Animal Nutrition 12 (2023) 345–359

Contents lists available at ScienceDirect

Animal Nutrition

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

Original Research Article

Rumen microbiota-host transcriptome interaction mediates the protective effects of *trans*-10, *cis*-12 CLA on facilitating weaning transition of lambs

Chunlei Yang [a], Xiangfei Deng [a], Peter Lund [b], Haixia Liu [c], Xingwang Ding [c], Zhengwei Fu [a], Naifeng Zhang [d], Jinjun Li [e, *], Lifeng Dong [d, *]

[a] College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou, 310014, China
[b] Department of Animal Science, Aarhus University, AU Foulum, DK-8830, Tjele, Denmark
[c] Jiangsu Agri-animal Husbandry Vocational College, Taizhou, 225300, China
[d] Institute of Feed Research, Chinese Academy of Agricultural Sciences/Sino-US Joint Lab on Nutrition and Metabolism of Ruminant, Beijing, 100081, China
[e] Institute of Food Sciences, Zhejiang Academy of Agricultural Sciences, Hangzhou, 310021, China

ARTICLE INFO

Article history:
Received 3 March 2022
Received in revised form
10 November 2022
Accepted 15 November 2022
Available online 21 November 2022

Keywords:
Trans-10,*cis*-12 conjugated linoleic acid
Rumen microbiota
Epithelial transcriptome
Peroxisome proliferator-activated receptor gamma
Weaning stress

ABSTRACT

Developing alternatives to antibiotics for prevention of gastrointestinal dysbiosis in early-weaning farmed animals is urgently needed. This study was to explore the potential effects of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) on maintaining ruminal homeostasis of young ruminants during the weaning transition period. Thirty neonatal lambs were selected (6 lambs per group) and euthanized for rumen microbial and epithelial analysis. The lambs were weaned at 28 d and experienced the following 5 treatments: euthanized on d 28 as the pre-weaning control (CON0), fed starter feed for 5 (CON5) or 21 (CON21) d, fed starter feed with 1% of CLA supplemented for 5 (CLA5) or 21 (CLA21) d. Results showed that the average daily weight gain and dry matter intake were significantly higher in CLA5 than CON5 group. As compared with the CON5 and CON21 group, the relative abundances of volatile fatty acid (VFA) producing bacteria including *Bacteroides*, *Treponema*, *Parabacteroides* and *Anaerovibrio*, as well as the concentrations of acetate, butyrate and total VFA were significantly increased in CLA5 and CLA21 group, respectively. Integrating microbial profiling and epithelial transcriptome results showed that 7 down-regulated inflammatory signaling-related host genes *IL2RA*, *CXCL9*, *CD4*, *CCR4*, *LTB*, *SPP1*, and *BCL2A1* with CLA supplementation were significantly negatively correlated with both VFA concentration and VFA producing bacteria, while 3 (*GPX2*, *SLC27A2* and *ALDH3A1*) and 2 (*GSTM3* and *GSTA1*) upregulated metabolism-related genes, significantly positively correlated with either VFA concentration or VFA producing bacteria, respectively. To confirm the effects of CLA on epithelial signal transduction, in vitro experiment was further conducted by treating rumen epithelial cells without or with IL-17A + TNF-α for 12 h after pretreatment of 100 μM CLA or not (6 replicates per treatment). The results demonstrated the anti-inflammatory effect of CLA via suppressing the protein expression of NF-κB p-p65/p65 with the activation of peroxisome proliferator-activated receptor gamma (PPARγ). In conclusion, CLA supplementation enhanced the ruminal microbiota-driven transcriptional regulation in healthy rumen epithelial development via rumen VFA production, and CLA may therefore serve as an alternative way to alleviate early-weaning stress and improve physiological and metabolic conditions of young ruminants.

1. Introduction

All mammals are associated with a vast microbial community that mainly consists of bacteria, but also archaea, eukaryotes and viruses. Microorganisms essentially cover all the mucosal surfaces of the host, although the majority residents within the gastrointestinal tract (Krautkramer et al., 2021). The gut microbiota plays a

* Corresponding authors.
E-mail addresses: lijinjun@zaas.ac.cn (J. Li), donglifeng@caas.cn (L. Dong).
Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2022.11.002

critical role in benefiting hosts such as participation in nutrient digestion and fermentation, sustaining the normal functions of the gastrointestinal mucosa, regulation of immune responses, and protection against pathogenic bacteria (Kundu et al., 2017). Recent research shows that the early neonatal period is critical for the development of microbial community, host–microbiota interaction, and immune system (Fulde et al., 2018). The disruption of the establishment of gut microbiota during early life even influences immune homeostasis and increases the susceptibility to metabolic and inflammatory diseases in later life (Fulde et al., 2018).

The suckling-to-weaning transition is a stressful and critical event in the life cycle of mammals due to the dramatic changes of diet, environment, and management in intensification production system, which usually requires short breeding cycle to reduce cost and improve productivity (Gresse et al., 2017). For example, the piglet behavior, as well as the physiology and function of their still-immature gastrointestinal tract were suppressed as the highly digestible liquid milk changed into more complex less-digestible solid feeds. This could be attributed to the increased intestinal inflammatory responses and permeability to toxins and antigens, induction of intestine atrophy and decreased villous height, as well as reduced nutrient absorption (Hu et al., 2018; Meng et al., 2020). Research in young ruminants has also revealed that weaning is associated with changes in growth performance, physiological parameters, rumen development, and gut integrity (Wood et al., 2015; Lin et al., 2019). Cui et al. (2018) found that the expression of proteins involved in immune system processes, nutrient metabolism, and cytoskeleton organization in jejunal epithelium of lambs were still significantly downregulated in lambs 15 d after weaning. Li et al. (2018) even revealed that the altered microbial taxa and gene expressions related to intestinal barrier function in lambs derived from weaning process could last for 84 d. Emerging evidences suggest these etiology of post-weaning enteric infections and dysfunctions are linked to the modifications in gut microbiota, due to in the neonatal period, the gut microbiota exhibits dynamic composition and diversity over time and along the gastrointestinal tract as multiple stressors existed (Yatsunenko et al., 2012). Antibiotic therapy is the most commonly used treatment to prevent gastrointestinal infections in young animals. However, given the significant role of antibiotics in gut microbial dysbiosis, as well as the rise of public health concern on the spread of multi-resistant bacteria, developing non-antibiotic alternatives to recover microbial balance and control gastrointestinal infections derived from weaning transition is urgent (Gresse et al., 2017).

As a mixture of positional and geometrical isomers, conjugated linoleic acid (CLA) possesses many health-promoting effects, such as immune enhancement and anti-carcinogenic and anti-obesogenic effects (Dilzer and Park, 2012). Previous studies demonstrated that supplementation of CLA can improve energy balance, regulate gut microbiota, and alleviate the onset of inflammation (Liu et al., 2020; Yang et al., 2021). In addition, besides the energetic effect and being a major component of cell membrane, fatty acids and their metabolites also play a key role as substrates in various biochemical pathways, and are known as immune modulators and cell signaling molecules (Lim et al., 2017; Sugihara et al., 2018). It is also known that CLA shows a high similarity with the ligand of peroxisome proliferator-activated receptor gamma (PPARγ) (Yuan et al., 2015), which is involved in the transductions of various inflammatory and nutritional signals (Daynes and Jones, 2002). Our previous study indicated that an isomer specific effect of CLA existed in ruminal epithelial cells, with *trans*-10, *cis*-12 CLA showing the best anti-inflammatory effect (Yang et al., 2021). However, the potential effect of *trans*-10, *cis*-12 CLA supplementation on gastrointestinal dysbiosis around weaning has not been clearly investigated.

Herein, we hypothesized that *trans*-10, *cis*-12 CLA supplementation can alleviate gastrointestinal dysbiosis around weaning by regulating the microbial composition and its associated fermentation metabolites, which coincides with the transcriptomic modulation of both key immune and physical signals in host epithelium.

2. Materials and methods

2.1. Animal ethical statement

The experiment was approved by the Animal Ethics Committee of the Chinese Academy of Agricultural Sciences (protocol number: 062-2021). The experiment was conducted at the farm of Jiangsu Xilaiyuan Ecological Agriculture Co., Ltd., Taizhou, Jiangsu, China.

2.2. Animal experiment and sampling

In total, 30 healthy neonatal Hu lambs (0-d-old) were used and fed with breast milk without starter feed until the age of 10 d, then starter feed was supplied daily ad libitum from 08:00 to 10:00 and from 15:00 to 17:00 respectively, to gradually adapt the lambs to solid feed. All the lambs were weaned at 28 d, and 6 lambs were euthanized on d 28 and used as the pre-weaning control group (CON0). Then, the remaining 24 lambs were randomly divided into 4 groups: 6 lambs were fed starter feed (the nutrient composition is provided in Table S1) for 5 d (CON5), 6 lambs were fed starter feed with 1% of CLA (*trans*-10, *cis*-12 CLA, purity $\geq$80.0%, Bide Pharmatech Ltd., Shanghai, China) supplemented for 5 d (CLA5), 6 lambs were fed starter feed for 21 d (CON21), 6 lambs were fed starter feed with 1% of CLA supplemented for 21 d (CLA21). The amounts of feed supply and residue were measured daily to calculate the dry matter intake (DMI). The CLA supplements were fed on a daily basis to ensure that it was consumed accurately. The nutritional composition of the starter feed met the nutrient requirement of Hu lambs (feeding standard for mutton sheep, NY/T 816-2004, Ministry of Agriculture of the People's Republic of China). Diet samples were analyzed for the DM (AOAC International, 2003; method 934.01), crude protein (AOAC International, 2003; method 954.01), ether extract (AOAC International, 2003; method 920.39), ash (AOAC International, 2003; method 942.05), and crude fiber using the method described by Van Soest et al. (1991). The body weight of the lambs was measured before morning feeding every week. All lambs had free access to fresh water and had no opportunity to get the ewes' feed. The rumen samples (content and tissue separately) were collected from pre-weaned lambs (CON0), and post-weaned lambs on d 5 (CON5 and CLA5) and d 21 (CON21 and CLA21) after weaning, respectively, and within 30 min after euthanization. Rumen contents (approximately 5 mL) were sampled in triplicates and snap-frozen in liquid nitrogen and then stored at -80 °C for microbial analysis. Another equal amount of rumen content from each animal was collected and screened through a four-layer cheesecloth to obtain the fluid, the fluid was then kept in 25% (wt/vol) metaphosphoric acid and mixed in a ratio of 5:1 and stored at -20 °C for volatile fatty acid (VFA) analysis. Simultaneously, rumen epithelial tissue (5 cm $\times$ 5 cm) from the bottom of the ventral sac was collected after detachment from the serosal and muscular layers by blunt dissection, and then snap-frozen in liquid nitrogen and stored at -80 °C for tissue RNA extraction.

2.3. Analysis of rumen content-associated microbiota using 16S rRNA gene sequencing

Total DNA was extracted from the rumen content per sample using the cetyltrimethylammonium bromide (CTAB) plus repeated bead-beating method (Gagen et al., 2010). The purity and concentration of extracted DNA were measured by agarose gel electrophoresis and Qubit 2.0 Fluorometer (Thermo Scientific, MA, USA) respectively. The specific primers (341F: 5′-CCTAYGGGRBGCASCAG-3′, 806R: 5′-GGACTACNNGGGTATCTAAT-3′) were used to target the V3-V4 region of the 16S rRNA gene of rumen bacteria, together with a 6 bp barcode that is unique to each sample (Lin et al., 2019). After PCR amplification, all the products were quantified and each product was pooled at an equal molar amount, then purified with GeneJETTM Gel Extraction Kit (Thermo Scientific, MA, USA) and used for library construction using Ion Plus Fragment Library Kit 48 rxns (Thermo Scientific, MA, USA). The quality of amplicon library was assessed using the Qubit 2.0 Fluorometer (Thermo Scientific, MA, USA) and finally sequenced on the NovaSeq6000 platform.

The obtained sequences were assembled using FLASH (v1.2.11) after the removal of barcode and primer sequences. Quality control was then conducted using fastp (v0.23.0) (Chen et al., 2018) and chimeras were deleted after the comparison with Silva 138.1 database using UCHIME2 algorithm in Usearch (v11.0.667) (Edgar et al., 2011). The cleaned sequences were then assigned to amplicon sequence variants (ASV) using the DADA2 plugin in QIIME2 (v2022.2) (Bolyen et al., 2019). Taxonomic assignment of each ASV was performed against the Silva 138.1 database using classify-sklearn algorithm in QIIME2. Determination of the alpha and beta diversities was further conducted using QIIME2 and displayed with R software (v4.2.0). PICRUSt2 was utilized to predict the functional profiles of the obtained 16S rRNA gene data (Douglas et al., 2020).

2.4. Analysis of the rumen epithelium transcriptome profiling using RNA-seq

Total RNA of the rumen epithelium from each sample was extracted using TRIzol (Takara Bio, Otsu, Japan). The NanoPhotometer spectrophotometer (IMPLEN, CA, USA) and Qubit RNA assay kit with a Qubit 2.0 fluorometer (Life Technologies, CA, USA) were used to measure the purity and concentration of the extracted RNA respectively. The RNA integrity was determined by the RNA Nano 6000 assay kit with Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total amount of 3 μg RNA per sample was used as input material for library preparation by using the NEBNext Ultra™ RNA library prep kit for Illumina (NEB, USA) according to the manufacturer's instructions. Finally, paired-end sequencing (150 bp) was conducted in the Illumina HiSeq4000 instrument, and a minimum depth of 40 million reads per sample were obtained. The sequencing work was supported by the Beijing Novogene Biological Information Technology Co., Ltd.

The obtained raw reads were performed with quality control and the reads containing adapter and poly-N sequences were removed. The clean data were then mapped to the reference genome and gene model annotation files of *Ovis aries* (ftp://ftp.ensembl.org/pub/release-95/fasta/ovis_aries/ and ftp://ftp.ensembl.org/pub/release-95/gtf/ovis_aries/) respectively using HISAT2 (v2.2.1) (Kim et al., 2015). The gene expression level of each sample was evaluated according to the fragments per kilobase of transcript per million fragments mapped (FPKM). The differential gene expression among the different groups were analyzed using the DESeq2 (v3.15) (Love et al., 2014). The Benjamini and Hochberg was used to control the adjusted false discovery rate (FDR). The genes with FDR <0.05 and fold change (FC) > 2 were considered as differentially expressed genes (DEG). Then, the DAVID (v6.8) (da Huang et al., 2009) and KOBAS (v3.0) software (Xie et al., 2011) were used to analyze the statistical enrichment ($P < 0.05$) of the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of the DEG, respectively. Further, the protein–protein interaction network of the DEG were tested using the STRING v10.5 database and hub genes (degree > 5) were determined by Cytoscape (v3.9.1) (Shannon et al., 2003).

2.5. VFA profile analysis

Approximately 1 mL of the mixed rumen fluid sample were homogenized and centrifuged at 6,000 × *g* and 4 °C for 10 min. The supernatant (0.3 mL) was combined with internal standard (0.4 mL of 25 μg/mL 2-methylvaleric acid) and 0.1 mL of 50% sulfuric acid, and then mixed uniformly. After ultrasonicating in ice water for 5 min, the mixture was centrifuged at 13,000 × *g* and 4 °C for 10 min, then the supernatant was transferred to fresh glass vial for gas chromatography-mass spectrometry measurement using an Agilent 7890 GC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a capillary column (HP-FFAP, 30 m × 250 μm × 0.25 μm, Agilent Technologies, Santa Clara, CA, USA) coupled with an Agilent 7000D mass spectrometer, as described by Bi et al. (2021).

2.6. Cell culture, quantitative real-time PCR and immunoblotting

The immortalized rumen epithelial cell line (REC) used in the present study was obtained and cultured in our research group (Yang et al., 2021). Generally, the REC was cultured in DMEM (Gibco, New York, USA) containing 2% FBS, 1% penicillin/streptomycin and 1% epithelial cell additive at 37 °C with 5% CO_2 as previously described (Yang et al., 2021). The cells were treated without (control group, CON) or with IL-17A (25 ng/mL), or IL-17A (25 ng/mL) + TNF-α (0.5 ng/mL) for 12 h after pretreatment of 100 μM *trans*-10, *cis*-12 CLA (purity ≥96.0%, Sigma–Aldrich, Shanghai, China) or not when REC monolayers reached 70% to 80% confluency. Each treatment contained 6 replicates.

After the treatment, the effects of IL-17A, IL-17A + TNF-α and *trans*-10, *cis*-12 CLA on cell viability in REC were performed by MTT bioassay (Sangon Biotech, Shanghai, China). Then, total RNA was extracted from the REC using TRIzol (Takara Bio, Otsu, Japan). cDNA was then synthesized using the PrimeScript RT Reagent Kit (Takara, Dalian, China). qPCR was then conducted using Roche LightCycler 480 System (Roche, Switzerland) with SYBR green and the specific primers for IL-1β and IL-6 as previously described (Yang et al., 2021). The gene expressions were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ). Then the relative expression was determined by the $2^{-\Delta\Delta Ct}$ method.

To obtain the total protein cell lysates, REC were homogenized and sonicated in lysis radio-immunoprecipitation assay buffer (RIPA) (Millipore, Billerica, MA) with protease and phosphatase inhibitors (Roche Diagnostics) supplemented. Then immunoblots were conducted as previously described (Ma et al., 2020). The primary antibodies used were anti-NF-κB p65 (phospho S536) (ab76302), anti-NF-κB p65 (ab32536), anti-PPAR gamma (ab178860) (Abcam) and monoclonal anti-β-actin (#4967) (Cell Signaling Technology).

2.7. Statistical analysis

Statistical analyses were conducted by SPSS (v22.0), GraphPad Prism (v9.3.0), R (v4.2.0), and Cytoscape (v3.9.1). The value of $P < 0.05$ was considered as statistically significant and the Benjamini-Hochberg method was used to adjust *P* values into FDR.

Unpaired Student's *t*-test was used for the comparison of two groups. ANOVA was applied for the comparison of multiple groups. Pairwise comparisons on Bray–Curtis distance were performed by the analysis of similarities (ANOSIM) using the "vegan" package of R. The biomarker analysis of bacterial taxa was conducted using linear discriminant analysis (LDA) effect size (LEfSe) with the LDA score >4 (Segata et al., 2011). The correlations among the VFA concentrations, bacterial abundances and gene expressions were identified according to Spearman rank correlation coefficient using "psych" package in the R software. Cytoscape was used for visualizing the protein–protein interaction network and determine the hub genes with a degree >5 (Han et al., 2019).

3. Results

3.1. Trans-10, cis-12 CLA prevented early weaning-related productivity reduction

To identify and validate the association of *trans*-10, *cis*-12 CLA with productivity resistance to early weaning, we supplemented *trans*-10, *cis*-12 CLA to lambs in CLA5 and CLA21 groups at weaning by mixing it into the feed. Supplementation of *trans*-10, *cis*-12 CLA significantly increased average daily weight gain (ADG) of the lambs during the first 5 d after weaning compared with the control group (Table 1, $P < 0.05$). Simultaneously, the average DMI of lambs during the first 5 d after weaning was also significantly higher after *trans*-10, *cis*-12 CLA supplementation compared to the control group (Table 1, $P < 0.05$).

3.2. Trans-10, cis-12 CLA induced a shift in the rumen microbiota composition

Using 16S rRNA gene sequencing analysis of rumen microbiota in lambs at 2 time points (d 5 and 21) after weaning, we investigated the rumen microbial alterations related to the potential enhanced resistance towards early weaning-induced productivity reduction in lambs when *trans*-10, *cis*-12 CLA was supplemented. Good's coverage revealed that nearly all bacterial species were detected in each of the rumen content samples collected (Fig. S1A). Our data indicated that *trans*-10, *cis*-12 CLA supplementation did not significantly affect the alpha diversity of rumen microbiota (Fig. 1A), while the shift of beta diversity was observed according to principal coordinates analysis (PCoA) based on unweighted UniFrac distance method (Fig. 1B). The Bray–Curtis dissimilarity analysis on beta diversity further revealed that the *trans*-10, *cis*-12 CLA supplementation for 5 or 21 d caused shifts of rumen microbial beta diversity in lambs after weaning (Table S2, $P < 0.05$). Microbial function assignment using the KEGG orthologs (KO) and their corresponding pathways revealed that the *trans*-10, *cis*-12 CLA supplementation induced a shift in the functional profiles of rumen bacterial communities according to the principal component analysis (PCA) plots (Fig. S1B). The KO abundances of beta-galactosidase and long-chain acyl-CoA synthetase were significantly increased after *trans*-10, *cis*-12 CLA supplementation (Fig. S1C, $P < 0.05$).

Table 1
Effects of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) supplementation for either 5 or 21 d on the growth performance of lambs after weaning.

Item	Days after weaning	CON[1]	CLA[2]	SEM	*P*-value
ADG, g/d	5	101.67[b]	151.66[a]	2.414	0.046
	21	152.10	167.50	1.198	0.064
DMI, g/d	5	148.41[b]	189.64[a]	1.501	0.004
	21	299.39	300.16	5.837	0.994

ADG = average daily weight gain; DMI = daily dry matter intake on DM basis.
[a,b] Within a row, a different superscript means significant difference ($P < 0.05$).
[1] CON, lambs were fed starter feed for 5 or 21 d after weaning ($n = 6$).
[2] CLA, lambs were fed starter feed with 1% of *trans*-10, *cis*-12 CLA supplemented for 5 or 21 d after weaning ($n = 6$).

3.3. Trans-10, cis-12 CLA prevented the dysbiosis of ruminal microbiota in early weaning lambs

To identify the specific rumen microbiota that improve the resistance to early weaning stress-induced rumen microbial dysbiosis, we compared the relative abundance of rumen microbial taxa identified in *trans*-10, *cis*-12 CLA supplemented lambs and control lambs during weaning transition. After comparing the groups of CON5 and CON21 with CON0, respectively, we found that early weaning significantly increased the relative abundance of Firmicutes (d 5) and decreased the relative abundance of Bacteroidetes (d 5 and 21) in lambs after weaning (Fig. 2A, $P < 0.05$). In addition, the relative abundances of several pathogenic bacteria including *Solobacterium*, *Haemophilus*, *Streptococcus* and *Veillonella* significantly increased, whereas the relative abundances of beneficial bacteria including *Prevotella*, *Bacteroides*, *Prevotellaceae_UCG-001*, *Prevotellaceae_UCG-003*, *Rikenellaceae_RC9_gut_group*, *Parabacteroides*, *UCG-005*, *Christensenellaceae_R-7_group* and *Saccharofermentans* significantly decreased on at least one time point post-weaning (d 5 or 21) (Fig. 2B, $P < 0.05$). However, after comparing the groups of CLA5 and CON5, CLA21 and CON21, respectively, the supplementation of *trans*-10, *cis*-12 CLA significantly decreased the relative abundance of Firmicutes and increased the relative abundance of Bacteroidetes in lambs, showing a restoring effect on the alterations in the abundances of these 2 phyla caused by early weaning (Fig. 2A, $P < 0.05$). Furthermore, at the genus level, the supplementation of *trans*-10, *cis*-12 CLA significantly increased the relative abundances of the beneficial bacteria including *Bacteroides*, *Treponema*, *Anaerovibrio*, *Parabacteroides* and *Oribacterium* in lambs, while decreased the gut dysbiosis-related bacteria including *Acinetobacter*, *Acidaminococcus*, *Solobacterium*, *Catenisphaera*, *Syntrophococcus* and *Weissella* at least on one sampled time point after early weaning (Fig. 2B, $P < 0.05$). The LEfSe analysis further confirmed that the bacterial taxa *Bacteroides*, *Anaerovibrio*, *Oribacterium* and Butyricicoccaceae were weighted significant to the differences between communities after *trans*-10, *cis*-12 CLA supplementation, with an absolute LDA score greater than 4 in CLA5 or CLA21 group (Fig. S2).

Based on the above results, we further identified all bacteria ASVs that were modulated by *trans*-10, *cis*-12 CLA supplementation after weaning. The Venn profile indicated that although 851, 679 and 754 ASVs were shared between the groups of CON5 and CON0, CLA5 and CON5, CLA21 and CON21 respectively, the supplementation of *trans*-10, *cis*-12 CLA for 5 d caused 77 ASVs (62.34% assigning to Bacteroidetes) inversely emerged that has missed in CON5 group as compared to CON0 group due to early weaning, as well as caused 253 ASVs (56.52% assigning to Firmicutes) missed that has emerged in CON5 group as compared with CON0 group (Fig. 3). When *trans*-10, *cis*-12 CLA supplementation lasted for 21 d, a total of 82 ASVs (59.76% assigning to Bacteroidetes) inversely emerged that has missed in CON5 group as compared with CON0 group, simultaneously 52 ASVs (71.15% assigning to Firmicutes) missed that has emerged in CON5 as compared with CON0 group (Fig. 3). These results confirmed the regulatory effect of *trans*-10, *cis*-12 CLA supplementation on Bacteroidetes and Firmicutes.

3.4. Trans-10, cis-12 CLA altered rumen VFA profile of post-weaning lambs

Rumen bacterial fermentation of carbohydrates results in the production of VFA such as acetate, propionate and butyrate. To

investigate whether the changes in ruminal microbiota were align with changes in VFA profile, we compared the concentration of ruminal VFA in *trans*-10, *cis*-12 CLA supplemented lambs and control lambs during weaning transition. The supplementation of *trans*-10, *cis*-12 CLA significantly increased the concentration of total VFA, as well as the concentration of acetate and butyrate in the rumen of lambs after early weaning on both d 5 and 21 (Fig. 1C, $P < 0.05$).

3.5. Trans-10, cis-12 CLA modified the transcriptome profile in rumen epithelium

Then, we investigated whether the protective effects of *trans*-10, *cis*-12 CLA on alleviating rumen dysfunction was associated with a regulation of the rumen epithelial transcriptome by using high-throughput RNA-seq-based transcriptome sequencing. PCA analysis indicated an obvious shift of all the gene expressions measured at different sampled time points according to the supplementation of *trans*-10, *cis*-12 CLA (Fig. S3A).

Among the encoded genes in rumen epithelium, there were 422 and 695 DEG (FC > 2, FDR <0.05) identified from comparison of the lambs supplemented with *trans*-10, *cis*-12 CLA for 5 and 21 d with the control, respectively. Among these DEG, there were 113 genes upregulated and 309 genes downregulated in the CLA5 group compared with the CON5 group, and 175 genes were upregulated and 520 genes downregulated in the CLA21 group compared with the CON21 group (Fig. S3B and C). To investigate the functional profile of these DEG due to *trans*-10, *cis*-12 CLA supplementation, GO and KEGG enrichment analysis was conducted to identify their biological processes. On d 5 and 21 after early weaning, there were 23 and 6 GO terms, such as "immune response", "cytokine receptor binding", "G-protein coupled receptor signaling pathway" and "tumor necrosis factor receptor binding" significantly down-regulated, while 18 and 36 GO terms, such as the ones related to amino acid and lipid metabolism and cell proliferation were significantly upregulated in rumen epithelium by supplementation of *trans*-10, *cis*-12 CLA (Fig. S4, $P < 0.05$). Furthermore, the inflammation-related KEGG pathways including "viral protein interaction with cytokine and cytokine receptor", "cytokine−cytokine receptor interaction", "chemokine signaling pathway", "NF-kappa B signaling pathway", "staphylococcus aureus infection", "Toll-like receptor signaling pathway", "IL-17 signaling pathway", "primary immunodeficiency" and "cell adhesion molecules" were significantly downregulated in the rumen epithelium of lambs at least on one sample time (d 5 or 21) after early weaning due to the supplementation of *trans*-10, *cis*-12 CLA (Figs. 4 and 5, $P < 0.05$). On the other hand, the supplementation of *trans*-10, *cis*-12 CLA significantly upregulated the metabolism-related pathways including "cortisol synthesis and secretion", "glutathione metabolism", "linoleic acid metabolism", "glycolysis/gluconeogenesis" and "arachidonic acid metabolism" in the rumen epithelium of lambs at least at one sample time after early weaning (Figs. 4 and 5, $P < 0.05$). In addition, the "PPAR signaling pathway" that has

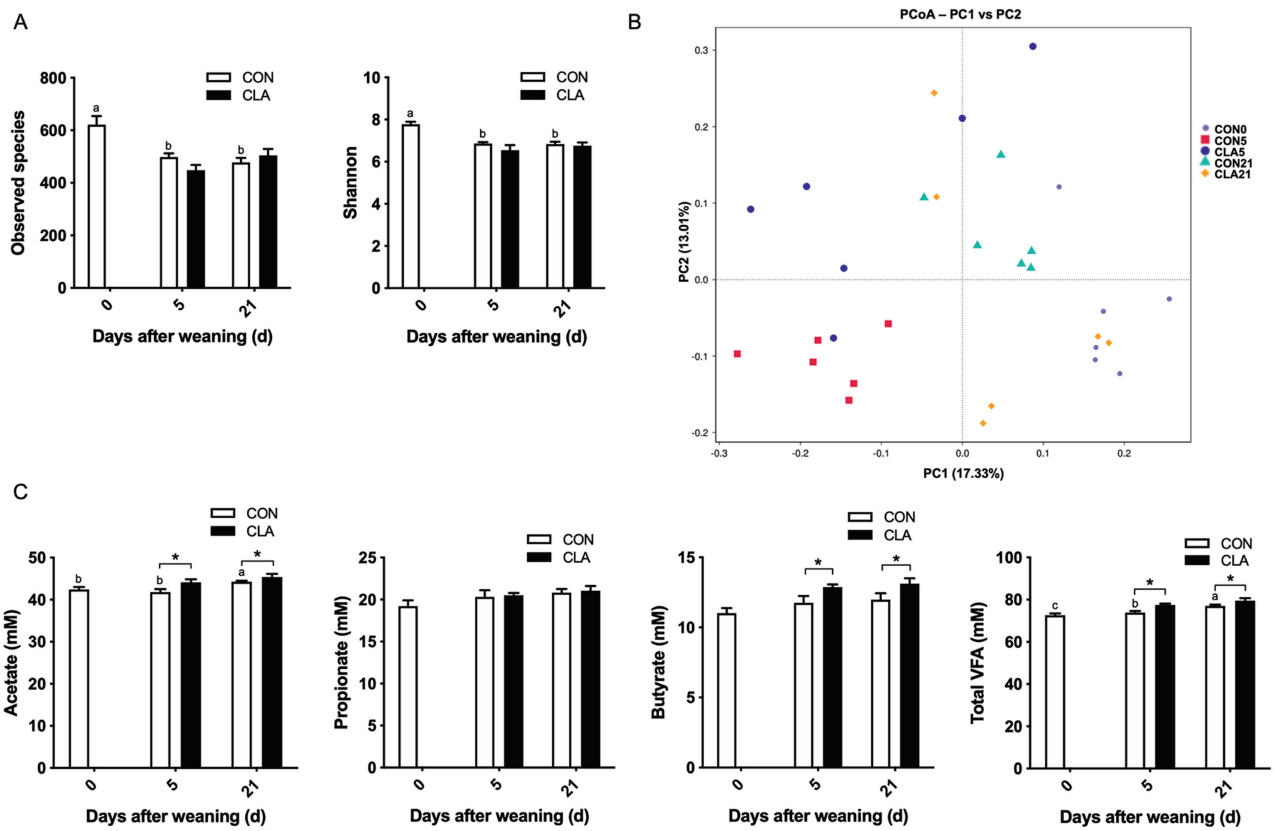

Fig. 1. Effects of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) supplementation for either 5 or 21 d on the ruminal bacterial diversity and fermentation parameter of lambs during weaning transition. (A) Rumen bacterial richness (number of observed species) and evenness (Shannon diversity index). (B) Principal coordinate analysis (PCoA) of ruminal bacterial diversity using the unweighted UniFrac metric. (C) The concentrations of acetate, propionate, butyrate and total VFA (acetate + propionate + butyrate) in the rumen. Data were presented as the mean ± SEM. CON0 ($n = 6$), pre-weaning control group; CON5 ($n = 6$) and CON21 ($n = 6$), lambs were fed starter feed for 5 or 21 d after weaning; CLA5 ($n = 6$) and CLA21 ($n = 6$), lambs were fed starter feed with 1% of *trans*-10, *cis*-12 CLA supplemented for 5 or 21 d after weaning. *$P < 0.05$. $^{a-c}$ Bars with a different superscript represent significant difference ($P < 0.05$). VFA = volatile fatty acid.

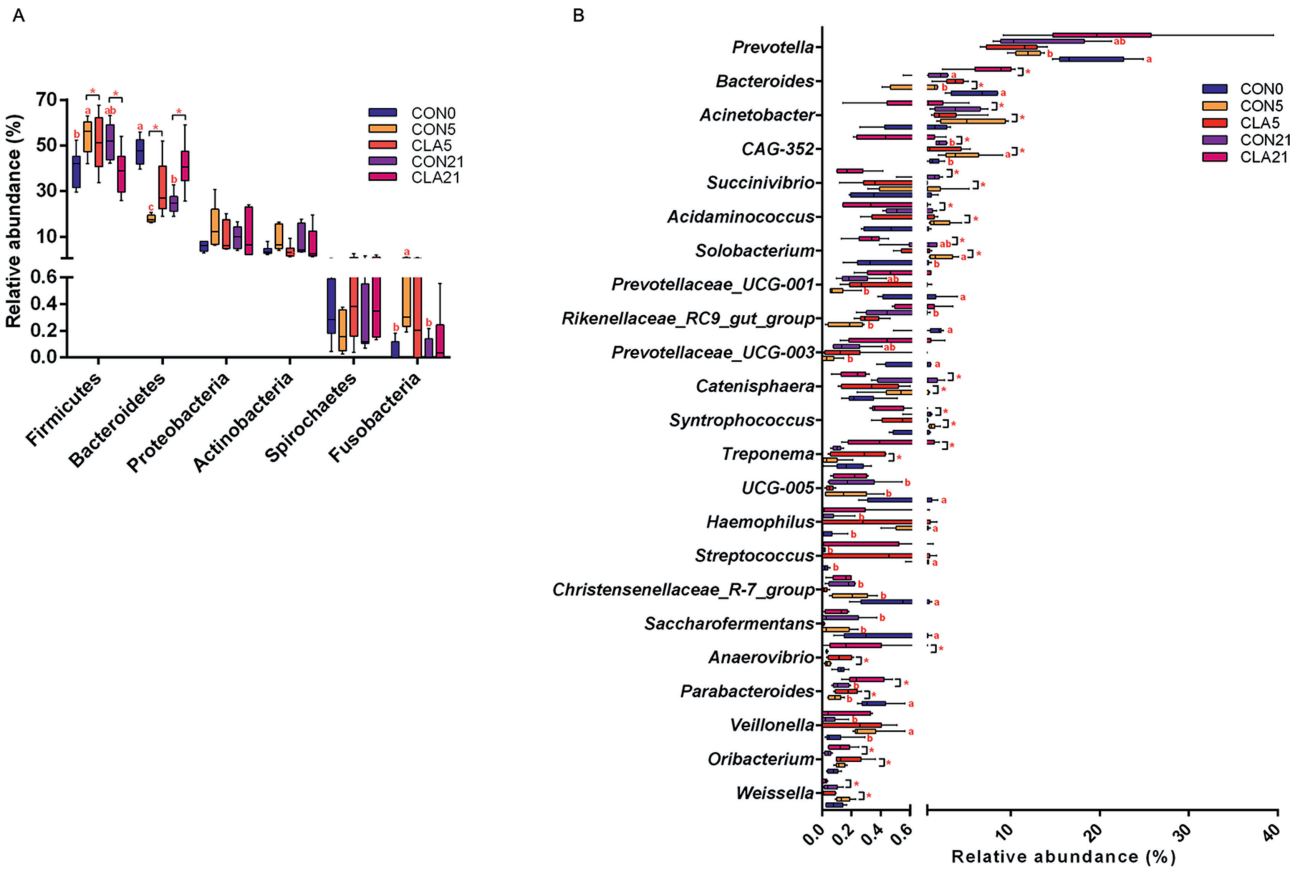

Fig. 2. *Trans*-10, *cis*-12 conjugated linoleic acid (CLA) supplementation altered the specific rumen bacterial compositions of lambs during weaning transition. (A) Dominant phyla of the rumen bacteria with a relative abundance more than 0.1% in at least one group. (B) Dominant genera of the rumen bacteria with a relative abundance more than 0.1% in at least one group. Data were shown using box and whiskers plot. CON0 ($n = 6$), pre-weaning control group; CON5 ($n = 6$) and CON21 ($n = 6$), lambs were fed starter feed for 5 or 21 d after weaning; CLA5 ($n = 6$) and CLA21 ($n = 6$), lambs were fed starter feed with 1% of *trans*-10, *cis*-12 CLA supplemented for 5 or 21 d after weaning. *$P < 0.05$. $^{a-c}$ Bars with a different superscript represent significant difference ($P < 0.05$).

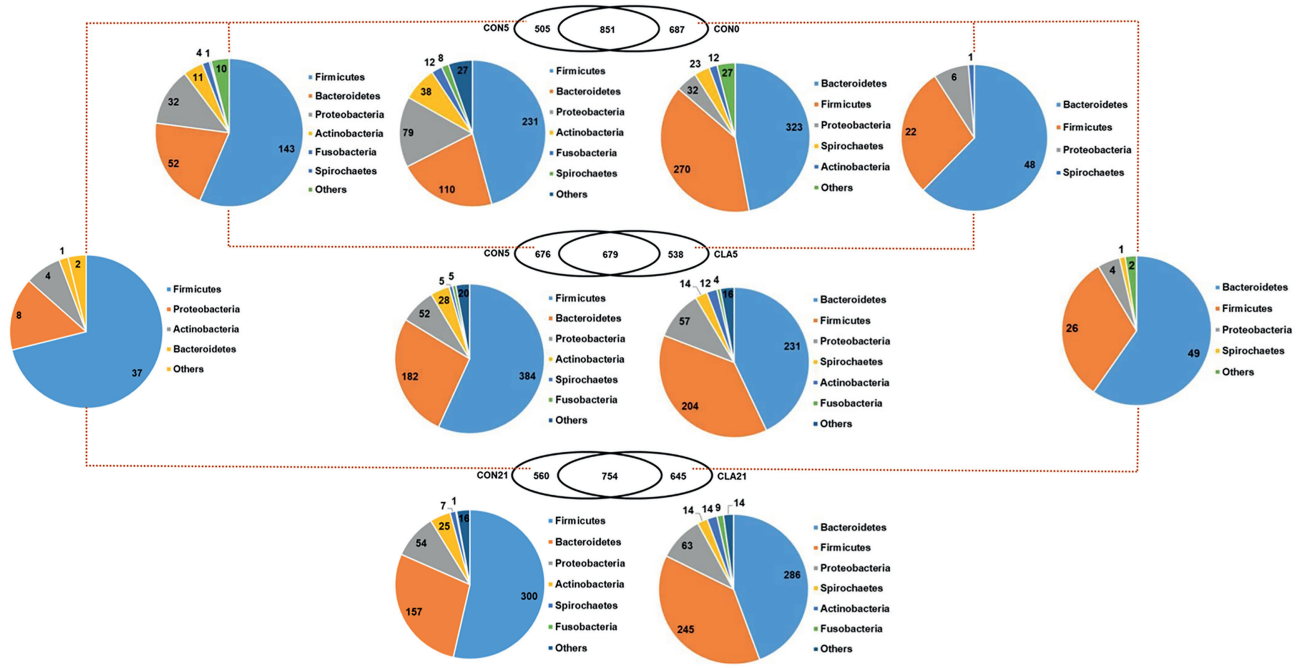

Fig. 3. *Trans*-10, *cis*-12 conjugated linoleic acid (CLA) supplementation altered the average reads of bacterial community in the rumen of lambs. Pie charts indicate the number of emerging and missing amplicon sequence variants (ASV) according to the ASV level of rumen bacteria. The different color of the parts means the different taxonomic distribution of the bacterial ASV at the phylum level. CON0 ($n = 6$), pre-weaning control group; CON5 ($n = 6$) and CON21 ($n = 6$), lambs were fed starter feed for 5 or 21 d after weaning; CLA5 ($n = 6$) and CLA21 ($n = 6$), lambs were fed starter feed with 1% of *trans*-10, *cis*-12 CLA supplemented for 5 or 21 d after weaning.

multiple roles such as lipid metabolism-, cell proliferation- and anti-inflammation was significantly upregulated in the rumen epithelium of post-weaning lambs on d 5 by *trans*-10, *cis*-12 CLA supplementation (Fig. 4, $P < 0.05$). Protein–protein interaction analysis further identified 69 genes as hub genes (degree >5), whose expressions have been significantly modified by the supplementation of *trans*-10, *cis*-12 CLA at least at one sample time after weaning, and close interactions among these hub genes, which are involved in either inflammation- or metabolism-related processes, have been detected (Fig. 6).

3.6. PPARγ mediated NF-κB p65 inhibition contributed to the protective effects of trans-10, cis-12 CLA in rumen epithelium

We further investigated the specific mechanisms by which *trans*-10, *cis*-12 CLA exerted anti-inflammatory effects in rumen epithelium using an IL-17A and TNF-α induced REC inflammation model in vitro. Both the treatment of IL-17A, IL-17A and TNF-α, as well as the *trans*-10, *cis*-12 CLA did not affect the cell viability after 12 h of incubation (Fig. 7A and B). The qPCR results indicated that the treatment of 100 μM *trans*-10, *cis*-12 CLA for 12 h significantly decreased the production of pro-inflammatory cytokines of IL-1β and IL-6 in REC after exposure to IL-17A and TNF-α (Fig. 7C, $P < 0.001$). The Western blot results further showed that the supplementation of 100 μM *trans*-10, *cis*-12 CLA significantly downregulated the protein expression of p-p65/p65 in REC upon IL-17A and TNF-α simulation, which plays a key role in the NF-κB signaling pathway (Fig. 7D, < 0.001). Furthermore, the protein expression of PPARγ that was supposed to be the target of CLA because of its high similarity with the PPARγ ligand significantly increased in REC after 100 μM *trans*-10, *cis*-12 CLA supplementation (Fig. 7D, $P < 0.01$). This supports that the PPARγ-mediated inhibition of NF-κB p65 signaling in REC contributed to the anti-inflammatory effects of *trans*-10, *cis*-12 CLA.

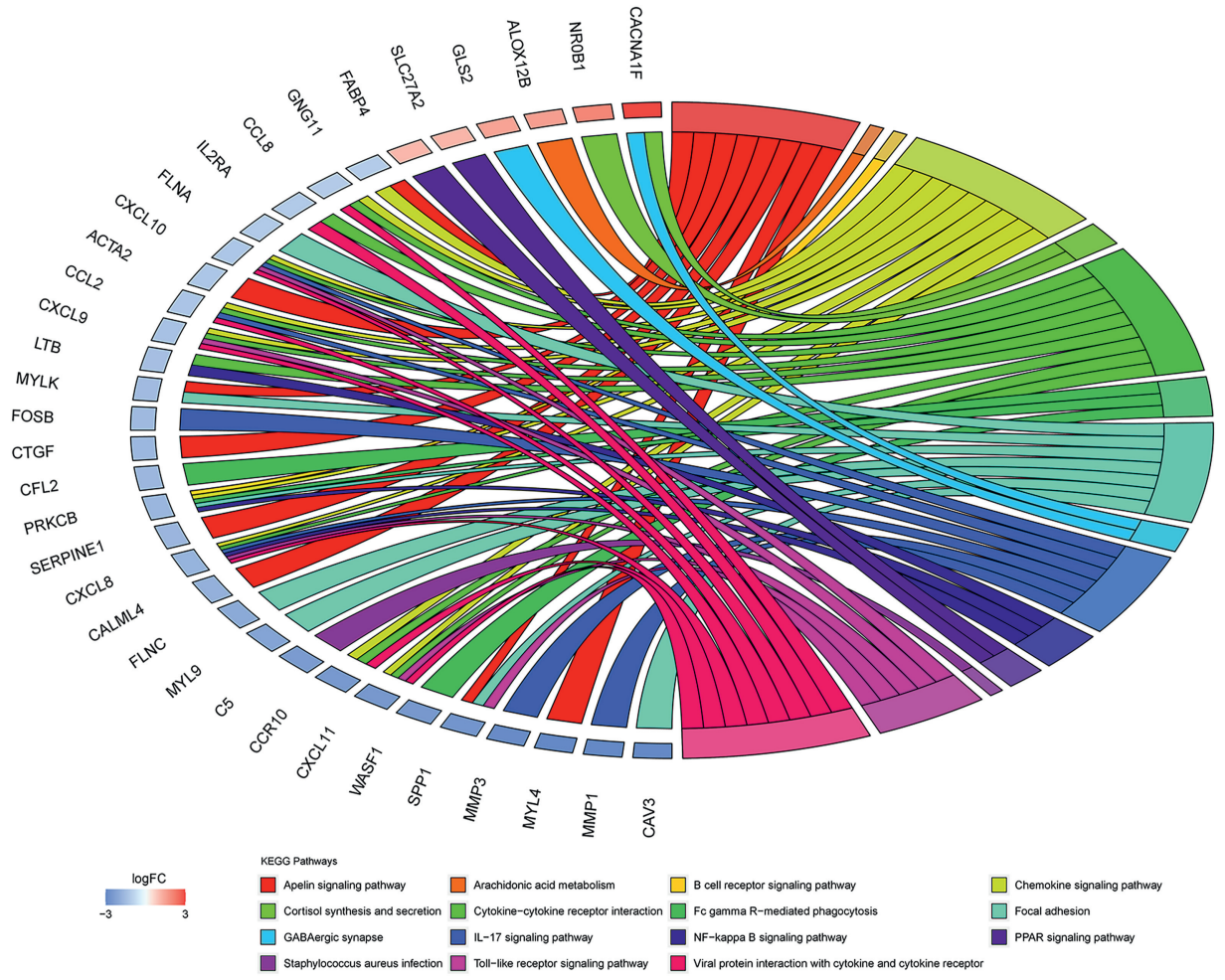

Fig. 4. The supplementation of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) for 5 d altered the gene encoding profiles, downregulated the inflammation-related signaling and upregulated the metabolism-related signaling in rumen epithelium of lambs after early weaning. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was conducted to compare the differentially expressed genes (FDR < 0.05, fold change >2) between the CLA5 and CON5 groups. Only the pathways with $P < 0.05$ were considered. CON5 ($n = 6$), lambs were fed starter feed for 5 d after weaning; CLA5 ($n = 6$), lambs were fed starter feed with 1% of *trans*-10, *cis*-12 CLA supplemented for 5 d after weaning. *ACTA2* = actin; *ALOX12B* = arachidonate 12-lipoxygenase (R-type); *CACNA1F* = voltage-dependent calcium channel L type alpha-1F; *CALML4* = calmodulin like 4; *CAV3* = caveolin 3; *CCL2* = C-C motif chemokine 2; *CCL8* = C-C motif chemokine 8; *CCR10* = C-C chemokine receptor type 10; *CFL2* = cofilin 2; *CTGF* = connective tissue growth factor; *CXCL8* = interleukin 8; *CXCL9* = C-X-C motif chemokine 9; *CXCL10* = C-X-C motif chemokine 10; *CXCL11* = C-X-C motif chemokine 11; *C5* = complement component 5; *FABP4* = fatty acid-binding protein 4; *FLNA* = filamin A; *FLNC* = filamin C; *FOSB* = FosB proto-oncogene, AP-1 transcription factor subunit; *GLS2* = glutaminase 2; *GNG11* = guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-11; *IL2RA* = interleukin 2 receptor alpha; *LTB* = lymphotoxin beta; *MMP1* = matrix metalloproteinase-1; *MMP3* = matrix metalloproteinase-3; *MYLK* = myosin-light-chain kinase; *MYL4* = myosin light chain 4; *MYL9* = myosin regulatory light chain 9; *NR0B1* = nuclear receptor subfamily 0 group B member 1; *PRKCB* = classical protein kinase C beta type; *SERPINE1* = plasminogen activator inhibitor 1; *SLC27A2* = solute carrier family 27 member 2; *SPP1* = secreted phosphoprotein 1; *WASF1* = WAS protein family member 1.

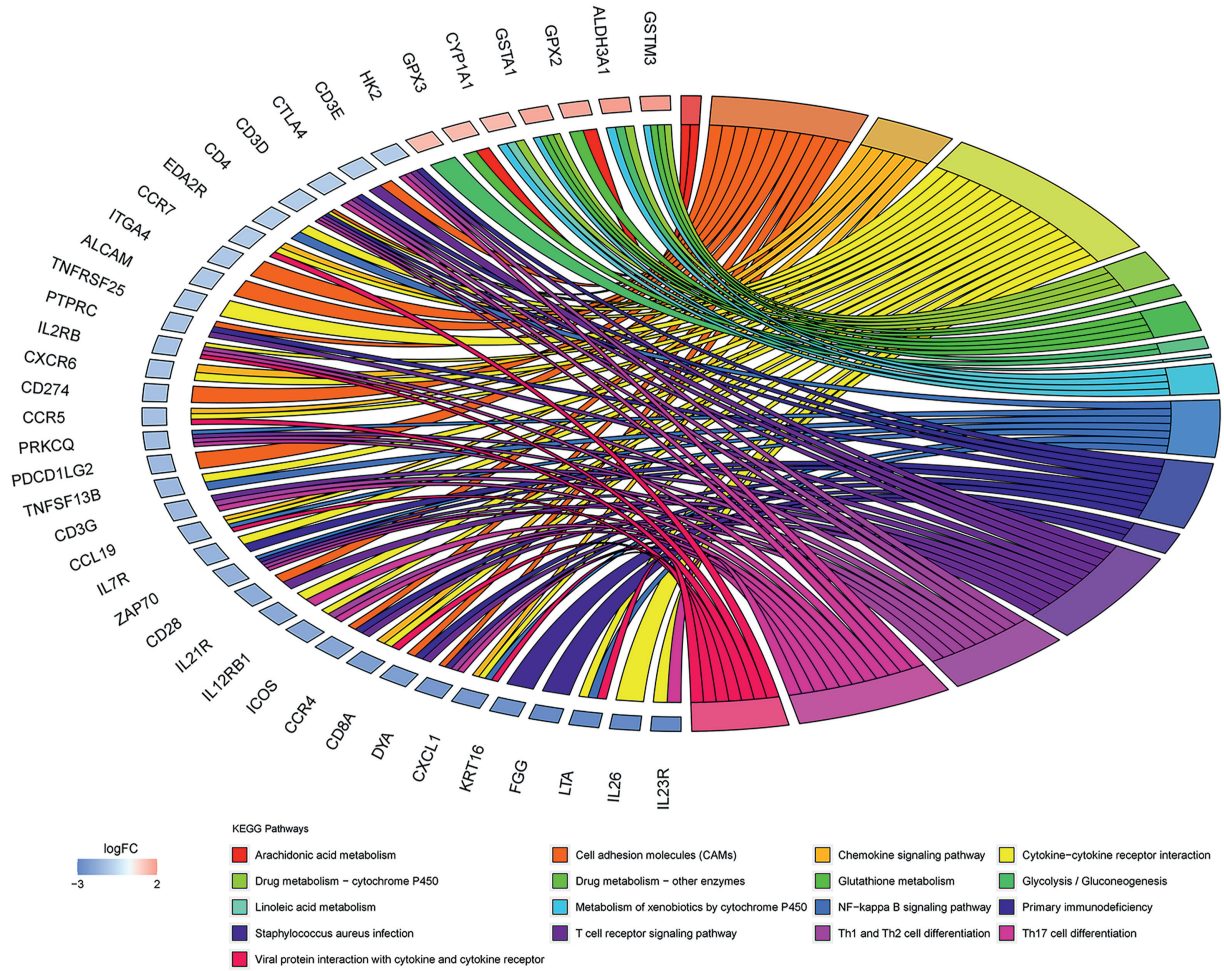

Fig. 5. The supplementation of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) for 21 d altered the gene encoding profiles, downregulated the inflammation-related signaling and upregulated the metabolism-related signaling in rumen epithelium of lambs after early weaning. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was conducted to compare the differentially expressed genes (FDR <0.05, fold change >2) between the CLA21 and CON21 groups. Only the pathways with $P < 0.05$ were considered. CON21 ($n = 6$), lambs were fed starter feed for 21 d after weaning; CLA21 ($n = 6$), lambs were fed starter feed with 1% of *trans*-10, *cis*-12 CLA supplemented for 21 d after weaning. *ALCAM* = activated leukocyte cell adhesion molecule; *ALDH3A1* = aldehyde dehydrogenase 3 family member A1; *CCL19* = C-C motif chemokine 19; *CCR4* = C-C chemokine receptor type 4; *CCR5* = C-C chemokine receptor type 5; *CCR7* = C-C chemokine receptor type 7; *CD3D* = T-cell surface glycoprotein CD3 delta chain; *CD3E* = T-cell surface glycoprotein CD3 epsilon chain; *CD3G* = T-cell surface glycoprotein CD3 gamma chain; *CD4* = T-cell surface glycoprotein CD4; *CD8A* = T-cell surface glycoprotein CD8 alpha chain; *CD28* = T-cell-specific surface glycoprotein CD28; *CD274* = programmed cell death 1 ligand 1; *CTLA4* = cytotoxic T-lymphocyte-associated protein 4; *CXCL1* = C-X-C motif chemokine ligand 1; *CXCR6* = C-X-C chemokine receptor type 6; *CYP1A1* = cytochrome P450 family 1 subfamily A1; *DYA* = HLA class II histocompatibility antigen, DQ alpha 2 chain; *EDA2R* = tumor necrosis factor receptor superfamily member 27; *FGG* = fibrinogen gamma chain; *GPX2* = glutathione peroxidase 2; *GPX3* = glutathione peroxidase 3; *GSTA1* = glutathione S-transferase alpha 1; *GSTM3* = glutathione S-transferase mu 3; *HK2* = hexokinase-2; *ICOS* = inducible T-cell co-stimulator; *IL2RB* = interleukin 2 receptor beta; *IL7R* = interleukin 7 receptor; *IL12RB1* = interleukin 12 receptor beta-1; *IL21R* = interleukin 21 receptor; *IL23R* = interleukin 23 receptor; *IL26* = interleukin 26; *ITGA4* = integrin alpha 4; *KRT16* = keratin 16; *LTA* = lymphotoxin alpha; *PDCD1LG2* = programmed cell death 1 ligand 2; *PRKCQ* = novel protein kinase C theta type; *PTPRC* = receptor-type tyrosine-protein phosphatase C; *TNFRSF25* = tumor necrosis factor receptor superfamily member 25; *TNFSF13B* = tumor necrosis factor ligand superfamily member 13B; *ZAP70* = tyrosine-protein kinase ZAP-70.

3.7. Trans-10, cis-12 CLA facilitated weaning transition of lambs via regulating microbiota-host interactions

Spearman rank correlation analysis was used to investigate the relationship among the relative abundance of rumen microbiota and fermentation parameters with the relative expression of inflammation- and metabolism-related hub genes in ruminal epithelium. According to Fig. 8A, the relative abundance of *Bacteroides* positively correlated with *GSTA1* ($P < 0.01$) and *GSTM3* ($P < 0.05$), whereas the abundance showed a negative correlation with *CXCL9* ($P < 0.05$), *CD4* ($P < 0.05$), *CCR4* ($P < 0.01$), *LTB* ($P < 0.05$), *SPP1* ($P < 0.05$) and *BCL2A1* ($P < 0.05$). Abundance of *Treponema* positively correlated with *GSTM3* ($P < 0.05$), and negatively correlated with *IL2RA* ($P < 0.05$). Abundance of *Parabacteroides* showed a positive correlation with *GSTM3* ($P < 0.05$). Abundance of *Anaerovibrio* negatively correlated with *CD4* ($P < 0.05$). Abundance of *Oribacterium* was negatively correlated *SERPINE1* ($P < 0.05$) and positively correlated with *SLC27A2* ($P < 0.05$). Abundance of both *Succinivibrio* and *Catenisphaera* was positively correlated with *SERPINE1* and *SPP1* ($P < 0.05$), and negatively correlated with *GPX2* ($P < 0.01$ and $P < 0.05$) and *GSTA1* ($P < 0.01$). Besides, abundance of *Catenisphaera* showed a negative correlation with *GSTM3* ($P < 0.05$). Some other significant correlations, such as a negative correlation between abundance of *Solobacterium* and *GSTM3* and *GSTA1* ($P < 0.01$), a positive correlation between abundance of *Syntrophococcus* and *CCR4* and *LTB* ($P < 0.05$), as well as a positive correlation between abundance of *Weissella* and *CCR4* ($P < 0.05$) were also identified.

As shown in Fig. 8B, both rumen acetate and total VFA concentrations were positively correlated with the expression of *ALDH3A1* ($P < 0.05$), whereas they were negatively correlated with the expression of *BCL2A1* ($P < 0.01$), *LTB* ($P < 0.01$), *CCR4* ($P < 0.05$ and

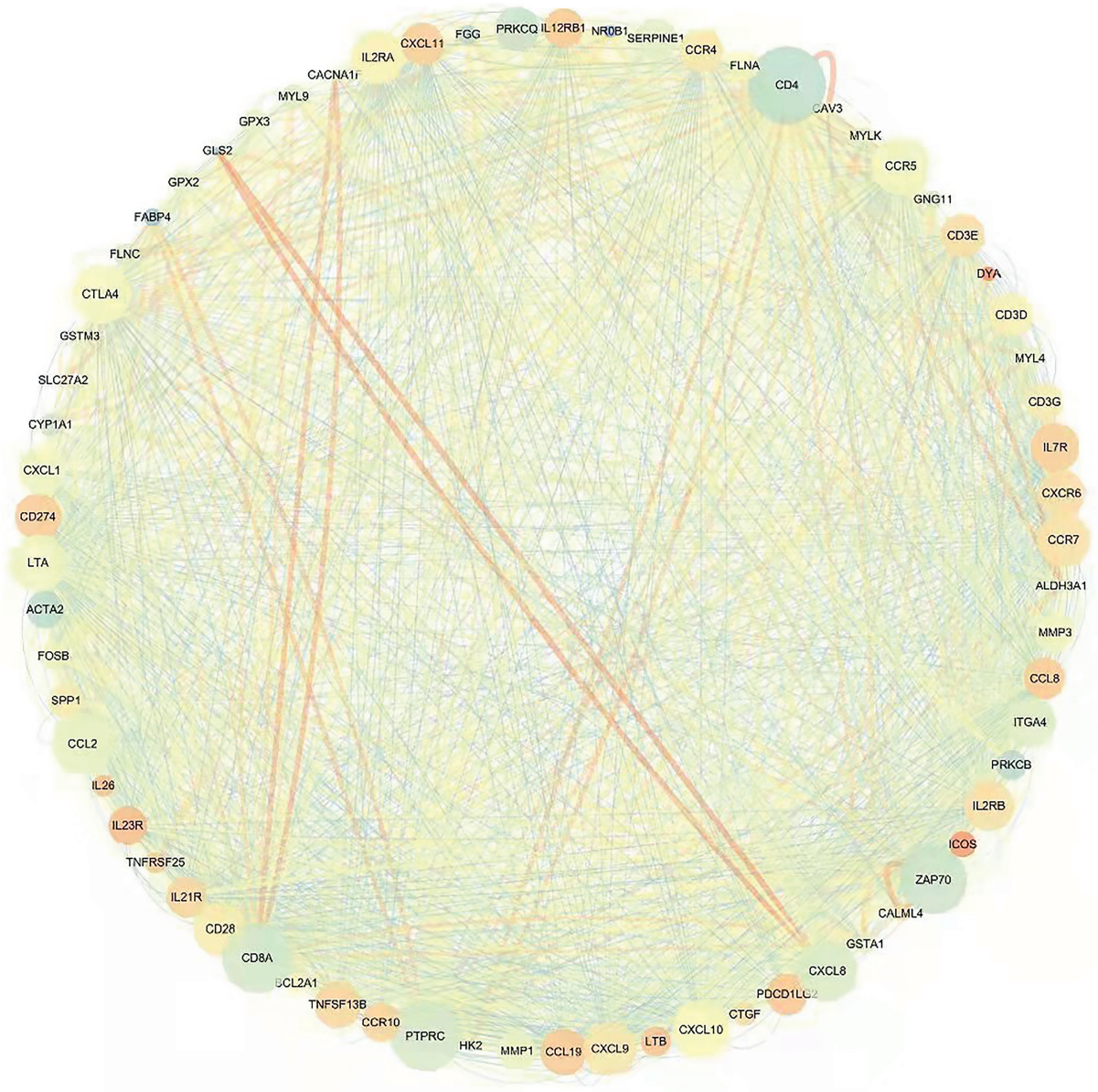

Fig. 6. Protein–protein interaction network analysis of the differentially expressed genes (DEG, FDR <0.05, fold change >2) due to *trans*-10, *cis*-12 conjugated linoleic acid (CLA) supplementation. All the DEG between CLA5 and CON5, CLA21 and CON21 were used for analysis. The node size and color indicate the number of degrees and the clustering coefficient (blue represents low values, red represents high values), respectively, the edge indicates the interaction (the thicker edge means the lower betweenness). Degree >5 represents hub genes. CON5 ($n = 6$) and CON21 ($n = 6$), lambs were fed starter feed for 5 or 21 d after weaning; CLA5 ($n = 6$) and CLA21 ($n = 6$), lambs were fed starter feed with 1% of *trans*-10, *cis*-12 CLA supplemented for 5 or 21 d after weaning. *ACTA2* = actin; *ALDH3A1* = aldehyde dehydrogenase 3 family member A1; *BCL2A1* = hematopoietic Bcl-2-related protein A1; *CACNA1F* = voltage-dependent calcium channel L type alpha-1F; *CALML4* = calmodulin like 4; *CAV3* = caveolin 3; *CCL2* = C-C motif chemokine 2; *CCL8* = C-C motif chemokine 8; *CCL19* = C-C motif chemokine 19; *CCR4* = C-C chemokine receptor type 4; *CCR5* = C-C chemokine receptor type 5; *CCR7* = C-C chemokine receptor type 7; *CCR10* = C-C chemokine receptor type 10; *CD3D* = T-cell surface glycoprotein CD3 delta chain; *CD3E* = T-cell surface glycoprotein CD3 epsilon chain; *CD3G* = T-cell surface glycoprotein CD3 gamma chain; *CD4* = T-cell surface glycoprotein CD4; *CD8A* = T-cell surface glycoprotein CD8 alpha chain; *CD28* = T-cell-specific surface glycoprotein CD28; *CD274* = programmed cell death 1 ligand 1; *CTGF* = connective tissue growth factor; *CTLA4* = cytotoxic T-lymphocyte-associated protein 4; *CXCL1* = C-X-C motif chemokine ligand 1; *CXCL8* = interleukin 8; *CXCL9* = C-X-C motif chemokine 9; *CXCL10* = C-X-C motif chemokine 10; *CXCL11* = C-X-C motif chemokine 11; *CXCR6* = C-X-C chemokine receptor type 6; *CYP1A1* = cytochrome P450 family 1 subfamily A1; *DYA* = HLA class II histocompatibility antigen, DQ alpha 2 chain; *FABP4* = fatty acid-binding protein 4; *FGG* = fibrinogen gamma chain; *FLNA* = filamin A; *FLNC* = filamin C; *FOSB* = FosB proto-oncogene, AP-1 transcription factor subunit; *GLS2* = glutaminase 2; *GNG11* = guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-11; *GPX2* = glutathione peroxidase 2; *GPX3* = glutathione peroxidase 3; *GSTA1* = glutathione S-transferase alpha 1; *GSTM3* = glutathione S-transferase mu 3; *ICOS* = inducible T-cell co-stimulator; *IL2RA* = interleukin 2 receptor alpha; *IL2RB* = interleukin 2 receptor beta; *IL7R* = interleukin 7 receptor; *IL12RB1* = interleukin 12 receptor beta-1; *IL21R* = interleukin 21 receptor; *IL23R* = interleukin 23 receptor; *IL26* = interleukin 26; *ITGA4* = integrin alpha 4; *HK2* = hexokinase-2; *LTA* = lymphotoxin alpha; *LTB* = lymphotoxin beta; *MMP1* = matrix metalloproteinase-1; *MMP3* = matrix metalloproteinase-3; *MYLK* = myosin-light-chain kinase; *MYL4* = myosin light chain 4; *MYL9* = myosin regulatory light chain 9; *NR0B1* = nuclear receptor subfamily 0 group B member 1; *PDCD1LG2* = programmed cell death 1 ligand 2; *PRKCB* = classical protein kinase C beta type; *PRKCQ* = novel protein kinase C theta type; *PTPRC* = receptor-type tyrosine-protein phosphatase C; *SERPINE1* = plasminogen activator inhibitor 1; *SLC27A2* = solute carrier family 27 member 2; *SPP1* = secreted phosphoprotein 1; *TNFRSF25* = tumor necrosis factor receptor superfamily member 25; *TNFSF13B* = tumor necrosis factor ligand superfamily member 13B; *ZAP70* = tyrosine-protein kinase ZAP-70.

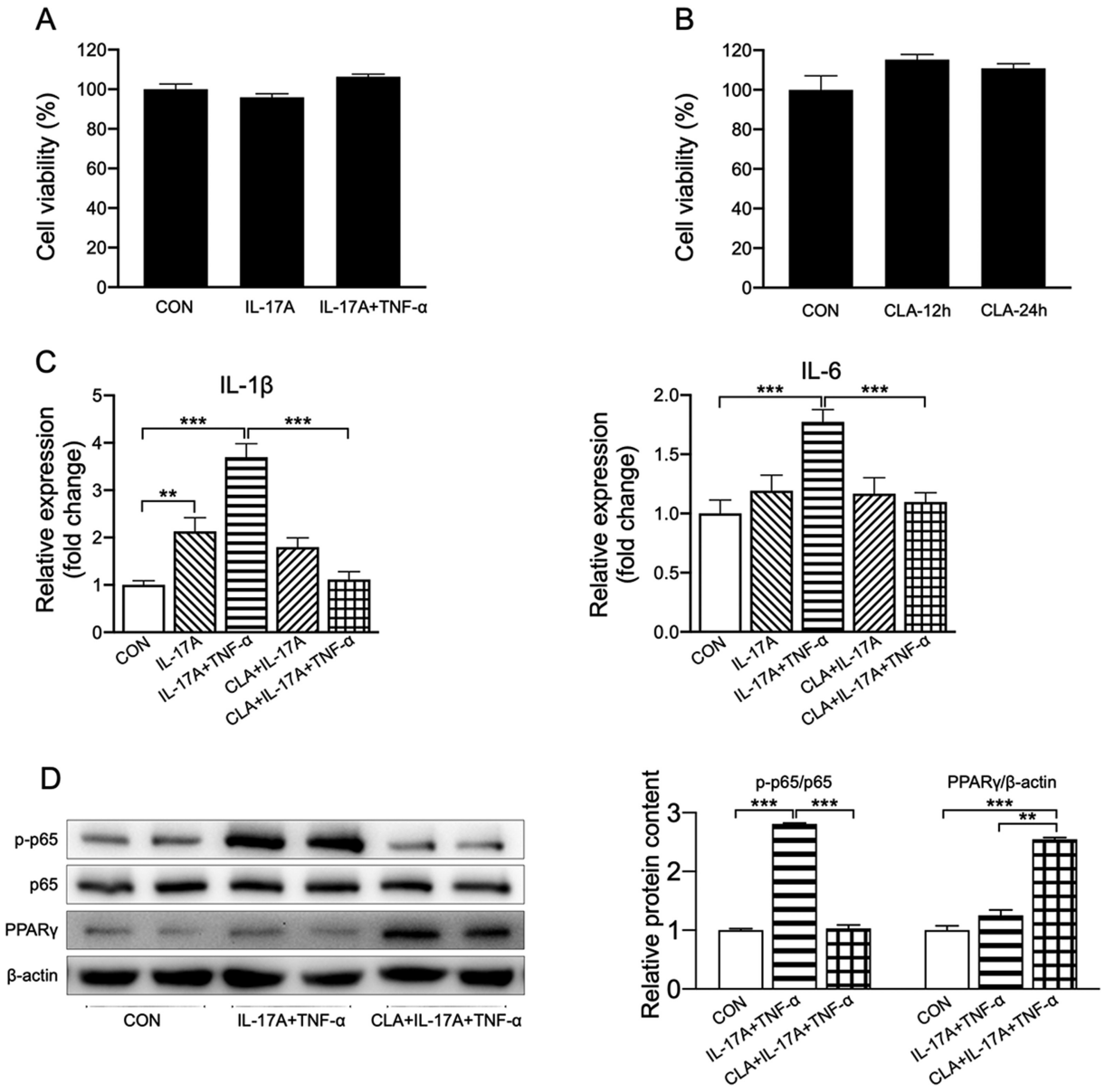

Fig. 7. Protective effects of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) on ruminal epithelial cells (REC) upon interleukin 17A (IL-17A) and tumor necrosis factor-α (TNF-α) stimulation. (A) The effect of IL-17A, IL-17A + TNF-α on cell viability after 12 h of treatment. (B) The effect of *trans*-10, *cis*-12 CLA on cell viability after 12 and 24 h of treatment, respectively. (C) The gene expression levels of IL-1β and IL-6 in REC among different treatments. (D) The protein expression levels of p-p65/p65 and PPARγ/β-actin in REC among different treatments. Beta-actin was used as reference protein. Data were presented as the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$. CON, control group ($n = 6$); IL-17A, REC were treated with IL-17A (25 ng/mL) for 12 h ($n = 6$); IL-17A + TNF-α, REC were treated with IL-17A (25 ng/mL) and TNF-α (0.5 ng/mL) for 12 h ($n = 6$); CLA + IL-17A, REC were treated with IL-17A (25 ng/mL) for 12 h after pretreatment of 100 μM *trans*-10, *cis*-12 CLA ($n = 6$); CLA + IL-17A + TNF-α, REC were treated with IL-17A (25 ng/mL) and TNF-α (0.5 ng/mL) for 12 h after pretreatment of 100 μM *trans*-10, *cis*-12 CLA ($n = 6$). *IL-1β* = interleukin 1 beta; *IL-6* = interleukin 6; p65 = NF-κB p65 subunit; p-p65 = phosphorylated p65; PPARγ = peroxisome proliferator-activated receptor gamma.

$P < 0.01$) and *IL2RA* ($P < 0.05$ and $P < 0.01$). Both rumen butyrate and total VFA concentration showed positive correlations with expression of *SLC27A2* ($P < 0.05$), and negative correlations with expression of *CCR4* and *CXCL9* ($P < 0.01$). Besides, some other significant correlations, such as the positive correlation between rumen butyrate concentration and expression of *GPX2* ($P < 0.05$), and the negative correlation between rumen acetate concentration and expression of *SPP1* ($P < 0.05$), and total VFA concentration and expression of *CD4* ($P < 0.01$), were also existed. Besides the microbiota as such, rumen VFA concentration, the predominant microbial fermentation product, also showed close correlations with the epithelial transcriptome. These changes in rumen microbiota-host interaction may account for the regulatory effects of *trans*-10, *cis*-12 CLA.

4. Discussion

The microbiota that gradually colonizes in the mammalian gut during the first days of life and its constant interaction with the host has long-term effects on animal performance and health conditions (Fulde et al., 2018). For ruminants, weaning is a very critical event in the life cycle and is frequently associated with enteric infections and productivity decline, due to the abrupt changes of diet and environment (Wood et al., 2015). Antibiotic

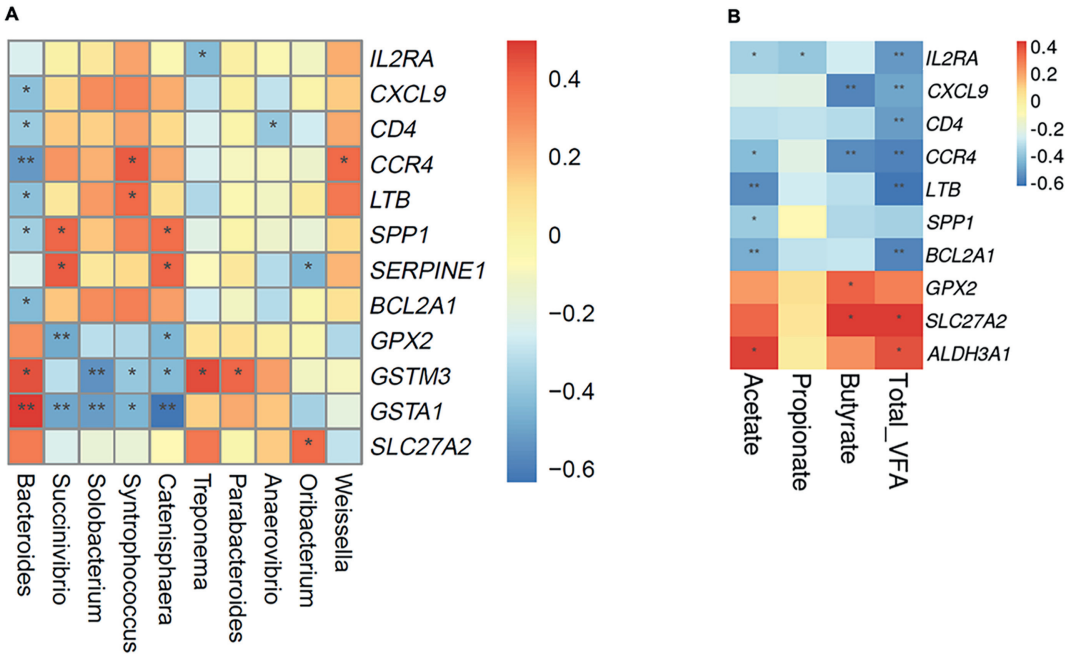

Fig. 8. The supplementation of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) alleviated early weaning stress induced rumen dysfunction of lambs via regulating microbe-host metabolic and signal interactions. (A) The correlations between the changes of bacterial abundance and the expression of hub genes that involve in inflammation and metabolism using Spearman correlation coefficient analysis. (B) The correlations between the changes of fermentation parameters and the expression of hub genes that involve in inflammation and metabolism using Spearman correlation coefficient analysis. Red indicates positive correlation; blue indicates negative correlation. *$P < 0.05$, **$P < 0.01$. *ALDH3A1* = aldehyde dehydrogenase 3 family member A1; *BCL2A1* = hematopoietic Bcl-2-related protein A1; *CCR4* = C-C chemokine receptor type 4; *CD4* = T-cell surface glycoprotein CD4; *CXCL9* = C-X-C motif chemokine 9; *GPX2* = glutathione peroxidase 2; *GSTA1* = glutathione S-transferase alpha 1; *GSTM3* = glutathione S-transferase mu 3; *IL2RA* = interleukin 2 receptor alpha; *LTB* = lymphotoxin beta; *SERPINE1* = plasminogen activator inhibitor 1; *SLC27A2* = solute carrier family 27 member 2; *SPP1* = secreted phosphoprotein 1; VFA = volatile fatty acid.

therapy is currently the most used treatment for weaning stress-related diseases; however, antibiotic resistance is a well-known threat for global health. Thus, developing antibiotic alternatives is urgently needed in livestock farming (Gresse et al., 2017). In the present study, we explored the potential effects of supplementing CLA, a natural rumen bacterial metabolite (Yuan et al., 2015), on alleviating weaning-related stress in lambs, and we integrated rumen taxonomic configurations and epithelial transcriptome profiling to investigate whether it can regulate the crosstalk between ruminal microbiota and host immunity.

Previous studies have revealed that during weaning, the rumen bacterial community experiences significant changes when animals just start to eat solid feed (Li et al., 2018; Lin et al., 2019). Our study confirmed this, as the rumen bacterial density significantly changed in lambs during the weaning transition period. In particular, an increased ratio of Firmicutes and a decreased abundance of Bacteroidetes were observed in lambs after weaning, which was consistent with the findings of Rey et al. (2014) and Palma-Hidalgo et al. (2021). Simultaneously, the relative abundance of *Bacteroides* and *Prevotella*, which are 2 main genera of Bacteroidetes, was significantly decreased after early weaning on d 5. The positive role of *Bacteroides* species in consuming milk oligosaccharides has previously been confirmed (Marcobal et al., 2011). *Prevotella* species are known to hold various ecological niches within the rumen as they have extremely diverse metabolic capabilities that are involved in the degradation of both non-structural carbohydrates and xylan (Accetto and Avguštin, 2019). In addition, the relative abundances of *Prevotellaceae_UCG-001* and *003* (Xiao et al., 2020), *Christensenellaceae_R-7_group* (Calderón-Pérez et al., 2020), *Rikenellaceae_RC9_gut_group* (Liang et al., 2022), and *Saccharofermentans* (Li et al., 2019), which are reported to be positively correlated with rumen carbohydrate fermentation and short chain fatty acid (SCFA) production was also significantly reduced in the rumen of lambs after early weaning. The unexpected decrease in abundances of these genera may partly reflect the early weaning stress-induced decline in rumen fermentation of lambs. Furthermore, the weaning transition also increased the abundances of several pathogenic bacteria such as *Haemophilus* (Duell et al., 2016), *Veillonella* (Chen et al., 2020b), *Streptococcus* (Segura et al., 2017) and *Solobacterium* (Lau et al., 2006), which consists with the findings that the young ruminants survive a risk for early life infections during the critical window like weaning as they lack developed gastric systems and immunity (Hulbert and Moisá, 2016).

As the major fermentation products of rumen microbiota, the importance of VFA in improving energy utilization and facilitating early rumen development has been extensively studied (Lin et al., 2019). Specially, butyrate and acetate have been indicated to inhibit the potential alternations of energy metabolism and epithelial structure and functions of the rumen epithelium otherwise caused by weaning stress due to their role as energy-supplying substrate for cell growth (Liu et al., 2019). In present study, the ADG, DMI and ruminal production of total VFA, as well as acetate and butyrate in lambs were both significantly improved after 5 d of weaning due to *trans*-10, *cis*-12 CLA supplementation, and this increase in VFA production even continued into 21 d after weaning, suggesting that the CLA may protect lambs against weaning-associated decline in growth performance by providing more available energy. In line with the elevated concentration of rumen VFA, our data also showed associated changes in the ruminal microbial taxonomic fingerprints. Phylogenetic analysis of the detectable rumen microbial genera indicated that the supplementation of *trans*-10, *cis*-12 CLA significantly increased abundances of *Treponema*, *Bacteroides*, *Anaerovibrio*, *Parabacteroides* and *Oribacterium* in the rumen of lambs after early weaning. These bacteria

are reported to positively involve in feed fermentation and VFA production. For example, *Treponema* species are known to encode a diversity of carbohydrate-active enzymes in the rumen and synergistically interact with cellulolytic bacteria in the degradation of pectin and cellulose to produce acetate (Liu et al., 2015). *Bacteroides* can degrade plant cell walls with the secretion of cellulases and hemicellulases, and convert the carbohydrates to acetate and succinate (Wang et al., 2019). *Anaerovibrio*, as an important rumen bacteria associated with lipid hydrolysis, can increase the production of glycerol and sugar that can be metabolized to VFA (Kim et al., 2020). The ability of *Parabacteroides* to ferment starch into acetate has also been certified, which was believed to positively correlate with its significant role in recovering unbalanced gut homeostasis (Lei et al., 2021). The positive correlation between *Oribacterium* and VFA production has been certified in the study of Zeng et al. (2019). The microbiota functional profiling data based on PICRUSt2 in our study further confirmed that *trans*-10, *cis*-12 CLA supplementation improved the rumen beta-galactosidase function in lambs during weaning transition, which was consistent with the enhanced VFA production from rumen microbial fermentation. The ability of CLA to change gut microbial composition and promote SCFA production have been previously verified in mouse study (Marques et al., 2015). The importance of utilizing feed additives to optimize rumen microbes and fermentation products to alleviate weaning stress of lambs has also been extensively reported (Belanche et al., 2019; Li et al., 2020a). For example, Izuddin et al. (2019) found that the enrichment of fiber degrading bacteria and enhancement of butyrate production in rumen could improve the growth performance of newly weaned lambs. Taken together, these findings suggest that supplementation of *trans*-10, *cis*-12 CLA stabilizes the rumen microbial ecosystem by regulating rumen microbial composition and fermentation to produce more acetate and butyrate, thus stimulating rumen development and relieving weaning transition related stresses of lambs.

The early weaning associated gut microbial dysbiosis has been demonstrated to result in gastrointestinal epithelial damage, which may further facilitate the occurrence of systemic inflammation (Gresse et al., 2017). In our study, the supplementation of *trans*-10, *cis*-12 CLA alleviated the rumen dysbiosis-induced epithelial injury of lambs through regulating the transcriptome profiling in host animals. Biological enrichment analysis of the rumen epithelial transcriptome revealed that the KEGG pathways related to inflammatory processes including "cytokine–cytokine receptor interaction", "NF-κB signaling pathway" and "chemokine signaling pathway" were significantly down-regulated in the rumen epithelium after the supplementation of *trans*-10, *cis*-12 CLA for 5 or 21 d. Inflammation is known to be a process characterized by the largely choreographed release of cytokines and the recruitment of immune effectors under the control of chemokine (Sokol and Luster, 2015). For example, the cytokines TNF-α, IL-1β and IL-6 can induce the activation of cytoplasmic NF-κB and its subsequent translocation into nucleus, then promote the expression and release of pro-inflammatory cytokines and further aggravate inflammation. On the other hand, the NF-κB can also induce the chemokine-signaling to attract and recruit more immune cells to the inflammatory site for defense (Marafini et al., 2019). Besides, the supplementation of *trans*-10, *cis*-12 CLA also exhibited some action against acute inflammation, for example, the "Toll-like receptor signaling pathway" and "IL-17 signaling pathway" in the rumen epithelium of lambs were only significantly down-regulated at 5 d after weaning with CLA supplemented. TLR4 is known to be the major pathogen-associated molecular receptor that induces various transcription factors involved in the initiation and regulation of inflammation (Kawai and Akira, 2011). This kind of transient dramatic response derived from weaning stress has also been found in other mammals. Deng et al. (2020) revealed that early weaning could increase the intestinal LPS production of piglets during the first 3 to 7 d.

The excess release of IL-17 is certified to be a significant contributor of inflammatory and autoimmune disorders via inducing the NF-κB signaling (Beringer et al., 2016). Thus, to verify the anti-inflammation effects of *trans*-10, *cis*-12 CLA on rumen epithelium, we further used in vitro cell culture experiment to explore the potent regulatory effects of *trans*-10, *cis*-12 CLA on alleviating REC's inflammation induced by pro-inflammatory cytokines. The results indicated that *trans*-10, *cis*-12 CLA can significantly suppress the production of IL-1β and IL-6 in REC when faced with inflammatory stimulation derived from IL-17A and TNF-α. The reason was certified to be its inhibiting effects on the activation of p65, which is an important regulatory protein of the NF-κB signaling system. It is known that in the rest state, the NF-κB family including the p65 exist in the cytoplasm that sustain as an inactive complex with the inhibitory protein IκB. Upon activation, the IκB such as IκBα undergoes degradation, then the NF-κB p65 is phosphorylated and translocated into the nucleus, where it further activates transcription (Oeckinghaus et al., 2011). In the present study, the protein expression of NF-κB p-p65/p65 in REC was significantly downregulated after *trans*-10, *cis*-12 CLA supplementation, which further supported the animal experiment results that the rumen epithelial NF-κB signaling pathway was suppressed in early weaned lambs with CLA supplementation. In addition, we also found the addition of *trans*-10, *cis*-12 CLA significantly upregulated the protein expression of PPARγ in REC when faced with stimulation, which is consist with its high similarity with the PPARγ ligand. The significant role of PPARγ in the control of inflammatory response has been suggested to largely rely on its ability to suppress the activities of many transcription factors including NF-κB, STAT and AP1 that are involved in the promotion of pro-inflammatory process (Daynes and Jones, 2002). Thus, *trans*-10, *cis*-12 CLA supplementation can alleviate the inflammation in REC via regulating PPARγ-dependent downregulation of NF-κB signaling.

Corresponding to the upregulated protein expression of PPARγ in REC after *trans*-10, *cis*-12 CLA supplementation, we also observed significantly upregulated "PPAR signaling" and metabolism-related KEGG pathways including "glutathione metabolism", "arachidonic acid metabolism", "metabolism of xenobiotics by cytochrome P450" and "glycolysis/gluconeogenesis" in the rumen epithelium of lambs. Glutathione is a tripeptide thiol antioxidant consisted of amino acids including glutamic acid, cysteine, and glycine, glutathione metabolism was indicated to play an important role in scavenging reactive oxygen species and maintaining redox homeostasis (Xiao and Meierhofer, 2019). In the concept of nutrition, arachidonic acid is an omega-6 polyunsaturated fatty acid that not only confers cell membranes with fluidity and flexibility, but also acts as lipid second messenger in various cell signaling such as immunity, inflammation, and redox homeostasis (Sonnweber et al., 2018). Cytochrome P450 enzymes are known to involve in numbers of reactions related to the metabolism of drugs, steroids and carcinogens, which contribute significantly to cellular metabolism and homeostasis (Guengerich et al., 2016). The glycolysis/gluconeogenesis, which play a critical role in energy metabolism, has been demonstrated to constantly involve in the response to stress (Dong et al., 2016). Therefore, on the other hand, the supplementation of *trans*-10, *cis*-12 CLA could potentially reduce weaning stress of lambs via linking metabolism to inflammation and immunity.

In consideration of the significant regulatory effects of *trans*-10, *cis*-12 CLA on rumen microbial composition, fermentation, and epithelial transcriptome, we further explored the potent correlation among them to better understand whether *trans*-10, *cis*- 12 CLA can alleviate early weaning stress-related rumen dysfunction

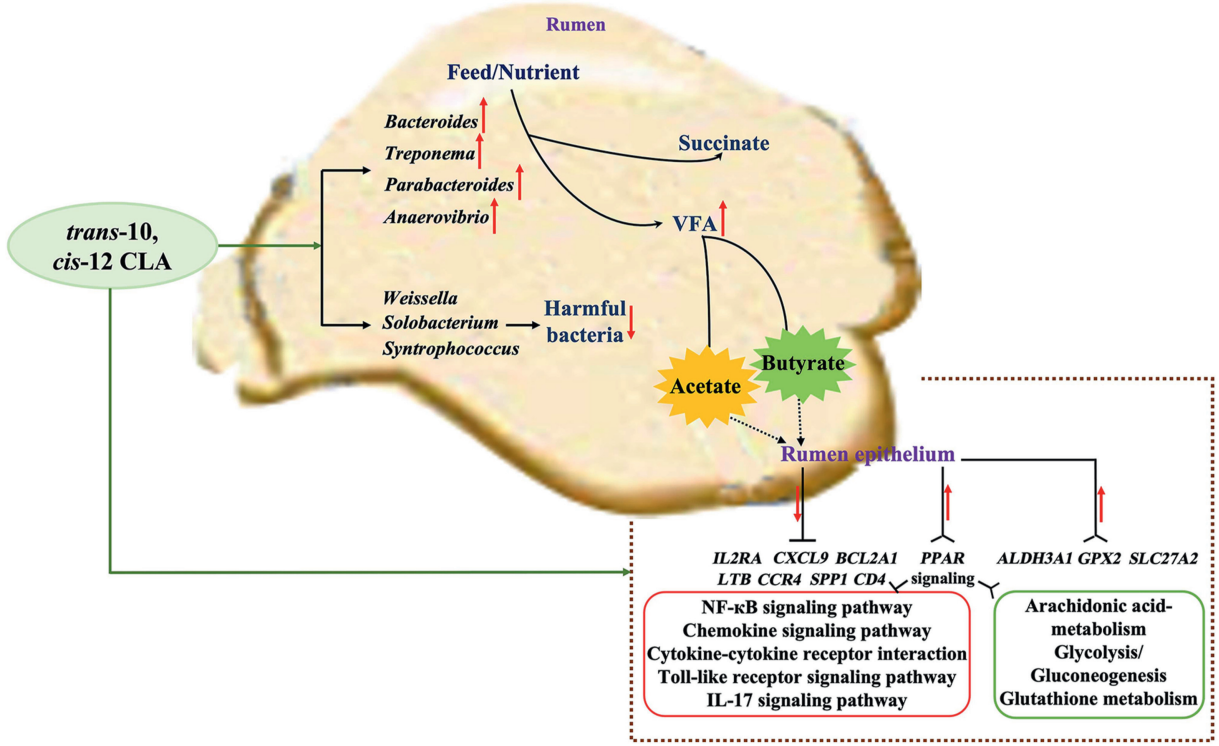

Fig. 9. The proposed regulatory mechanisms of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) on alleviating early weaning induced stress of lambs. The supplementation of *trans*-10, *cis*-12 CLA during weaning transition enriched the relative abundance of volatile fatty acid (VFA) producing bacteria and promoted the rumen microbiota-driven production of VFA, which were indicated to negatively correlated with the inflammatory genes and associated pathways, while positively correlated with metabolic genes and associated pathways in rumen epithelium. In vitro experiment further certified the anti-inflammatory effect of CLA was through suppressing the NF-κB-mediated proinflammatory responses with the activation of peroxisome proliferator-activated receptor (PPAR) signaling. The branch represents activation, the inverted-T represents inhibition. *ALDH3A1* = aldehyde dehydrogenase 3 family member A1; *BCL2A1* = hematopoietic Bcl-2-related protein A1; *CCR4* = C-C chemokine receptor type 4; *CD4* = T-cell surface glycoprotein CD4; *CXCL9* = C-X-C motif chemokine 9; *GPX2* = glutathione peroxidase 2; *IL2RA* = interleukin 2 receptor alpha; *LTB* = lymphotoxin beta; *SLC27A2* = solute carrier family 27 member 2; *SPP1* = secreted phosphoprotein 1.

via a microbe-host metabolic and signal interactions. Particularly interesting, our results revealed that the relative abundances of *Bacteroides*, *Treponema*, *Parabacteroides* and *Anaerovibrio*, which were significantly enriched by *trans*-10, *cis*-12 CLA supplementation and suggested to be important VFA producers, showed clear negative correlations with the expression of genes that are regarded as important biomarkers of systemic inflammation mediated diseases (Ramirez et al., 2012; Minutolo et al., 2021; Souza et al., 2021), such as *IL2RA*, *CXCL9*, *CCR4*, *CD4*, *LTB*, *SPP1* and *BCL2A1*. In addition, these inflammation-related genes also showed significant negative correlations with the enhanced production of ruminal total VFA, acetate or butyrate when *trans*-10, *cis*-12 CLA was supplemented. Furthermore, the *GPX2* encodes glutathione peroxidases (Li et al., 2020b), *SLC27A2* encodes fatty acid transporter (Chen et al., 2020a), and *ALDH3A1* encodes aldehyde dehydrogenase (Koppaka et al., 2016), also showed positive correlations with the improved production of VFA. While the *GSTA1* (Michaud et al., 2019) and *GSTM3* (Llavanera et al., 2020) encode glutathione-S-transferase showed positive correlations with the increased abundances of *Bacteroides*, *Parabacteroides* and *Treponema*. Simultaneously, these metabolism-related genes also showed negative correlations with the pathogenic bacteria. Taken together, the protective effects of *trans*-10, *cis*-12 CLA on alleviating weaning stress-related rumen dysfunction of lambs were suggested to be its alternation of microbiome-driven production of VFA, which further mediated the expression of inflammatory response- and metabolism-related genes and their associated signaling pathways in rumen epithelium.

5. Conclusion

In conclusion, the supplementation of *trans*-10, *cis*-12 CLA may protect lambs against early weaning induced stresses by enhancing the ruminal microbiota-driven transcriptional regulation in healthy rumen epithelial development via VFA (Fig. 9). These findings suggest that a nutritional intervention that facilitates weaning transition of young ruminants by regulating microbiota-host metabolic and signal interactions is a valuable tool for improving animal health.

Data statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. The 16S rRNA and RNA-Seq data was deposited in the Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra) under the accession number of PRJNA784343 and PRJNA784167, respectively.

Author contributions

Chunlei Yang and **Lifeng Dong:** conceived the study, curated and visualized the data, and wrote the original manuscript. **Xiangfei Deng**, **Haixia Liu** and **Xingwang Ding:** performed the experiments. **Peter Lund**, **Zhengwei Fu**, **Naifeng Zhang** and **Jinjun Li:** supervised, edited and revised the manuscript.

Declaration of competing interest

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

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 31702133), and the Central Public-interest Scientific Institution Basal Research Fund of Chinese Academy of Agricultural Sciences (No. Y2021GH18-2).

Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2022.11.002.

References

Accetto T, Avguštin G. The diverse and extensive plant polysaccharide degradative apparatuses of the rumen and hindgut *Prevotella* species: a factor in their ubiquity? Syst Appl Microbiol 2019;42:107–16.
Belanche A, Yáñez-Ruiz DR, Detheridge AP, Griffith GW, Kingston-Smith AH, Newbold CJ. Maternal versus artificial rearing shapes the rumen microbiome having minor long-term physiological implications. Environ Microbiol 2019;21:4360–77.
Beringer A, Noack M, Miossec P. IL-17 in chronic inflammation: from discovery to targeting. Trends Mol Med 2016;22:230–41.
Bi Y, Tu Y, Zhang N, Wang S, Zhang F, Suen G, et al. Multiomics analysis reveals the presence of a microbiome in the gut of fetal lambs. Gut 2021;70:853–64.
Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol 2019;37:852–7.
Calderón-Pérez L, Gosalbes MJ, Yuste S, Valls RM, Pedret A, Llauradó E, et al. Gut metagenomic and short chain fatty acids signature in hypertension: a cross-sectional study. Sci Rep 2020;10:6436.
Chen L, Vasoya RP, Toke NH, Parthasarathy A, Luo S, Chiles E, et al. HNF4 regulates fatty acid oxidation and is required for renewal of intestinal stem cells in mice. Gastroenterology 2020a;158:985–99. e9.
Chen S, Zhou Y, Chen Y, Gu J. fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 2018;34:i884–90.
Chen X, Li P, Liu M, Zheng H, He Y, Chen MX, et al. Gut dysbiosis induces the development of pre-eclampsia through bacterial translocation. Gut 2020b;69:513–22.
Cui K, Wang B, Zhang N, Tu Y, Ma T, Diao Q. iTRAQ-based quantitative proteomic analysis of alterations in the intestine of Hu sheep under weaning stress. PLoS One 2018;13:e0200680.
Daynes RA, Jones DC. Emerging roles of PPARs in inflammation and immunity. Nat Rev Immunol 2002;2:748–59.
da Huang W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009;4:44–57.
Deng Q, Tan X, Wang H, Wang Q, Huang P, Li Y, et al. Changes in cecal morphology, cell proliferation, antioxidant enzyme, volatile fatty acids, lipopolysaccharide, and cytokines in piglets during the postweaning period. J Anim Sci 2020;98:skaa046.
Dilzer A, Park Y. Implication of conjugated linoleic acid (CLA) in human health. Crit Rev Food Sci Nutr 2012;52:488–513.
Dong S, Chen QL, Song YN, Sun Y, Wei B, Li XY, et al. Mechanisms of CCl4-induced liver fibrosis with combined transcriptomic and proteomic analysis. J Toxicol Sci 2016;41:561–72.
Douglas GM, Maffei VJ, Zaneveld JR, Yurgel SN, Brown JR, Taylor CM, et al. PICRUSt2 for prediction of metagenome functions. Nat Biotechnol 2020;38:685–8.
Duell BL, Su YC, Riesbeck K. Host-pathogen interactions of nontypeable Haemophilus influenzae: from commensal to pathogen. FEBS Lett 2016;590:3840–53.
Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 2011;27:2194–200.
Fulde M, Sommer F, Chassaing B, van Vorst K, Dupont A, Hensel M, et al. Neonatal selection by Toll-like receptor 5 influences long-term gut microbiota composition. Nature 2018;560:489–93.
Gagen EJ, Denman SE, Padmanabha J, Zadbuke S, Al Jassim R, Morrison M, et al. Functional gene analysis suggests different acetogen populations in the bovine rumen and tammar wallaby forestomach. Appl Environ Microbiol 2010;76:7785–95.
Gresse R, Chaucheyras-Durand F, Fleury MA, Van de Wiele T, Forano E, Blanquet-Diot S. Gut microbiota dysbiosis in postweaning piglets: understanding the keys to health. Trends Microbiol 2017;25:851–73.
Guengerich FP, Waterman MR, Egli M. Recent structural insights into cytochrome P450 function. Trends Pharmacol Sci 2016;37:625–40.
Han J, Shao J, Chen Q, Sun H, Guan L, Li Y, et al. Transcriptional changes in the hypothalamus, pituitary, and mammary gland underlying decreased lactation performance in mice under heat stress. Faseb J 2019;33:12588–601.
Hu J, Ma L, Nie Y, Chen J, Zheng W, Wang X, et al. A microbiota-derived bacteriocin targets the host to confer diarrhea resistance in early-weaned piglets. Cell Host Microbe 2018;24:817–32. e8.
Hulbert LE, Moisá SJ. Stress, immunity, and the management of calves. J Dairy Sci 2016;99:3199–216.
Izuddin WI, Loh TC, Samsudin AA, Foo HL, Humam AM, Shazali N. Effects of postbiotic supplementation on growth performance, ruminal fermentation and microbial profile, blood metabolite and GHR, IGF-1 and MCT-1 gene expression in post-weaning lambs. BMC Vet Res 2019;15:315.
Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity 2011;34:637–50.
Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. Nat Methods 2015;12:357–60.
Kim H, Kim B, Cho S, Kwon I, Seo J. Dietary lysophospholipids supplementation inhibited the activity of lipolytic bacteria in forage with high oil diet: an *in vitro* study. Asian-Australas J Anim Sci 2020;33:1590–8.
Koppaka V, Chen Y, Mehta G, Orlicky DJ, Thompson DC, Jester JV, et al. ALDH3A1 plays a functional role in maintenance of corneal epithelial homeostasis. PLoS One 2016;11:e0146433.
Krautkramer KA, Fan J, Bäckhed F. Gut microbial metabolites as multi-kingdom intermediates. Nat Rev Microbiol 2021;19:77–94.
Kundu P, Blacher E, Elinav E, Pettersson S. Our gut microbiome: the evolving inner self. Cell 2017;171:1481–93.
Lau SK, Teng JL, Leung KW, Li NK, Ng KH, Chau KY, et al. Bacteremia caused by Solobacterium moorei in a patient with acute proctitis and carcinoma of the cervix. J Clin Microbiol 2006;44:3031–4.
Lei Y, Tang L, Liu S, Hu S, Wu L, Liu Y, et al. Parabacteroides produces acetate to alleviate heparanase-exacerbated acute pancreatitis through reducing neutrophil infiltration. Microbiome 2021;9:115.
Li C, Wang W, Liu T, Zhang Q, Wang G, Li F, et al. Effect of early weaning on the intestinal microbiota and expression of genes related to barrier function in lambs. Front Microbiol 2018;9:1431.
Li C, Zhang Q, Wang G, Niu X, Wang W, Li F, et al. The functional development of the rumen is influenced by weaning and associated with ruminal microbiota in lambs. Anim Biotechnol 2020:1–17.
Li F, Dai L, Niu J. GPX2 silencing relieves epithelial-mesenchymal transition, invasion, and metastasis in pancreatic cancer by downregulating Wnt pathway. J Cell Physiol 2020b;235:7780–90.
Li L, Qu M, Liu C, Pan K, Xu L, OuYang K, et al. Expression of a recombinant Lentinula edodes cellobiohydrolase by Pichia pastoris and its effects on *in vitro* ruminal fermentation of agricultural straws. Int J Biol Macromol 2019;134:146–55.
Liang J, Fang W, Chang J, Zhang G, Ma W, Nabi M, et al. Long-term rumen microorganism fermentation of corn stover *in vitro* for volatile fatty acid production. Bioresour Technol 2022;358:127447.
Lim GH, Singhal R, Kachroo A, Kachroo P. Fatty acid- and lipid-mediated signaling in plant defense. Annu Rev Phytopathol 2017;55:505–36.
Lin L, Xie F, Sun D, Liu J, Zhu W, Mao S. Ruminal microbiome-host crosstalk stimulates the development of the ruminal epithelium in a lamb model. Microbiome 2019;7:83.
Liu J, Pu YY, Xie Q, Wang JK, Liu JX. Pectin induces an *in vitro* rumen microbial population shift attributed to the pectinolytic *Treponema* group. Curr Microbiol 2015;70:67–74.
Liu L, He Y, Wang K, Miao J, Zheng Z. Metagenomics approach to the intestinal microbiome structure and function in high fat diet-induced obesity in mice fed with conjugated linoleic acid (CLA). Food Funct 2020;11:9729–39.
Liu L, Sun D, Mao S, Zhu W, Liu J. Infusion of sodium butyrate promotes rumen papillae growth and enhances expression of genes related to rumen epithelial VFA uptake and metabolism in neonatal twin lambs. J Anim Sci 2019;97:909–21.
Llavanera M, Delgado-Bermúdez A, Mateo-Otero Y, Padilla L, Romeu X, Roca J, et al. Exploring seminal plasma GSTM3 as a quality and *in vivo* fertility biomarker in pigs-relationship with sperm morphology. Antioxidants (Basel) 2020;9:741.
Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014;15:550.
Ma L, Ni Y, Wang Z, Tu W, Ni L, Zhuge F, et al. Spermidine improves gut barrier integrity and gut microbiota function in diet-induced obese mice. Gut Microb 2020;12:1–19.
Marafini I, Sedda S, Dinallo V, Monteleone G. Inflammatory cytokines: from discoveries to therapies in IBD. Expet Opin Biol Ther 2019;19:1207–17.
Marcobal A, Barboza M, Sonnenburg ED, Pudlo N, Martens EC, Desai P, et al. Bacteroides in the infant gut consume milk oligosaccharides via mucus-utilization pathways. Cell Host Microbe 2011;10:507–14.
Marques TM, Wall R, O'Sullivan O, Fitzgerald GF, Shanahan F, Quigley EM, et al. Dietary *trans*-10, *cis*-12-conjugated linoleic acid alters fatty acid metabolism and microbiota composition in mice. Br J Nutr 2015;113:728–38.

Meng Q, Luo Z, Cao C, Sun S, Ma Q, Li Z, et al. Weaning alters intestinal gene expression involved in nutrient metabolism by shaping gut microbiota in pigs. Front Microbiol 2020;11.
Michaud V, Tran M, Pronovost B, Bouchard P, Bilodeau S, Alain K, et al. Impact of GSTA1 polymorphisms on busulfan oral clearance in adult patients undergoing hematopoietic stem cell transplantation. Pharmaceutics 2019;11:440.
Minutolo A, Potestà M, Roglia V, Cirilli M, Iacovelli F, Cerva C. Plant microRNAs from *Moringa oleifera* regulate immune response and HIV infection. Front Pharmacol 2021;11:620038.
Oeckinghaus A, Hayden MS, Ghosh S. Crosstalk in NF-κB signaling pathways. Nat Immunol 2011;12:695–708.
Palma-Hidalgo JM, Jiménez E, Popova M, Morgavi DP, Martín-García AI, Yáñez-Ruiz DR, et al. Inoculation with rumen fluid in early life accelerates the rumen microbial development and favours the weaning process in goats. Anim Microbiome 2021;3:11.
Ramirez TA, Jourdan-Le Saux C, Joy A, Zhang J, Dai Q, Mifflin S, et al. Chronic and intermittent hypoxia differentially regulate left ventricular inflammatory and extracellular matrix responses. Hypertens Res 2012;35:811–8.
Rey M, Enjalbert F, Combes S, Cauquil L, Bouchez O, Monteils V. Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. J Appl Microbiol 2014;116:245–57.
Segata N, Izard J, Waldron L, Biology DGJG. Metagenomic biomarker discovery and explanation. Genome Biol 2011;12:R60.
Segura M, Fittipaldi N, Calzas C, Gottschalk M. Critical Streptococcus suis virulence factors: are they all really critical? Trends Microbiol 2017;25:585–99.
Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 2003;13:2498–504.
Sokol CL, Luster AD. The chemokine system in innate immunity. Cold Spring Harbor Perspect Biol 2015;7:a016303.
Souza FDS, Freitas NL, Gomes YCP, Torres RC, Echevarria-Lima J, da Silva-Filho IL, et al. Following the clues: usefulness of biomarkers of neuroinflammation and neurodegeneration in the investigation of HTLV-1-associated myelopathy progression. Front Immunol 2021;12:737941.
Sonnweber T, Pizzini A, Nairz M, Weiss G, Tancevski I. Arachidonic acid metabolites in cardiovascular and metabolic diseases. Int J Mol Sci 2018;19:3285.
Sugihara K, Morhardt TL, Kamada N. The role of dietary nutrients in inflammatory bowel disease. Front Immunol 2018;9:3183.
Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci 1991;74:3583–97.
Wang L, Zhang G, Xu H, Xin H, Zhang Y. Metagenomic analyses of microbial and carbohydrate-active enzymes in the rumen of Holstein cows fed different forage-to-concentrate ratios. Front Microbiol 2019;10:649.
Wood KM, Palmer SI, Steele MA, Metcalf JA, Penner GB. The influence of age and weaning on permeability of the gastrointestinal tract in Holstein bull calves. J Dairy Sci 2015;98:7226–37.
Xiao S, Liu C, Chen M, Zou J, Zhang Z, Cui X, et al. Scutellariae radix and coptidis rhizoma ameliorate glycolipid metabolism of type 2 diabetic rats by modulating gut microbiota and its metabolites. Appl Microbiol Biotechnol 2020;104:303–17.
Xiao Y, Meierhofer D. Glutathione metabolism in renal cell carcinoma progression and implications for therapies. Int J Mol Sci 2019;20:3672.
Xie C, Mao X, Huang J, Ding Y, Wu J, Dong S, et al. KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. Nucleic Acids Res 2011;39:W316–22.
Yang C, Zhu B, Ye S, Fu Z, Li J. Isomer-specific effects of *cis*-9,*trans*-11- and *trans*-10,*cis*-12-CLA on immune regulation in ruminal epithelial cells. Animals (Basel) 2021;11:1169.
Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. Nature 2012;486:222–7.
Yuan G, Chen X, Li D. Modulation of peroxisome proliferator-activated receptor gamma (PPARγ) by conjugated fatty acid in obesity and inflammatory bowel disease. J Agric Food Chem 2015;63:1883–95.
Zeng H, Guo C, Sun D, Seddik HE, Mao S. The ruminal microbiome and metabolome alterations associated with diet-induced milk fat depression in dairy cows. Metabolites 2019;9:154.