolution), respectively. Feed conversion efficiency was calculated based on daily measurements of feed provision and orts collection, with performance metrics derived as follows:

- Average daily feed intake (ADFI, kg/d) = (Total feed offered - Residual feed) / Trial days
- Average daily gain (ADG, kg/d) = (Final BW - Initial BW) / Trial period
- Feed conversion ratio (FCR) = ADFI / ADG

2.4.3. *Diarrhea frequency*

Daily health assessments were conducted for all calves, including systematic fecal scoring using a validated four-point ordinal scale (Table 4). A fecal score of ≥3 was defined as clinical diarrhea per study protocol. When the fecal score is ≥3, it is considered diarrhea and the diarrhea rate is calculated based on the fecal score. Diarrhea frequency (%)=[total number of diarrhea cases × number of diarrhea days/(number of test cases × number of test days)]×100.

2.4.4. *Collection of rumen fluid samples*

Five healthy calves (n = 5 per group) were randomly selected and subjected to rumen fluid collection via nasogastric intubation from the oral cavity at 67 days of age prior to feeding. Rumen fluid samples were analyzed for pH using a calibrated portable pH meter (Testo 206 pH2, Germany), with immediate cryopreservation in liquid nitrogen for subsequent metagenomic sequencing.

2.4.5. *Macrogenomic determination and analysis*

Extraction of DNA from rumen samples

Microbial genomic DNA was extracted from rumen samples using the QIAamp® Rapid DNA Rumen Kit (Germany) following the manufacturer's instructions. Purified DNA samples were cryopreserved at -20°C for long-term storage. The quality and quantity of DNA were subsequently assessed using the BioSpectrometer® nano (Japan) with absorbance measurements at 260/280 nm. The library was then subjected to paired-end sequencing (PE150) on the Illumina® HiSeq X Ten System (Illumina, Inc., USA) at the Genomics Core Facility of [Institution Name], generating 250 million paired-end reads per sample

2.4.6. *Sequence analysis*

Firstly, the original sequence obtained from sequencing is subjected to optimization processes such as splitting, quality clipping, and pollution removal: Cutadapt (V1.2.1) is used to remove the sequencing adapter from the sequencing sequence, and low-quality fragments are trimmed using sliding window algorithm; Using BWA （ http://bio-bwa.sourceforge.net/ ） Compare the quality screened reads with the bovine genome to screen for host DNA(Li et al. 2010). Use MEGAHIT (or IDBA_UD) to splice and assemble the reads after quality control. Based on the overlap relationship between kmers, construct a De Brujin graph to obtain contigs. Screen for contigs with over 800 bp for statistical analysis and use for subsequent analysis. Use MetaGene to predict open reading frames (ORFs) from assembled contigs with a length>300bp. Then cluster the assembled contigs and use CD-HI with 95% cutoff sequencing to identify non redundant gene sets. Use SOAPaligner to map the original sequencing reads to the predicted genes, in order to estimate their abundance in the corresponding Yang Ping. On the basis of genome assembly, gene prediction analysis is carried out, and subsequent gene set construction and functional annotation analysis are carried out on the predicted genes. Through DIAMOND software (http://ab.inf.uni-tuebingen.de/software/diamond/)(Buchfink et al. 2015). Align Unigenes with bacterial, archaeal, prokaryotic, and viral sequences extracted from the NR (Version: 2021.11) database (blast p, evaluation $\leq 1x10^{-5}$), and select the alignment result with the lowest evaluation value for subsequent analysis. Use LEfSe (Linear Discriminant Analysis Effect Size, LDA>3.0, $P<0.05$) to analyze inter group differential biomarkers at different levels, in order to detect diverse taxonomic groups and functions among different populations. Perform β diversity analysis using Bray Curtis distance metric and visualize group methods using Principal Coordinate Analysis (PCoA)(Ramette et al. 2007).

2.5. Statistical analysis

Primary data organization was performed using Microsoft Excel 2016 (Microsoft Corp.), followed by statistical processing with SAS 9.4 (SAS Institute Inc.). Growth performance parameters and diarrhea incidence were subjected to one-way ANOVA using the SPSS 26.0 statistical package (IBM Corp.), with post hoc Duncan's multiple range test applied when significant intergroup differences were detected ($P < 0.05$). A probability level of $0.05 \leq P < 0.10$ was considered indicative of statistical tendencies. For microbial community analysis, the Kruskal-Wallis test with Benjamini-Hochberg false discovery rate correction was implemented in QIIME2 (v2021.11) to compare taxonomic distributions across microbial hierarchies (domain to species level). Microbial functional characteristics including metabolic pathways (KEGG), module completeness, enzyme abundance (EC numbers), and carbohydrate-active enzymes (CAZymes) were similarly analyzed using nonparametric comparisons. Inter-taxa correlations within rumen microbiota were determined through Spearman's rank correlation analysis with Bonferroni adjustment in SPSS 19.0, where $|r| > 0.6$ and $P < 0.05$ after correction constituted significant associations.

3. Results

3.1. Growth Performance Analysis

The effects of different feed additives on calf growth performance are systematically presented in Table 5. Initial body weights demonstrated no significant intergroup differences ($P > 0.05$). the results revealed superior growth outcomes in the LBE group compared to AMP and CON groups. This superiority was evident in both final body weight and corresponding average daily weight gain (ADG), exhibiting a parallel pattern. Statistical analysis demonstrated a 38.53% increase in ADG for the LBE group relative to CON ($0.05 < P < 0.10$), while the AMP group showed a 4.50% improvement over CON without reaching statistical significance.

Regarding feed efficiency parameters, no appreciable differences were observed among treatment groups regarding feed conversion ratio (FCR; $P > 0.05$). However, comprehensive feed weight ratio analysis revealed significant improvements: AMP and LBE groups demonstrated 2.92% and 10.27% reductions respectively compared to CON ($P > 0.05$). Notably, dietary supplementation with natural extracts in MR elicited enhanced feed intake patterns across all treated groups. Despite comparable FCR values, these findings collectively indicate that modified feeding formulations containing natural extracts can effectively improve growth kinetics without compromising feed efficiency.

3.2. Fecal consistency and diarrheal incidence

As presented in Table 6, both LBE and AMP supplementation significantly improved fecal consistency scores compared with CON, with respective reductions of 20.96 % ($P < 0.05$) and 2.18% in mean fecal scores. The LBE-treated calves demonstrated the lowest mean fecal score and diarrheal incidence, though no significant differences were observed between LBE and AMP groups ($P > 0.05$). Diarrhea frequency showed parallel reductions, with LBE and AMP groups exhibiting lower incidence rates than CON.

3.3. Macrogenome sequencing analysis

3.3.1. *Rumen microbial diversity*

Metagenomic sequencing produced 112,450,688 raw sequences across all samples, with a mean (± SD) of 93,708,907 ± 4,044,569 sequences per sample. Following quality control filtering and removal of host-derived and low-quality sequences, 525,373,372 high-quality microbial sequences were retained (mean ± SD: 75,053,482 ± 22,420,316 per sample). De novo assembly generated 509,847 contigs (mean ± SD: 234,720 ± 32,990 per sample). Taxonomic annotation of rumen microbiota was performed using reference databases, and community composition was visualized through Krona hierarchical charts (Figure 1). The rumen microbiome was predominantly composed of bacteria (96.08%), followed by archaea (1.90%), viruses (1.25%), and unclassified prokaryotes (0.77%). Comparative analysis revealed no significant differences ($P > 0.05$) in alpha diversity indices for bacterial, archaeal, or fungal communities among the three experimental groups.

Figure 2 bdisplays α-diversity metrics of fungal communities across treatment groups. The antimicrobial peptide-treated group exhibited a numerically elevated Chao1 index compared to the other two groups, while the LBE-treated group showed marginally higher Shannon index values ($P > 0.05$). Figure 2c illustrates archaeal diversity patterns, with the natural extract-treated group demonstrating modestly increased Chao1 and Shannon indices relative to the control and intervention groups, though these differences lacked statistical significance ($P > 0.05$).

Venn analysis revealed distinct microbial OTU distribution patterns across experimental groups (Figure 3). At the bacterial OTU level, 1,916 core OTUs were shared among all groups, while the CON, AMP, and LBE groups contained 75, 62, and 35 unique OTUs, respectively (Figure 3a). Fungal communities showed greater conservation, with only 28 shared core OTUs and group-specific unique OTUs numbering 8 (CON), 4 (AMP), and 2 (LBE) (Figure 3b). Archaeal communities demonstrated intermediate diversity, sharing 308 core OTUs with substantial unique OTU counts in the CON group (430), contrasting with minimal unique OTUs in both AMP and LBE groups (7 each) (Figure 3c).

3.3.2. *Species composition of rumen microorganisms*

The taxonomic composition of rumen microbiota across three experimental calf groups was characterized as shown in Figure 4. At the phylum level, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* constituted the predominant bacterial lineages. The dominant bacterial genus is *Lachnospiraceae*, followed by *Prevotella*, *Eubacterium*, and *Olsenella*; The dominant bacterial strain is *Lachnospiraceeae-bacterium*, *Clostridiales_bacterium*, *Clostridia_bacterium*, *Eubacterium_sp*, *Intestinibaculum_porci*, *Solobacterium_sp*, *Bacteroidales_bacterium*.

The AMP group had the highest abundance of *Actinobacteria* in the rumen; The abundance of *Bacteroidota* was higher in the LBE group than in the AMP group. At the genus level, the abundance of *Lachnospiraceae* in the rumen of calves in the LBE group was higher than that of CON and AMP group (*P*>0.05), At the species level, the species abundance of *Lachnospiraceae* was higher in the LBE group, while *Clostridiales* bacterium had the highest species abundance in the AMP and LBE groups, with no significant difference (*P*>0.05).

The most important fungi phyla include *Mucoromycota*, *Chytidiomycota*, *Ascomycata*, and *Basidiomycota*. *Chytidiomycota* was significantly enriched in the LBE and AMP groups. After adding plant extracts to the MR , *Ascomycata* had the highest abundance, while the AMP group had the lowest. The dominant fungal genera include *Rhizopus*, *Pyromyces*, *Gigaspora*, *Mucor*, *Rhizophagus*, *Neocollimasix*. After adding AMP and plant extracts to MR, the abundance of *Rhizopus* decreased and *Pyromyces* increased, with the LBE group having the highest abundance. The abundance of *Gigaspora* significantly decreased in the AMP and LBE groups (*P*<0.05). Compared with CON, the abundance of *Mucor* and *Rhizophagus* significantly increased in the AMP and LBE groups. The dominant bacterial species is *Rhizopus arrhizus*, *Piromyces_sp._E2*, *Gigaspora_margarita*, *Mucor_ambiguus*, *Neocallimastix_sp._JGI-202a.* The trend is consistent with the genus level (Fig.5).

For archaea, the dominant phyla are *Euryarchaeota* and *Thermoplasmatota*, and the difference between the three groups is not significant (*P*>0.05). The dominant genus is *Methanobrevibacter*, *Methanosphaera*, *Methanocorpusculum*. There was no significant difference in the abundance of *Metanobrevibacter* and *Metanosphaera* among the three groups (*P*>0.05), while the abundance of *Metanocorpusculum* was significantly higher in the AMP group than in the other two groups (*P*<0.05). The dominant strain is *Metanosphaera_sp.SHI1033*, *Methanobrevibacter_wolinii*, *Methanobrevibacter_boviskoreani*, *Methanobrevibacter_sp._AbM4*. The trend of this dominant bacterial species is similar to that of the bacterial genus (Fig.6).

3.3.3. *Microbial markers analysis in the rumen*

At the phylum level, LEfSe analysis was used to further determine the differential bacteria between groups. This study found that at the phylum level, *Acidiminococcaceae* was significantly enriched in the AMP group, and *Christensenellaceae* was significantly enriched in the LBE group. At the genus level, *Acidaminococcus*, *Denitrobacterium*, *Megasphaera*, *Mycoavidus*, *Tractidigestivor, Synergistaseae* were significantly enriched in the AMP group. *Pullilachnospira*, *Christensella*, *Desulfobacula*, *Eggerthellaceae* were significantly enriched in the LBE group.

In terms of fungi, at the phylum level, *Agaricaceae* and *Glomeraceae* were significantly enriched in the AMP group, while at the genus level, *Agricus* and *Rhizophagus* were significantly enriched. After adding licorice with bitter wood extracts to the MR, no bacterial enrichment was observed.

For archaea, at the phylum level, *Euryarchaeota* and *Nanoarchaeota* are significantly enriched in the AMP group, while at the genus level, *Methanothyrophilaceae*, *Euryarchaeota*, and *Nanoarchaeota* are significantly enriched. No archaea are enriched in the CON and LBE groups.

3.3.4. *KEGG analysis of rumen microbiota*

Through metagenomics KEGG analysis, the addition of AMP and licorice with bitter wood extracts to MR can significantly enrich the Isoleucine biosynthesis, Valine/isoleucine biosynthesis, and Molybdenim cofactor biosynthesis pathways (*P*<0.05).

3.3.5. *Analysis of rumen microbiota CAZymes*

An analysis was conducted on the differences in the CAZymes family in the rumen of experimental calves, searching for a total of 505 genes encoding CAZymes, including 19 co activity (AA), 60 carbohydrate binding modules (CBM), 16 carbohydrate esterases (CE), 246 glycoside hydrolases (GH), 94 glycosyltransferases (GT), and 86 polysaccharide lyases (PL).

Among them, AA6, GT92, GT25, GT33, PL5, GH13_12 were significantly enriched in the AMP group, and GH36, CBM13, GH114, and GH5_46 were significantly enriched in the LBE group.

4. Discussion

4.1. Effects of LBE and AMP on the growth performance of calves

Growth performance serves as a critical biomarker for assessing developmental trajectories in ruminants. Our findings align with established metabolic principles where increased weaning weight, elevated average daily gain (ADG), and reduced feed-to-gain ratio (F/G) collectively enhance growth potential while optimizing feed efficiency - key determinants of economic viability in calf production systems. The biological activity of plant extracts may be attributed to their characteristic phytochemical profiles. Sweet licorice extracts contain glycyrrhizic acid, polysaccharides, flavonoids, and triterpenoid saponins, while bitter wood derivatives are enriched with alkaloids, triterpenoids, and specific bitter principles(Mou et al. 2024). These bioactive compounds exhibit documented antimicrobial, anti-inflammatory, and antioxidant properties (Citation (Xu et al. 2024)), potentially mediating their growth-promoting effects through gastrointestinal microbiota modulation. Notably, our results resonate with previous findings in monogastric species. Zhu et al. (2024) demonstrated that dietary supplementation with 400 g/t licorice extract improved growth performance in weaned piglets, significantly enhancing nutrient utilization efficiency. Parallel studies in poultry revealed licorice polysaccharides (optimal dose: 1,500 mg/kg) upregulate growth-related gene expression, including growth hormone, myostatin, and myogenic differentiation factors in broiler skeletal muscle (Li et al. 2024). While Zhang et al.(2009) reported non-significant effects on Tan lamb weight parameters, all growth metrics consistently outperformed control groups, suggesting potential dose-dependent responses. Complementary evidence from beef cattle trials indicates bitter wood extract supplementation improves feed efficiency compared to conventional regimens(Wei et al.2006).The microbial-derived antimicrobial peptide employed in this study, produced via Saccharomyces cerevisiae fermentation, represents a novel approach to growth promotion. Its mechanism likely differs from phytogenic additives through targeted modulation of rumen microbial ecology rather than direct nutrient absorption enhancement.

AMP exhibit distinct advantages in livestock production, including rapid propagation kinetics, short fermentation cycles, and cost-effective biosynthesis. Their broad-spectrum antibacterial activity, particularly against Gram-negative pathogens, enhances host disease resistance while optimizing production efficiency through improved feed conversion ratios. Current evidence suggests AMP can effectively substitute antibiotics in swine production, as demonstrated by improved growth performance in weaned piglets (Wang et al. 2020; Lai et al. 2023), reduced diarrheal incidence and enhanced nutrient digestibility acid detergent fiber. Our findings align with these observations, though with notable contextual differences. Supplementation of MR with AMP increased ADG by 4.50% compared to the CON group, albeit without statistical significance. Intriguingly, the combination treatment LBE yielded numerically higher ADG values, suggesting potential synergistic effects. The growth-promoting properties of phytogenic compounds may be mediated through bioactive constituents such as polysaccharides, flavonoids, and triterpenoid saponins, which enhance nutrient utilization via gastrointestinal tract modulation. Through the results of feed intake, we can also see that LBE can improve the feeding performance of calves. This indicates that sweet and bitter extracts can have a positive effect on the gastrointestinal tract of calves and effectively utilize dietary energy. The reason for the insignificant difference may be the influence of environmental temperature, as the hot weather causes heat stress in calves.

Notably, while extant research predominantly focuses on monogastric and aquatic species , ruminant applications remain underexplored, particularly in pre-weaned calves, which is still a blank and requires further in-depth research.

4.2. Effects of LBE and AMP on calf fecal consistency scores and diarrhea incidence

Current rearing practices for young ruminants remain predominantly based on traditional management systems, which are associated with elevated neonatal mortality rates. Growth performance during the lactation period serves as a critical indicator of calf development quality and profoundly influences future production potential.

In addition to exhibiting antioxidant and immune-enhancing properties, licorice flavonoids demonstrate potent antibacterial and antiviral activities, directly or indirectly inhibiting viral and bacterial pathogens. Yin et al.(2021) isolated pathogenic bacteria from diarrheic calf feces and demonstrated that 5% licorice extract effectively suppressed Escherichia coli biofilm formation. Bai et al. (2024) further established through in vitro and in vivo studies that licorice extract exerts inhibitory effects against porcine epidemic diarrhea virus. Notably, dietary supplementation with 400 g/t Sophora flavescens extract significantly reduced diarrhea incidence in piglets while improving growth performance(Li et al. 2014; Li et al. 2015).

The present study demonstrated that supplementation of MR with licorice and Sophora flavescens extracts significantly reduced diarrhea incidence in preweaning calves. This therapeutic effect may be attributed to the dual bacteriostatic activity of licorice polysaccharides against Staphylococcus aureus and E. coli, mediated through disruption of bacterial cell wall integrity and/or inhibition of protein synthesis(Palevich et al. 2019). Furthermore, licorice-Sophora flavescens complexes exhibit mucosal protective properties, as evidenced by their capacity to repair gastric ulcers, restore colonic lesions(Li et al. 2009), and form a continuous protective film along the gastrointestinal tract. These mechanisms collectively enhance digestive mucosal barrier function, thereby providing prophylactic and therapeutic benefits against diarrhea in neonatal calves(Wallace et al. 2003). AMP and proteins (AMPs) are evolutionarily conserved defense moleculessynthesized by multicellular organisms as primary components of innate immunity. These bioactive compounds exhibit broad-spectrum antimicrobial activity, including direct bactericidal effects against Escherichia coli and Staphylococcus aureus, fungistatic properties, and antiviral/anticancer potential(Feng et al. 2020). Studies have demonstrated that AMP supplementation effectively mitigates postweaning diarrhea in piglets through enhanced intestinal barrier integrity, modulated immune responses, and reduced inflammatory markers (Feng et al. 2020). Consistent with these findings, our results revealed that dietary supplementation of AMPs in MR significantly reduced diarrhea incidence (9.06% reduction), indicating their therapeutic potential in neonatal calf management.

Among experimental groups, calves receiving LBE demonstrated the most pronounced anti-diarrheal efficacy. The cost-effectiveness and biological activity of licorice-derived compounds position LBE as a promising candidate for developing natural antidiarrheal agents. This aligns with current industry demands for sustainable alternatives to antibiotic growth promoters in dairy calf production systems.

4.3. Effects of LBE and AMP on rumen microbiota development in preweaning calves

The rumen serves as the primary digestive organ in ruminants, housing complex microbial communities that directly influence host nutrient metabolism and health status (Kim et al. 2024; Hu et al. 2024). To evaluate microbial modulation strategies, we supplemented MR with LBE and AMP in preweaning calves. Rumen fluid samples obtained via esophageal catheterization at 67 days of age underwent metagenomic sequencing to characterize bacterial and fungal community structures.

Microbial diversity analysis revealed no significant intergroup differences in bacterial alpha diversity indices. For fungi, the Chao index of the AMP group was the highest, followed by the LBE group. The Shannon index is the opposite, with LBE higher than the AMP group, but the difference is not significant, indicating that adding AMP and licorice with bitter wood extracts to the MR can change the abundance of rumen fungi in calves.

4.4. Effects of AMP and LBE on the levels of microbiota in the rumen of calves

4.4.1. *Bacterial phylum-level composition*

The rumen microbiome comprises approximately total number of bacteria in each gram of rumen fluid is 10^{10}-10^{11}, the number of protozoa is 10^{5}-10^{6}, and the number of anaerobic fungi is 10^{3}-10^{5}(Williams et al. 2020). The microbial community can regulate the physiological processes of host nutrition digestion and absorption, metabolism, body immunity, and even host behavior and cognitive function(Hu et al. 2022). At the phylum level, *Firmicutes*, *Bacteroidota*, and *Actinobacteria* dominated the bacterial community, consistent with established profiles of ruminant gastrointestinal ecosystems (de Oliveira et al. 2013; Wang et al. 2017). These core phyla are functionally linked to dietary fiber fermentation, lipid metabolism, and energy harvest efficiency (Bi et al. 2018).

No intergroup differences were observed in the relative abundances of *Firmicutes*, *Actinobacteria*, or *Bacteroidota*. The AMP group had the highest abundance of *Actinobacteria*, followed by the LBE group. The study by Shaodan Wang et al. showed that dietary antimicrobial peptide supplements can reduce the abundance of *Clostridium*, while increasing the proportion of *Actinobacteria*, which is consistent with the results of this experiment. *Bacteroidota* has a significant ability to ferment various plant oligosaccharides and polysaccharides, host derived polysaccharides, and breast milk oligosaccharides (Adamberg et al. 2018). In this experiment, the CON group had the highest abundance of *Bacteroidota*, followed by the LBE group, and the AMP group had the lowest abundance. Qiao et al.(2022) found that adding licorice polysaccharides to the diet of broiler chickens significantly reduced the abundance of *Bacteroidetes*, consistent with the results of this experiment. Based on the results of growth performance and diarrhea frequency, we can speculate that adding AMP, licorice, and bitter wood extracts to the MR can to some extent regulate the rumen microbiota to improve the growth performance of calves.

Using LEfSe analysis to further determine the differential bacteria between groups, this study found that at the phylum level, *Acidaminococcaceae* was significantly enriched in the AMP group, *Selenomonadaceae* and *Aurantimonadaceae* were significantly enriched in the CON, and *Christensenaceae* was significantly enriched in the LBE group. Acidaminococcaceae has the ability to decompose amino acids, and through enzymatic reactions, it degrades amino acids into intermediate metabolites, further participating in some metabolic pathways such as tricarboxylic acid metabolism. In the gastrointestinal tract of the body, it participates in the host's nutrient absorption and metabolic processes(Morotomi et al. 2007; Savin et al. 2022).

The *Christensenellaceae*, a recently described family in the phylum *Firmicutes*, is emerging as an important player in human and animal health. Research shows that *Christensenellaceae* is related to some metabolic diseases, such as hypertension, diabetes, etc(Waters et al. 2019; Tavella et al. 2021), and also to the reduction of the risk of intestinal inflammatory diseases. It can affect the level of inflammation by regulating the intestinal immune system. *Christensenellaceae* can produce α - arabinosidase, β - glucosidase and β - galactosidase related to feed conversion efficiency(Ducker et al. 2017). *Christensenaceae* contains genes for cellulase and hemicellulase secretase, which can enhance the ability of ruminants to degrade cellulose and obtain energy from recalcitrant polysaccharides, and is positively correlated with body weight(Williamson et al. 2022; Kim et al. 2001). Wang et al.(2023) observed a positive correlation between the relative abundance of *Christensenellaceae* and ADG. The results of this experiment are consistent with this. When LBEs were added to the MR, it was observed that *Christensenaceae* was significantly enriched in this group, and the abundance of *Christensenaceae* was positively correlated with ADG. The side view indicates that the addition of LBEs reduces the occurrence of diseases, and further research is needed on the relevant mechanisms in calf rearing(Li et al. 2020).

4.4.2. *Bacterial genus level*

At the genus level, the dominant genera are *Lachnospiraceae*, *Prevotella*, *Eubacteria*, and *Olsenella*; Adding AMP and licorice with bitter wood extracts to MR resulted in an increase in the abundance of *Lachnospiraceae*, *Eubacteria*, and *Olsenella*, while the abundance of *Prevotella* was consistent with that of *Bacteroidetes*.

Tan et al.(2023) found that the relative abundance of *Lachnospiraceae* in the feces of piglets fed with a diet supplemented with AMP increased, and it can reduce diarrhea, improve nutrient digestion and immunity, and increase the abundance of beneficial bacteria in the weaned autogenous gut. The use of Shaoyao Gancao Tang can significantly increase the relative abundance of *Lachnospiraceae*, and the results of this experiment are consistent with this. *Lachnospiraceae,* as a butyrate producing bacterium, has a certain correlation between its abundance and the concentration of volatile fatty acids in the rumen(Yin et al. 2021). It plays an important role in normal fermentation of the rumen and is significantly correlated with fat degradation(Kang et al. 2017). Adding licorice and bitter wood to MR can significantly improve the apparent digestibility of crude fat, which may be the reason for the significant increase in the relative abundance of the genus *Trichospiraceae*. Meanwhile, it can exert anti-inflammatory effects, which is beneficial for maintaining gastrointestinal health.

Eubacterium is one of the core bacterial genera in the human and animal gastrointestinal tract. It was first proposed in 1938 to exist in the rumen and internal environment of ruminants and is the main species that produces butyric acid. Short chain fatty acids are generally considered to have multiple important roles in maintaining human health, such as serving as special nutrients and energy components in the intestinal epithelium, protecting the intestinal mucosal barrier, reducing inflammation levels, and enhancing gastrointestinal motility. The breakdown of plant polysaccharides by microorganisms in the rumen is the basis for digestion in ruminant livestock(Mukherjee et al.2020). Adding licorice and bitter wood to MR can promote the effectiveness of Olsenella. Olsenella is an important degrader and user of lignocellulosic plant materials. These bacteria degrade polysaccharides and ferment the released monosaccharides, producing short chain fatty acids for ruminant growth and the production of meat, milk, and fiber products(Palevich et al. 2019). This is also positively correlated with the daily weight gain of restructured calves.

4.4.3. *Microbial Community Analysis*

LEfSe analysis revealed significant enrichment of six bacterial genera in the AMP group compared to controls. including *Acidaminococcus*, *Denitrobacterium*, *Megasphaera*, *Mycoavidus*, *Tractidigestivor*, and *Synergistaceae*. Notably, *Acidaminococcus*, a Gram-negative anaerobic bacterium prevalent in the gastrointestinal tract of ruminants (Hamada et al.2023; Takakura et al.2019), demonstrated unique metabolic characteristics. This genus primarily utilizes amino acids (particularly glutamate) rather than carbohydrates for energy production, with only 40% of strains exhibiting limited glucose metabolism(Hamada et al. 2023). During amino acid fermentation, Acidaminococcus generates acetic and butyric acids at a 2:1 molar ratio, accompanied by CO_2 production, while neither hydrogen nor propionic acid is synthesized(Takakura et al. 2019). These metabolic outputs align with its recognized role in intestinal health maintenance through short-chain fatty acid (SCFA) production and energy provision to host epithelial cells(Abdugheni et al. 2023).The phytochemical additives (LBE) in AMP formula likely contributed to rumen microbiota modulation by suppressing pathogenic bacteria while promoting beneficial taxa colonization. This microbial shift correlated with improved nutrient digestibility, enhanced intestinal immune barrier function, and reduced diarrhea incidence. Of particular interest, *Denitrobacterium* and *Tractidigestivor* demonstrated potential biotechnological relevance through their capacity to produce specialized metabolites with applications in pharmaceutical development and environmental management(Benlaifaoui et al. 2023; Gargari et al. 2024; Kwon et al. 2023; Anderson et al. 2000).

Megasphaera exhibited dual functional characteristics: while its fructose and lactate fermentation capabilities may contribute to rumen acidosis mitigation via lactic acid metabolism(Arik et al. 2019), certain species have been associated with diarrheal conditions. This genus' net beneficial impact in our trial may stem from its synergistic interactions with Acidaminococcus in maintaining ruminal pH homeostasis, particularly under high-concentrate dietary conditions.

The AMP supplementation significantly increased *Megasphaera* abundance, consistent with its documented role in rumen acidosis mitigation. Arik et al. (2019)demonstrated that ruminal inoculation of *Megasphaera* alleviates subacute rumen acidosis in cattle, while Carey et al. (2021) reported an inverse relationship between *Megasphaera* abundance (class: Clostridia) and Cryptosporidium-associated diarrhea severity. Specifically, low *Megasphaera* levels correlated with clinical diarrhea during Cryptosporidium infection, suggesting its metabolites or immunomodulatory properties may ameliorate pathogen-induced outcomes. Our findings extend these observations by demonstrating that AMP-mediated *Megasphaera* enrichment contributes to diarrhea reduction in calves, likely through dual mechanisms of lactic acid metabolism and immune regulation.

Mycoavidus, first taxonomically characterized by Buttner et al. (2021), exhibited AMP-induced enrichment despite lacking prior mechanistic studies in this context. This genus biosynthesizes bioactive compounds including macrolides, neoximes C/D, and symbiosin, which demonstrate nematocidal activity and function as biosurfactants to potentiate fungal virulence against pathogens. While the precise interaction between AMP and Mycoavidus colonization remains uncharacterized, our results establish AMPs as effective modulators of its proliferation. This enrichment suggests a synergistic mechanism whereby AMPs simultaneously promote beneficial bacterial taxa (*Mycoavidus*, *Megasphaera*) while suppressing enteric pathogens, thereby maintaining gut microenvironment homeostasis.

4.4.4. *Phytochemical Modulation of Gut Microbiota*

Dietary supplementation with licorice-bitter wood extract blend significantly enriched beneficial gastrointestinal taxa in the AMP group, including *Pullilachnospira*, *Christensenella*, *Desulfobacula*, and *Eggerthellaceae*. Licorice-derived bioactive compounds (flavonoids and triterpenoid saponins) demonstrated digestostimulatory properties, while bitter wood constituents (alkaloids and bitter principles) exhibited dual functionality: (1) antimicrobial activity against enteropathogens, and (2) prolonged intestinal chyme retention through motility modulation. The enriched Eggerthellaceae (family: *Coriobacteriaceae*) demonstrated critical metabolic functions through carbohydrate fermentation, generating short-chain fatty acids (SCFAs) that enhance intestinal epithelial energy metabolism, mucosal integrity maintenance, and immunoregulatory signaling(Gupta et al. 2013; Zhang et al. 2022). Notably, this family counteracts *Clostridium* proliferation and ameliorates diet-induced gastrointestinal dysbiosis, particularly constipation-associated symptoms(Li et al. 2023).

Pullilachnospira (phylum: *Actinobacteria*) formed synergistic consortia with *Eggerthellaceae*, collectively contributing to host nutrient assimilation and xenobiotic metabolism. *Christensenella* emerged as a keystone probiotic taxon, with its AMP-induced abundance correlating with improved gut microbiome resilience and metabolic homeostasis. The observed microbial shifts align with Desulfobacula's documented role in sulfate reduction and mucosal layer stabilization, suggesting comprehensive phytochemical-driven microbiota restructuring.

4.5. Fungal Community Modulation in Calf Rumen

4.5.1. *Phylum-Level Composition*

Rumen fungi, constituting 8-20% of total ruminal biomass, enhance dietary fiber degradation through lignocellulolytic enzyme production, thereby optimizing rumen digestion efficiency(Wallace et al. 2003). Our analysis identified four dominant fungal phyla: *Mucoromycota*, *Chytridiomycota*, Ascomycota, and *Basidiomycota*. Antimicrobial peptide (AMP) supplementation exhibited dose-dependent inhibition of Mucoromycota, consistent with previous reports that 14.3 kDa grass carp scale-derived AMPs exert additive antifungal effects against this phylum(Wang et al.2020).

Both AMP and licorice-bitter wood extract (LBE) treatments significantly increased the relative abundance of *Chytridiomycota* and *Basidiomycota* compared to control. The ecological significance of this shift is twofold: 1) *Basidiomycota* enhances lignin decomposition through peroxidase secretion, and 2) *Chytridiomycota* contributes to chitinrich substrate degradation. LEfSe analysis further revealed phylum-level enrichment of *Glomeromycota* (arbuscular mycorrhizal fungi) and Agaricomycetes (wood-decay specialists) in treatment groups, suggesting enhanced plant cell wall deconstruction capacity.

4.5.2. *Microbial funi genus level*

At the genus level, adding AMP, licorice, and bitter wood extracts to MR can reduce the abundance of *Rhizopus*, *Gigaspora*, *Mucor*, and *Rhizophagus*, while increasing the abundance of *Pyromyces*. As part of the rumen microbial community, *Pyromyces* may have specific metabolic pathways and enzyme systems to adapt to the degradation of high fiber substrates. The study by Xufeng Dong et al(Dong et al. 2023). showed that AMP have high expression activity in *Pyromyces*, and the study by De Mei Meng et al(Meng et al. 2017). also showed that when *Pyromyces* is used as a cell vector, AMP can effectively express and significantly inhibit the growth of *Escherichia coli* and *Staphylococcus aureus*. This is the first report on the heterologous expression of PaDef in Pichia pastoris, and the described method has broad prospects in the development of antibacterial drugs. However, research on the effects of LBE on *Pyromyces* is still blank, and the associated mechanisms need to be further explored. From the results of this experiment, it can be inferred that adding LBEs to MR has the strongest promoting effect on *Pyromyces*. The use of LEfSe analysis to further determine the differential bacteria between groups showed that *Agricus* and *Rhizophagus* were significantly enriched in the AMP group. It can be seen that AMP help to enhance the richness level of fungi, which is consistent with the alpha diversity Chao index.

4.6. Effects of AMP and LEB on branched chain amino acids and *carbohydrate metabolic* enzymes

4.6.1. *KEGG Pathway Enrichment Analysis*

Supplementation of MR with AMP and LBE significantly upregulated the biosynthesis pathways of isoleucine , valine， leucine and molybdenum cofactor. The increase of branched chain amino acids containing valine, leucine, and isoleucine can affect the expression of albumin genes, thereby affecting albumin synthesis. The study by Liang et al.(2021) showed that Isoleucine biosynthesis can improve the growth performance and red blood cell and plasma protein concentrations of piglets, which can serve as an effective strategy to protect weaned piglets from pathogen infection through immune regulation. The licorice with bitter wood extracts used in this experiment are widely used Chinese herbal medicines, containing various bioactive components such as glycyrrhetinic acid and glycyrrhizin. These ingredients have various biological functions such as anti-inflammatory, antioxidant, and immune regulation. In animal husbandry, licorice extract may indirectly promote carbon metabolism by regulating the physiological state of animals, improving the digestibility and utilization of feed. Kumu, also a plant with medicinal value, may contain active ingredients such as alkaloids and flavonoids in its extracts. These ingredients have antibacterial, antiviral, and immunomodulatory effects.

In animal husbandry, the addition of bitter wood extract may help improve the intestinal health of animals, reduce the growth of harmful bacteria, improve the absorption and utilization efficiency of nutrients, and promote the strengthening of carbon metabolism pathways. Isoleucine biosynthesis, Valine/isoleucine biosynthesis, Molybdenim cofactor biosynthesis pathways, these amino acids are key components of protein synthesis, capable of oxidizing energy supply, promoting protein synthesis, and enhancing immunity. Therefore, sufficient supply of valine, leucine, and isoleucine is crucial for the normal growth and development of ruminants. In the rumen fermentation process of ruminants, the synthesis of these amino acids is also closely related to microbial activity. The microorganisms in the rumen can decompose protein and non protein nitrogen-containing compounds in feed, producing nutrients such as amino acids. These amino acids can not only be utilized by host organisms, but also serve as nitrogen sources for microorganisms themselves, participating in their growth and metabolic activities. Therefore, the biosynthesis of valine, leucine, and isoleucine interacts with the fermentation of rumen microorganisms, jointly maintaining the nutritional balance and health status of ruminants(Xue et al. 2023). The addition of AMP and licorice with bitter wood extracts may directly affect enzymes or metabolic pathways related to amino acid synthesis. For example, AMP may activate or enhance the activity of certain key enzymes, which play a crucial role in the biosynthesis of valine, leucine, and isoleucine. By regulating the activity of these enzymes, AMP can promote the synthesis process of amino acids. It can also promote the conversion of carbohydrates into volatile fatty acids such as propionic acid, enhancing the pathway of propionic acid metabolism.

4.6.2. *CAZy Analysis*

Based on *CAZy Analysis*, AA6, GH153, GT92, GH13:32, GT33, PL5, and GT25 were significantly enriched in the AMP group, and these gene families were involved in the synthesis of enzymes encoding plant cell wall degradation, such as endo-1,4- β - xylanase (EC 3.2.1.8) and N-glycan core α-1,6-fucoside β-1,4-galactosyltransferase (EC 2.4.1. -); β-1,4-galactan β -1,4-galactosyltransfer/β-1,4-galactan synthesis (EC 2.4.1. -); β-1,4-galactan β -1,4-galactosyltransfer/β-1,4-galactan synthesis (EC 2.4.1. -); while GH36, GH114, GH5_8, CBM13, and GH133 were significantly enriched in the LBE group. These genes encode genes involved in Stachyose synthesis/galactinol raffinose galactosyltransfer (EC 2.4.1.67), Raffinose synthesis/galactinol sucrosse galactosyltransfer (EC 2.4.1.82), alpha galactosaminidase (EC 3.2.1. -), Blood group B α -1,3-galactosamine (EC 3.2.1. -), endo - α -1,4-galactosamine (EC 3.2.1.109). Among them, only one CAZymes gene involved in auxiliary enzyme activity was enriched in the antimicrobial peptide group. Among CAZymes genes involved in structural carbohydrates (including cellulose, hemicellulose, starch, protein, and lignin), one was significantly enriched in the LBE group, and in the GH group, two were significantly enriched in the AMP group, one was significantly enriched in the CON group, and four were significantly enriched in the LBE group. Glycosyltransfers (GT) are a class of enzymes widely present in organisms, with 3 significantly enriched in the AMP group. Polysaccharide Lyases (PL) are a class of enzymes that can catalyze the non hydrolytic cleavage of glycosidic bonds in polysaccharide chains (usually through a β - elimination mechanism). Among the CAZymes genes, only one is significantly enriched in the AMP group.

Under the conditions of this experiment, in the rumen of calves in the LBE group, compared with the CON and Z groups, the genes encoding the enzymes that hydrolyze glycosidic bonds in the GH (GH36, GH114, GH5_8, GH13_3) family of CAZymes were significantly enriched in the LBE group, which enriched the KEGG function of carbohydrate degradation, including "galactose degradation", "starch and sucrose metabolism", and the downstream pathway of "glycolysis" that converts glucose to pyruvate. This indicates that the LBE group may produce more hydrolysis products and pyruvate, as the carbohydrate degradation ability is higher. This result indicates that the addition of licorice and bitter wood to MR improved the calf's ability to degrade carbohydrates. The ability of cows to degrade complex substrates, CON and AMP do not affect the abundance of plasmin genes encoded by the GH family genes. Firmicutes play an important role in the degradation of carbohydrates, including starch, cellulose, hemicellulose, and oligosaccharides(Wang et al. 2023). *Lachnospiraceae* belongs to a genus of *Firmicutes*, which is involved in the degradation of starch, hemicellulose, and pectin(Vacca et al. 2020). There are reports(Park et al. 2020)that cows fed high-energy starchy feed are rich in Firmicutes. Similarly, in this study, the GH family related to starch biodegradation mainly belonged to the phyla *Firmicutes*, *Lachnospiraceae*, and *Eubacteria*. *Bacteroidetes*, *Actinobacteria*, and others are major contributors to CAZymes and have been found in the GH, GT, CBM, CE, and PL families, playing important roles in the rumen of calves(Wang et al. 2019).

5. Conclusion

Based on the experimental findings, the following conclusions were drawn:

1. Growth and Health Parameters:

Supplementation of MR and starter with LBE combined with AMP significantly enhanced growth performance, reduced diarrhea incidence, and improved immune function in preweaned calves.

2. Rumen Microbiota Modulation:

Dietary inclusion of LBE induced marked shifts in rumen microbial ecology. At the phylum level, we observed increased abundance of *Christensenellaceae*, a taxon associated with enhanced gut health. Genus-level analysis revealed elevated proportions of *Pulilachnospira*, *Christensenella*, *Desulfobacula*, and *Eggerthellaceae*. These microbial changes correlated with functional enrichment of key metabolic pathways, including isoleucine biosynthesis, valine/isoleucine biosynthesis and molybdenum cofactor biosynthesis

The modified microbiota profile demonstrated three synergistic benefits:

(a) Enhanced nutrient absorption efficiency through improved gastrointestinal metabolism
(b) Suppression of pathogenic bacterial colonization
(c) Increased average daily gain (ADG) via optimized rumen function

Practical Implications:

Licorice-Bitter Wood Extracts supplementation in MR demonstrated optimal efficacy in calf rearing programs. This natural growth-promoting strategy shows promise as a sustainable alternative to antibiotic growth promoters, with particular value for improving gastrointestinal health and production parameters in intensive calf production systems.

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7. CRediT author statement

I also declare that all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

8. CRediT authorship contribution statement

Zhang Chuntao: Investigation, Validation, Writing - original draft, Writing - review&editing. **Xing Zhongying:** Investigation, Validation, Writing - original draft. **Xu Xiang:** Conceptualization, Investigation, Writing-review & editing. **Tu Yan** and **Diao Qiyu:** Conceptualization, Investigation, Project administration, Writing-review & editing.

9. Declaration of competing interest

The authors declare that they have no conflict of interest

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11. Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

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Table 1. The content of main active ingredients in Licorice-Bitter Wood Extracts(LBE)

Items	Ingredients	Contents
Total Flavonoids	Flavonoids	10.29, mg/g
Soluble sugar	Sucrose（Suc）	183.38, ug/mg
	Glucose（Glu）	65.09, ug/mg
	Fructose（Fru）	27.49, ug/mg
	Maltose（Mal）	5.91, ug/mg
Polyphenol	Isoliquiritigenin	25.65, ng/mg
	4-Hydroxybenzoic acid	19.97, ng/mg
	3,4-Dihydroxybenzoic acid	14.26, ng/mg
	Salicylic acid	11.96, ng/mg
	Vanillic acid	10.90, ng/mg
	Quercetin 3-β-D-glucoside	9.01, ng/mg
	Rutin	7.31, ng/mg
	Naringenin Chalcone	7.31, ng/mg
	Benzoic acid	5.77, ng/mg
	Genistin	4.88, ng/mg
	Naringenin	4.40, ng/mg
	Apigenin	4.33, ng/mg
	Daidzein	4.29, ng/mg

Table 2. Nutrient levels of milk replacer (%, DM)

Items	Contents$^{1)}$
DM	95.01
CP	24.07
EE	18.28
Ash	4.62
NDF	0.99
Ca	0.81
P	0.43

[1] Nutrient levels were measured values.

Table 3. Composition and nutrient levels of the starter (%, DM)

Items	Contents$^{1)}$
Ingredients	
Soybean meal	24.00
Steam-flaked corn	43.00
Lucerne	10.00
Wheat bran	8.50
Milk powder	10.00
Salt	0.50
Premixed feed	4.00
Nutrition levels	
DM	88.70
CP	18.27
Ash	6.91
EE	3.59
NDF	19.84
ADF	9.33
Ca	0.88
P	0.51

[1] The premix provided the following per kg of the starter: VA 15800 IU, VD3 2880 IU, VE 28 IU, Fe 64 mg, Cu 11 mg, Mn52 mg, Zn 92 mg, Se 0.6 mg, I 0.8 mg, Co 0.3 mg.

[2]The calves in the experimental group were added with 150mg/kg of starter feed.

[3]Nutrient levels were measured values.

Table 4. Fecal scoring criteria

Score	Appearance
1	Normal, Forming
2	Semi formed, Paste like
3	Loose, but still on the surface of the mat
4	Water like, under the mat grass

Table 5. The effect of different additives on the growth performance of calves

Items	Groups			SEM	*P*-value
	CON	AMP	LBE		
Initial weight/kg	42.58	42.48	42.19	0.73	0.97
Final weight/kg	64.36	65.24	72.36	1.87	0.06
ADG/g	363.05	379.38	502.95	23.78	0.08
ADFI/g	932.59	910.58	1124.20	58.62	0.16
F/G	2.47	2.40	2.24	0.11	0.64

Table 6. The effect of different additives on fecal score and diarrhea rate of lactation calves

Items	Treatments			SEM	*P*-value
	CON	AMP	LBE		
Fecal score	2.29^{a}	2.24^{a}	1.81^{b}	0.07	0.003
Diarrhea frequency/%	52.71^{a}	48.33^{ab}	31.67^{b}	3.01	0.008

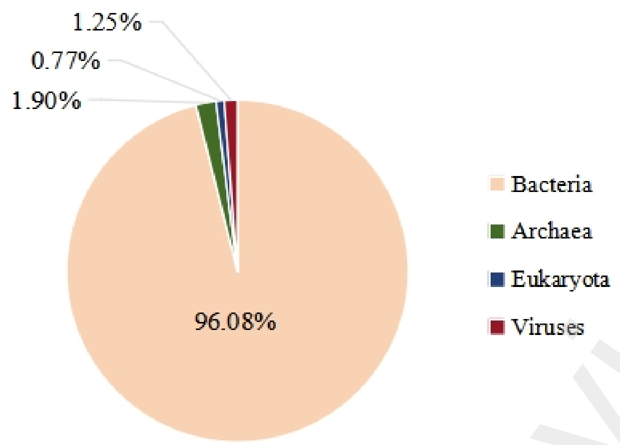
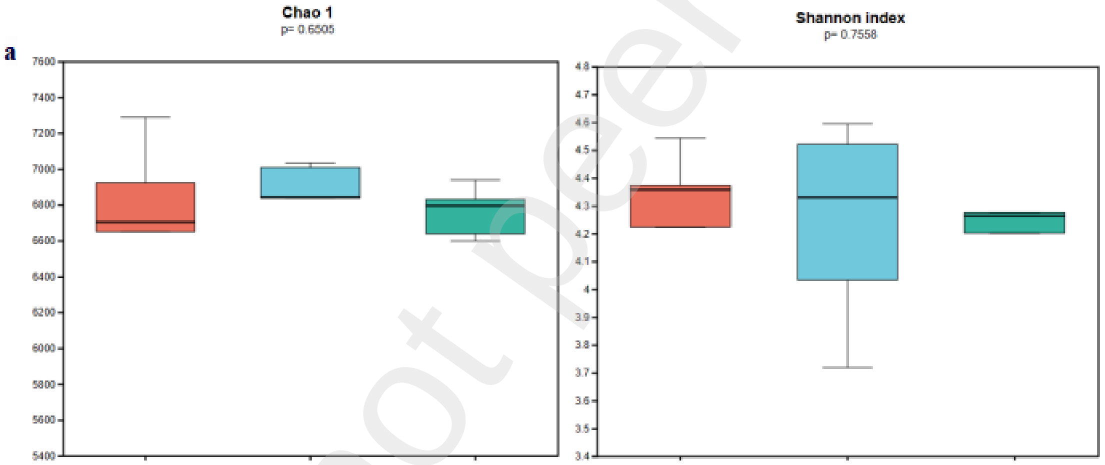
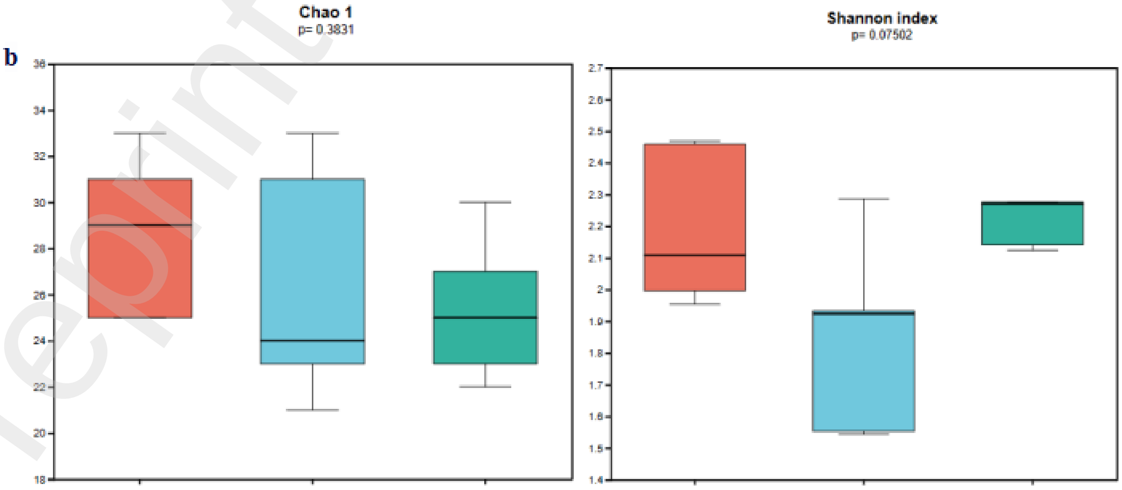


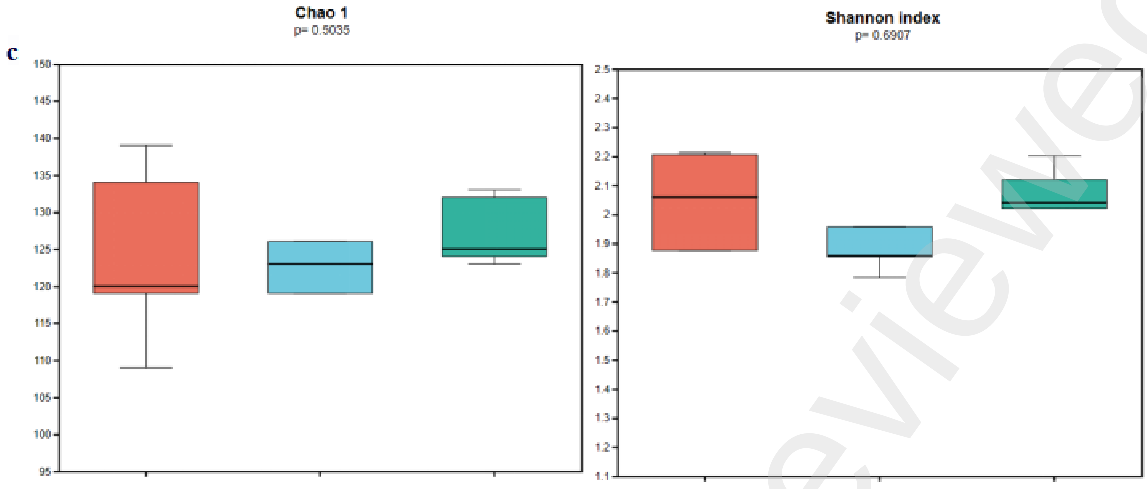
Fig: 1. Basic composition of rumen microbiota identified by metagenomic analysis



Bacteria

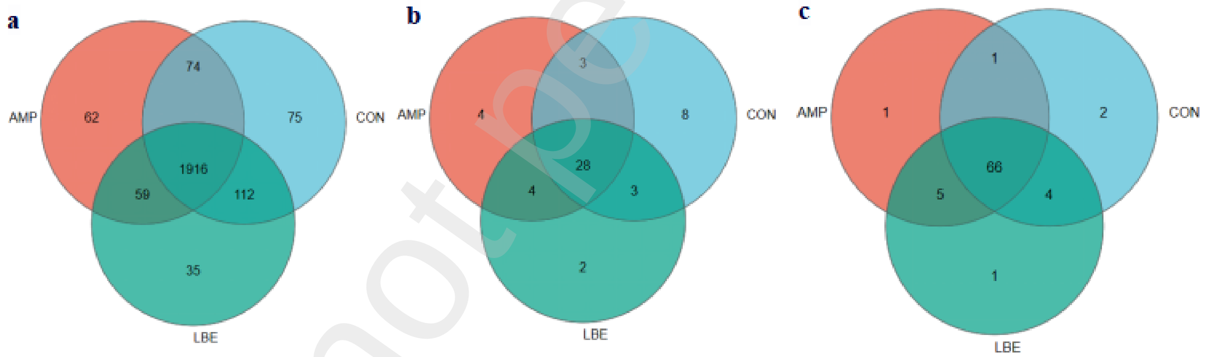


Fungi



Archaea

Fig: 2. The diversity of rumen microbial communities in calves. a α-diversity of Bacterial in the rumen; b α-diversity of Eukaryota in the rumen; c α-diversity of Fungi in the rumen.



Bacteria **Fungi** **Archaea**

Fig: 3. Venn diagram of OTUs in three groups. The difference among three groups was identified by Kruskal–Wallis multiple comparisons, and asterisk indicated the significant difference (*P*<0.05).

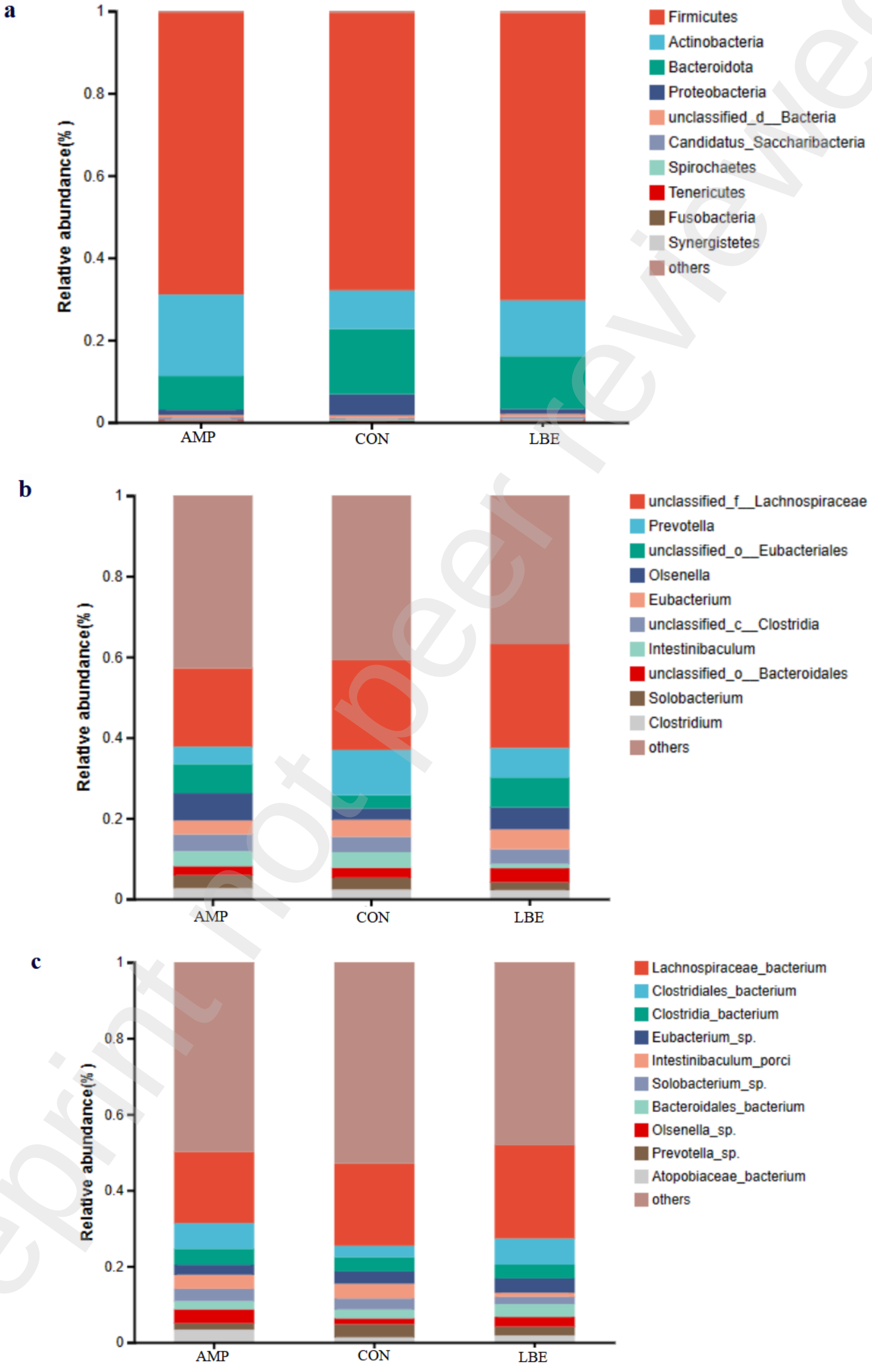
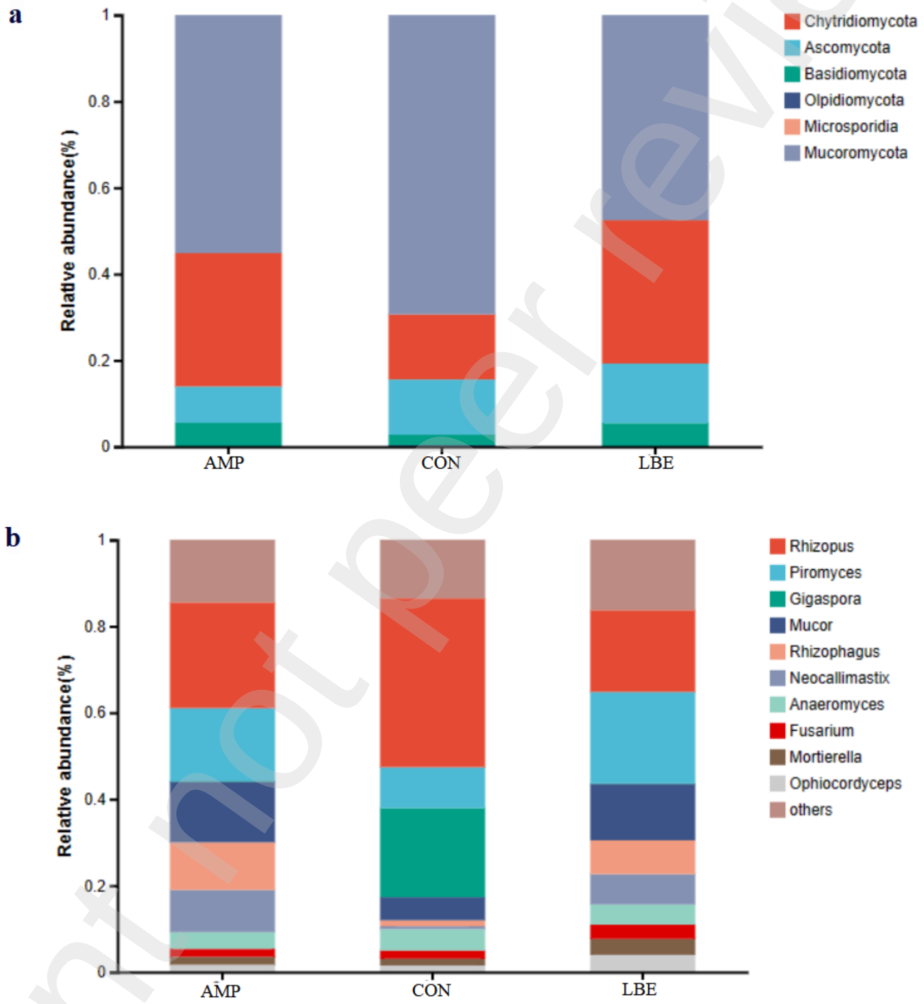

a
Relative abundance(%)
1
0.8
0.6
0.4
0.2
0
AMP
CON
LBE
Firmicutes
Actinobacteria
Bacteroidota
Proteobacteria
unclassified_d__Bacteria
Candidatus_Saccharibacteria
Spirochaetes
Tenericutes
Fusobacteria
Synergistetes
others
b
Relative abundance(%)
1
0.8
0.6
0.4
0.2
0
AMP
CON
LBE
unclassified_f__Lachnospiraceae
Prevotella
unclassified_o__Eubacteriales
Olsenella
Eubacterium
unclassified_c__Clostridia
Intestinibaculum
unclassified_o__Bacteroidales
Solobacterium
Clostridium
others
c
Relative abundance(%)
1
0.8
0.6
0.4
0.2
0
AMP
CON
LBE
Lachnospiraceae_bacterium
Clostridiales_bacterium
Clostridia_bacterium
Eubacterium_sp.
Intestinibaculum_porci
Solobacterium_sp.
Bacteroidales_bacterium
Olsenella_sp.
Prevotella_sp.
Atopobiaceae_bacterium
others

Fig: 4. The relative abundances of bacteria in rumen. **a** Relative abundances of bacterial communities at the phylum level. **b** Relative abundances of bacterial communities at the genus level. **c** Relative abundances of bacterial communities at the species level. The difference among three groups was identified by Kruskal–Wallis multiple comparisons, and asterisk indicated the significant difference (*P*<0.05).



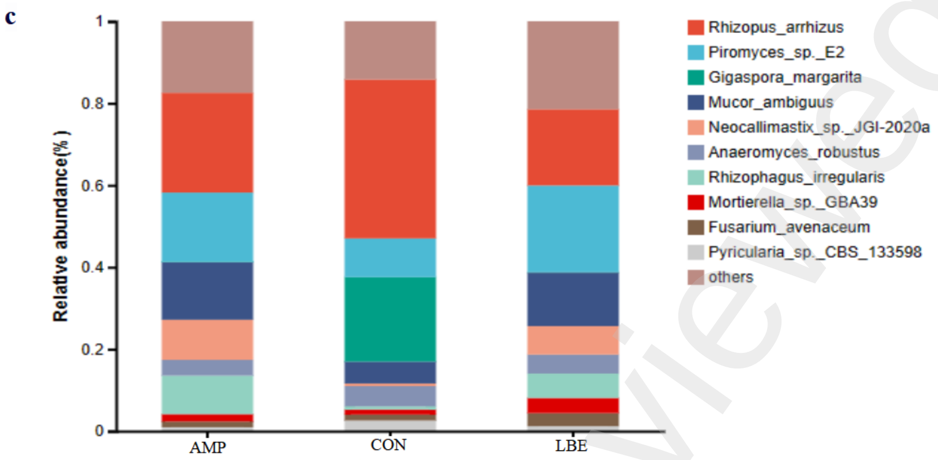
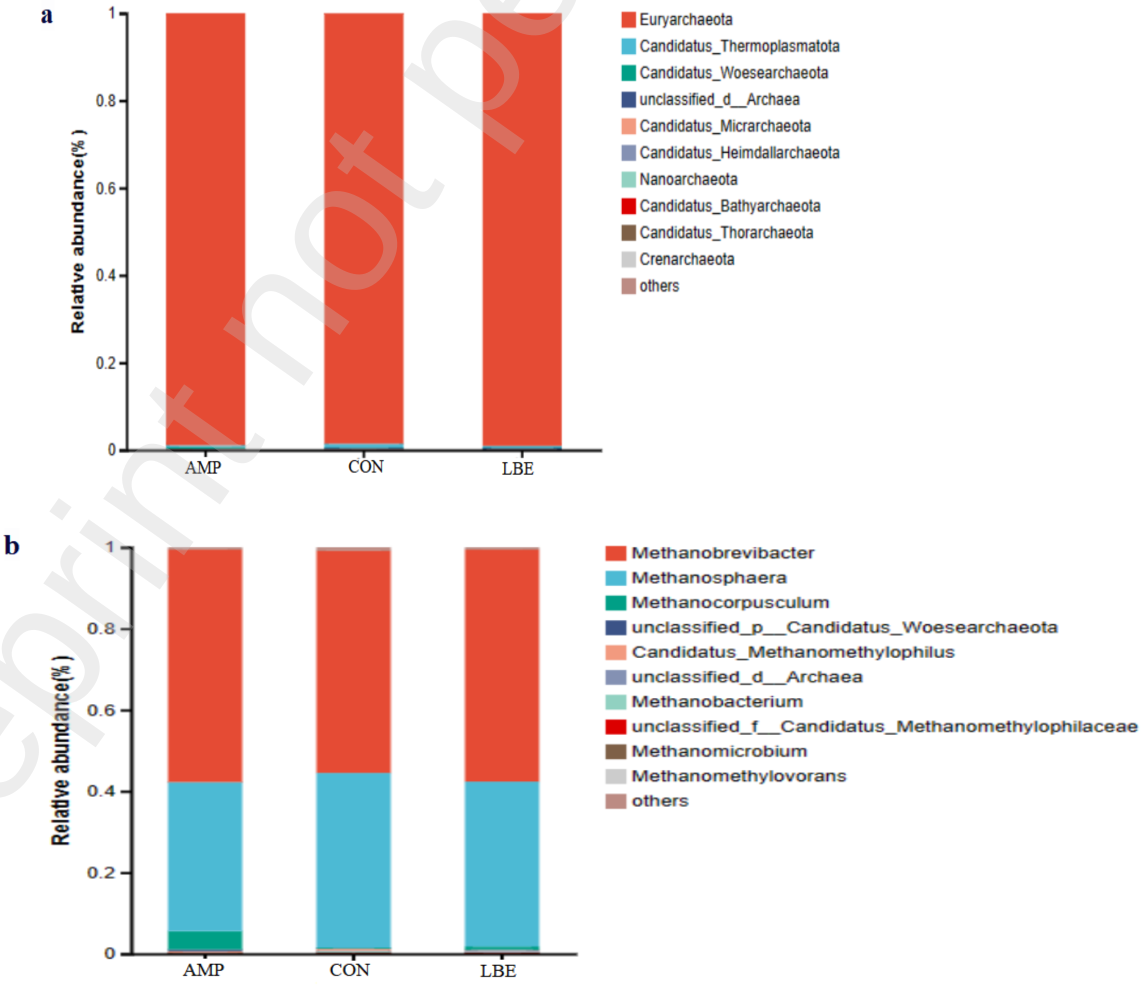


Fig: 5. The relative abundances of fungi in rumen. **a** Relative abundances of fungi communities at the phylum level. **b** Relative abundances of fungi communities at the genus level. **c** Relative abundances of fungi communities at the species level. The difference among three groups was identified by Kruskal–Wallis multiple comparisons, and asterisk indicated the significant difference ($P<0.05$).



c

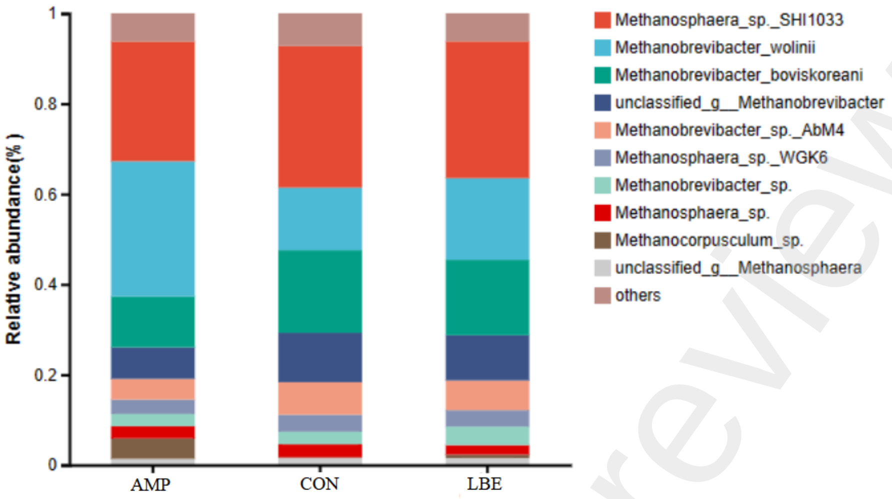


Fig: 6. The relative abundances of archaea in rumen. **a** Relative abundances of archaea communities at the phylum level. **b** Relative abundances of archaea communities at the genus level. **c** Relative abundances of archaea communities at the species level. The difference among three groups was identified by Kruskal–Wallis multiple comparisons, and asterisk indicated the significant difference (*P*<0.05).

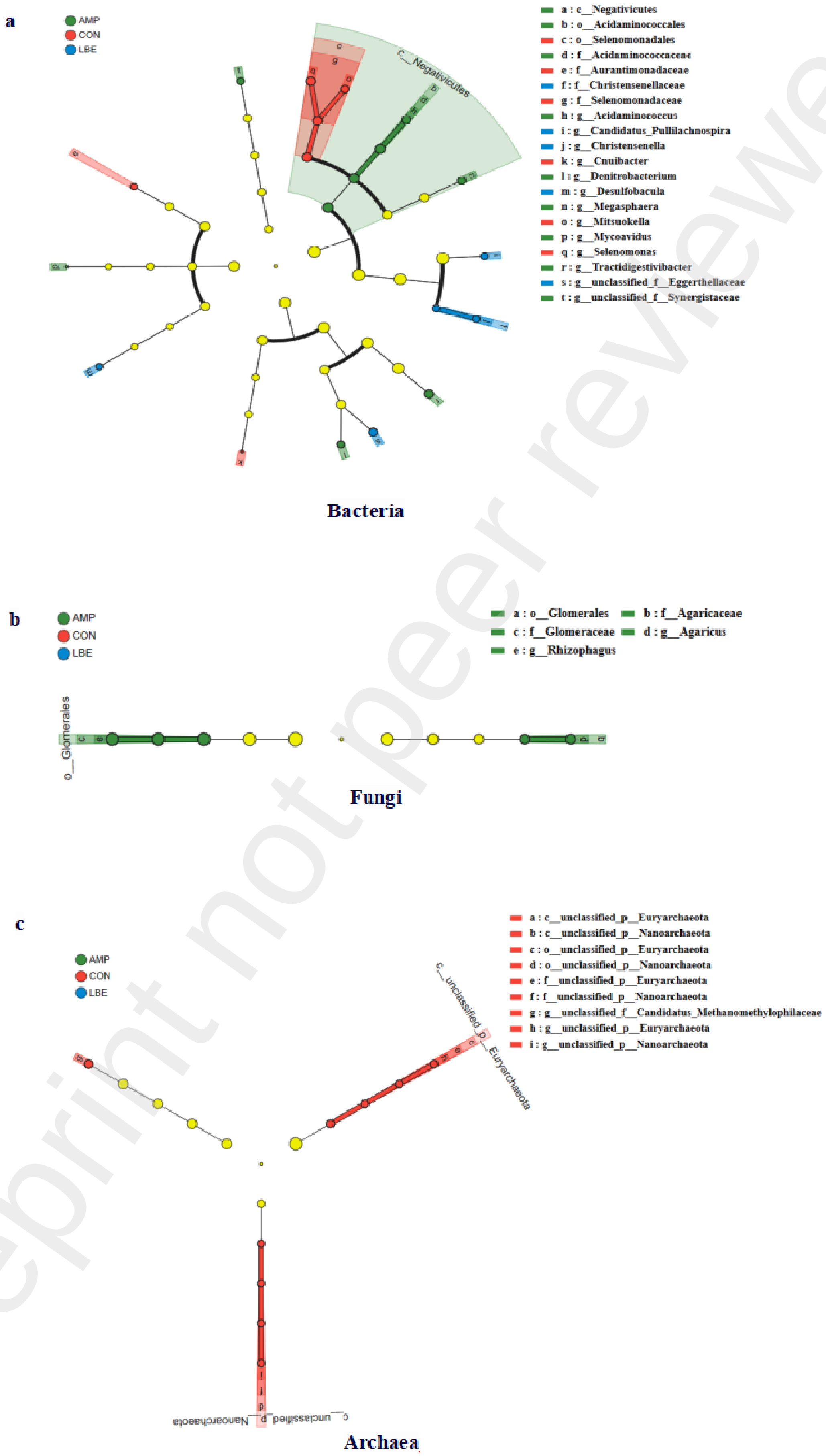

a
AMP
CON
LBE
c__Negativicutes
a : c__Negativicutes
b : o__Acidaminococcales
c : o__Selenomonadales
d : f__Acidaminococcaceae
e : f__Aurantimonadaceae
f : f__Christensenellaceae
g : f__Selenomonadaceae
h : g__Acidaminococcus
i : g__Candidatus_Pullilachnospira
j : g__Christensenella
k : g__Cnuibacter
l : g__Denitrobacterium
m : g__Desulfobacula
n : g__Megasphaera
o : g__Mitsuokella
p : g__Mycoavidus
q : g__Selenomonas
r : g__Tractidigestivibacter
s : g__unclassified_f__Eggerthellaceae
t : g__unclassified_f__Synergistaceae
Bacteria
b
AMP
CON
LBE
o__Glomerales
a : o__Glomerales
b : f__Agaricaceae
c : f__Glomeraceae
d : g__Agaricus
e : g__Rhizophagus
Fungi
c
AMP
CON
LBE
c__unclassified_p__Euryarchaeota
c__unclassified_p__Nanoarchaeota
a : c__unclassified_p__Euryarchaeota
b : c__unclassified_p__Nanoarchaeota
c : o__unclassified_p__Euryarchaeota
d : o__unclassified_p__Nanoarchaeota
e : f__unclassified_p__Euryarchaeota
f : f__unclassified_p__Nanoarchaeota
g : g__unclassified_f__Candidatus_Methanomethylophilaceae
h : g__unclassified_p__Euryarchaeota
i : g__unclassified_p__Nanoarchaeota
Archaea

Fig: 7. The different colored nodes in the figure represent microbial communities that are significantly enriched in the corresponding groups and have a significant impact on inter group differences; The light yellow nodes represent microbial groups that have no significant differences in different groups (*P*>0.05), or have no significant impact on inter group differences (*P*>0.05).

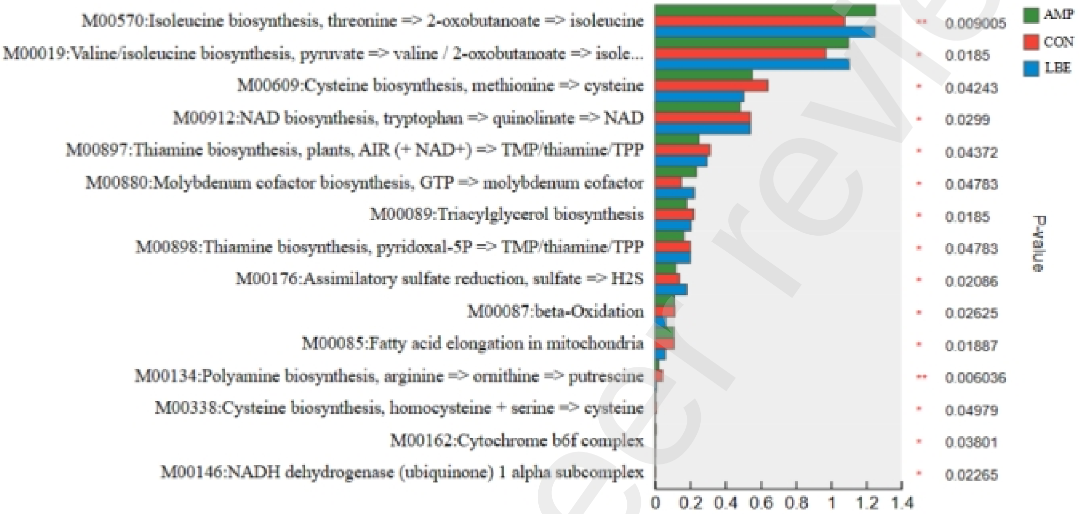


Fig: 8. The vertical axis represents the KEGG functional names at different classification levels, the horizontal axis represents the percentage value of a certain KEGG functional abundance in the sample, and different colors represent different groups. The bar chart displays the differences in average relative abundance of the same function between different groups, and annotates whether the differences are significant (*P*<0.05, represents significant differences).

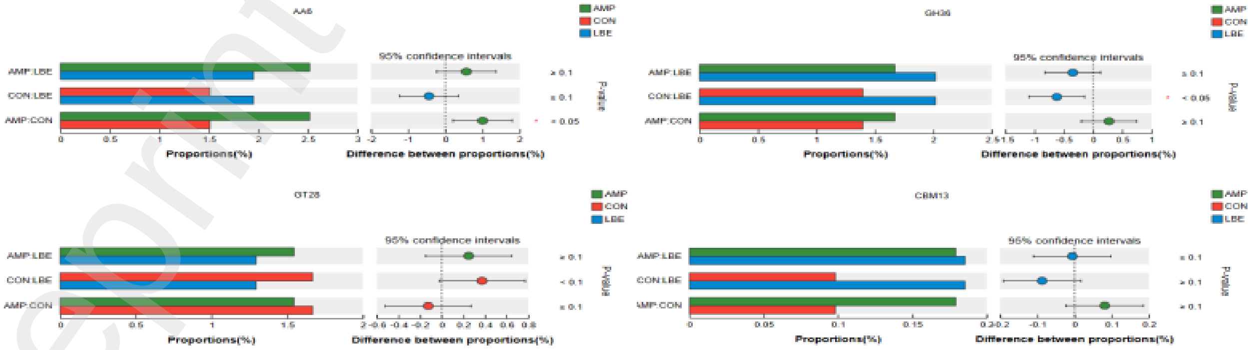


Fig: 9. The vertical axis represents the CAZy functional names at different classification levels, the horizontal axis represents the percentage value of a certain CAZy functional abundance in the sample, and different colors represent different groups Indicates significant difference (0.01<$P \le 0.05$), indicates extremely significant difference ($P \le$

0.01).