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Original Research Article

# Proteomic identification of ruminal epithelial protein expression profiles in response to starter feed supplementation in pre-weaned lambs

Daming Sun [a, b, c], Shengyong Mao [a, b, c], Weiyun Zhu [a, b, c], Junhua Liu [a, b, c, *]

[a] Jiangsu Key Laboratory of Gastrointestinal Nutrition and Animal Health, Laboratory of Gastrointestinal Microbiology, College of Animal Science and Technology, Nanjing Agricultural University, Nanjing 210095, Jiangsu Province, China
[b] National Center for International Research on Animal Gut Nutrition, Nanjing Agricultural University, Nanjing 210095, China
[c] National Experimental Teaching Demonstration Center of Animal Science, Nanjing Agricultural University, Nanjing 210095, China

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ABSTRACT

The present study aimed to comparatively characterize the ruminal epithelial protein expression profiles in lambs fed ewe milk or milk plus starter diet using proteome analysis. Twenty new-born lambs were randomly divided into a group receiving ewe milk (M, $n = 10$) and a group receiving milk plus starter diet (M + S, $n = 10$). From 10 d old, M group lambs remained with the ewe and suckled ewe milk without receiving the starter diet. The lambs in the M + S group were separated from the ewe and received starter feed. All lambs were slaughtered at 56 d old. Eight rumen epithelia samples (4 per group) were collected to characterize their protein expression profiles using proteomic technology. Proteome analysis showed that 31 upregulated proteins and 40 downregulated proteins were identified in the rumen epithelium of lambs in response to starter diet supplementation. The results showed that starter feeding regulates a variety of biological processes in the epithelium, especially blood vessel development and extracellular matrix protein expression. Meanwhile, the expression of proteins associated with synthesis and degradation of ketone bodies, butanoate metabolism, and citrate cycle signaling transduction pathway were upregulated in the group with starter diet supplementation, including 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMGCS2, fold change [FC] = 1.93), 3-hydroxybutyrate dehydrogenase 1 (BDH1, FC = 1.91), and isocitrate dehydrogenase 1 (IDH1, FC = 8.12). The metabolic processes associated with ammonia detoxification and antioxidant stress were also affected by starter diet supplementation, with proteins, microsomal glutathione S-transferase 3 (MGST3, FC = 2.37) and IDH1, linked to the biosynthesis of glutamate and glutathione metabolism pathway being upregulated in the group with starter diet supplementation. In addition, starter feeding decreased the expression of Ras-related protein rap-1A (RAP1A, FC = 0.48) enriched in Rap1 signaling pathway, Ras signaling pathway, cyclic adenosine monophosphate (cAMP) signaling pathway, and mitogen-activated protein kinase (MAPK) signaling pathway. In summary, starter feed supplementation changed the expression of proteins related to energy production, ammonia detoxification, antioxidant stress, and signaling pathways related to proliferation and apoptosis, which facilitates the rumen epithelia development in lambs. The results provide new insights into the molecular adaptation of rumen epithelia in response to starter diet supplementation at the protein level in lambs.



* Corresponding author.
*E-mail address:* liujunhua0011@163.com (J. Liu).
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## 1. Introduction

The pre-weaning stage of the young ruminants' life is the key period for the development of the ruminal epithelial function, which could affect animal health and performance in adults (Khan et al., 2007; Chai et al., 2015). Ruminal epithelium needs to adapt to

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solid feed, a process by which molecular adaption includes absorption, transportation, pH regulation, and immune function (Steele et al., 2016). Therefore, implementing nutritional interventions to improve gastrointestinal adaptability at an early age is important for reducing weaning stress. One commonly used nutritional intervention is supplementation of a concentrate starter with breast milk feeding in ruminants, which could enhance gastrointestinal fermentation and promote overall gastrointestinal development (Jiao et al., 2015; Wang et al., 2016). Laarman et al. (2012) found that feeding a calf starter affected the expression of genes involved in epithelial intracellular pH regulation and butyrate metabolism in ruminal epithelium using quantitative real-time PCR (qRT-PCR) technology. Our recent study also demonstrated that starter feeding promoted ruminal epithelial morphological development and altered expression of genes related to cell cycle and metabolism in lambs (Sun et al., 2018). However, there was no study that systematically and completely investigated the molecular adaptation of rumen epithelial protein in response to starter diet supplementation using proteomic technology.

Proteome analysis is able to detect all expressed proteins in cells, tissue, or an organism that is in the specific physical or pathological course. Proteomics is a subject that aims to analyze the constituent parts, expressed situation and modification of all proteins, and realize the reaction and change rule among proteins (Azad et al., 2006). Although proteome analysis has been widely used in the field of bioscience, the application of proteomics in animal science is still in a start-up phase. The main directions of research are in the production traits (Eckersall et al., 2012) and epidemic diseases (Turk et al., 2012; Piras et al., 2015). The use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the present technology is one of the important tools in the separation and analysis of proteome. Liquid chromatogram (LC) can separate different proteins based on its different partition coefficient between the mobile phase and stationary phase. It separates and authenticates mixed proteins and then quantifies expressions of peptide fragments and proteins through the intensity of separated mass spectrum peak signals. Although LC makes it possible to rapidly uncover the differences in protein expression among different samples, its application in proteomics is seldom used in the analysis of rumen tissue of young ruminants.

Therefore, the objective of this study was to investigate the effect of starter feeding on the rumen epithelial morphology and molecular adaptation of rumen epithelial protein in response to starter diet supplementation using proteome technology.

## 2. Materials and methods

The experimental design and procedures were approved by the Animal Care and Use Committee of Nanjing Agricultural University, in compliance with the Regulations for the Administration of Affairs Concerning Experimental Animals (the State Science and Technology Commission of P. R. China, 1988).

### 2.1. Experimental design

The details of the animal experimental design have been reported in a previous study (Lin et al., 2019). Briefly, 20 new-born lambs were selected in this study and randomly divided into milk group (M, $n = 10$) and milk plus starter group (M + S, $n = 10$). From 10 d old, M group lambs, without receiving starter feed, remained with the ewe and suckled ewe milk ad libitum. The M + S group lambs were separated from the ewe and were administered starter feed in an individual fold from 04:00 to 19:00 every day. During this period, lambs in the M + S group were fed with ewe's milk for 1 h at a fixed time (06:30, 10:30, and 15:30). The amount of dry matter intake of the starter feed was targeted to 200 g/d per M + S group lambs. When the dry matter intake of the lambs' starter reached 200 g/d per lamb, the amount of starter did not rise any further. The lambs in both groups received water and oat hay (28.71% crude fiber, 10.05% crude protein) ad libitum. The body weight of lambs was measured before the morning feeding at 56 d of age and the average daily gain was calculated accordingly. The nutrient composition of the starter diet was shown in Appendix Table 1.

### 2.2. Sample collection

The lambs in the 2 groups were slaughtered at 56 d of age. According to the animal protection law of China, lambs were terminated by way of exsanguination and captive bolt. After slaughter, at least 10 mL of representative rumen liquid from each lamb was immediately collected and stored at −20 °C for the analysis of ammonia nitrogen ($NH_3$−N) level according to the method of Weatherburn (1967) and the results were presented in Appendix Fig. 1. Within 5 min of slaughter, the rumen epithelial tissue was collected from the ventral sac of the rumen as described previously (Sun et al., 2018; Lin et al., 2019). One portion of the rumen epithelia samples were cut to approximately 1.0 × 1.0 cm and fixed in 4% paraformaldehyde (Sigma, MO, USA) under the preparation of paraffin section. Another portion of the rumen epithelia samples were cut to approximately 0.5 cm × 0.5 cm and directly dipped into liquid nitrogen for protein extraction.

### 2.3. Histological measurements

The rumen epithelia sample was embedded with paraffin, then sliced (6 μm) and stained by eosin and haematoxylin. One slide was prepared for each lamb, with each slide capturing 2 images and each group measuring a total of 20 replicates per measurement. Using a 40 × objective lens, the thickness of stratum basale and stratum spinosum (SB + SS), stratum granulosum (SG), and corneum (SC) of ruminal epithelia were measured as described previously (Malhi et al., 2013). During the analysis of rumen epithelia histomorphometry, the microscopist was blind to the treatment group.

### 2.4. Sample preparation and mass spectrometry

Four rumen epithelia samples per group were selected to conduct proteome analysis. The total proteins in rumen epithelia samples was extracted with radio immunoprecipitation assay lysis buffer (Beyotime, Shanghai, China). Precipitated proteins were prepared as described previously (Guo et al., 2019). Briefly, proteins were dissolved in 50 mmol/L Tris−HCl (pH 8.0) with 8 mol/L urea and incubated for 60 min in 60 °C and alkylated with 1 mol/L iodoacetamide. Then, the samples were incubated at room temperature for 45 min. Finally, proteins on the membrane were dissolved in 50 mmol/L $NH_4HCO_3$ (pH 7.8). The digested protein by trypsin was desalted using a C18 column and then freeze-dried before sample analysis.

The liquid chromatography-mass spectrometry (LC-MS) system was used for the mass spectrometry analysis according to the method described by Yu et al. (2016). In short, the peptides were first dissolved in buffer A (0.1% formic acid). A 15-cm analytical column (C18, 3 μm, 100 A) was applied in LC separation. The elution of peptides used a 2% to 95% gradient of buffer B (aqueous 80% acetonitrile in 0.08% formic acid) with a total run time of 2 h. The peptides were ionized by nano-electrospray and subsequent tandem mass spectrometry (MS/MS) on a Q Exactive Plus (Thermo, CA, USA) was performed with the electrospray voltage set to 2.2 kV and the capillary temperature set to 240 °C. The Orbitrap was

performed with a full scan MS spectra (m/z from 350 to 1,800) with a resolution of 60,000.

### 2.5. Data analysis and bioinformatic analysis

The original data was analyzed by Proteome Discoverer (Version 1.4, Thermo Fisher Scientific, Waltham, MA, United States). Based on the *Q*-value, we verified the results of protein identification to ensure that the error detection rate was less than 1%. The SIEVE software (Version 2.1 Thermo Scientific, San Jose, CA, United States) was used to analyze 2 original files for each group by ChromAlign. When alignment scores aligned by retention time and mass was higher than 0.75, it was regarded as a further quantitative analysis. The area under the curve for each group was calculated. Identification of proteins was performed using Sequest HT engine against the UniprotKB *Ovis aries* database. In the present study, a multi-omics data analysis tool, OmicsBean (http://www.omicsbean.cn/), was used for the kyoto encyclopedia of genes and genomes (KEGG) pathway, the principal components analysis (PCA), and hierarchical clustering analysis of whole protein expressed. The gene ontology (GO) enrichment of differentially expressed proteins (DEP) was performed using DAVID (Version 6.8) (Huang et al., 2009). The protein–protein interactions (PPI) network analysis of DEP was performed using STRING database version 11.0 (Szklarczyk et al., 2019) and visualized by Cytoscape (Version 3.7.2) (Shannon et al., 2003). The proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD025197.

### 2.6. Statistical analysis

Independent sample *t*-test in SPSS software packages (Version 20.0.1; SPSS Inc., Chicago, IL, USA) was used to assess statistical significance. $P < 0.05$ was considered as a significant difference between 2 groups. The DEP were selected based on the criteria false discovery rate (FDR) < 0.05 (Benjamini-Hochberg) and fold change (FC) [(M + S)/M] > 1.8 (upregulated) or < 0.56 (downregulated).

## 3. Results

### 3.1. Animal performance, rumen fermentation, and morphology of rumen papillae

There was no significant difference in final body weight at 56 d of age (10.68 ± 0.80 vs. 10.71 ± 0.56 kg, $P = 0.976$) and average daily gain (0.18 ± 0.02 vs. 0.19 ± 0.01 kg, $P = 0.687$) between the 2 groups. In the current study, starter diet supplementation significantly ($P < 0.05$) increased the length, width, and surface area of rumen papillae, but did not affect the density of the rumen papillae ($P = 0.406$) (Appendix Table 2) (Lin et al., 2019). As shown in Appendix Fig. 1, starter diet supplementation increased the concentration of $NH_3$–N in the rumen of lambs. As shown in Table 1 and Fig. 1, starter diet supplementation significantly ($P < 0.05$) increased the thickness of SC, SG and total epithelia of rumen epithelia, but did not significantly ($P = 0.195$) change the thickness of the SS + SB.

### 3.2. Kinds and number of differently expressed proteins

In the present study, a total of 1,186 proteins were identified and 71 kinds of DEP were found in the rumen epithelium between the 2 groups after analysis and identification (Fig. 2A, Table 2). The volcano map presented a clear visual of the relationship between the FDR and FC for all proteins, enabling us to view expression levels quickly (Fig. 2A). A total of 31 proteins were upregulated, and 40 proteins were downregulated. We performed Principal Component Analysis (PCA) across the whole protein expression on each sample. The x-axis represents principal component 1 (PC1), with 67.27% variation; y-axis represents principal component 2 (PC2), 11.63% variation. PCA plots showed an obvious separation between 2 groups (Fig. 2B). The results of hierarchical clustering of the global protein expression data showed clusters of proteins with different pattern of expression between the 2 groups, and the distance between the 2 conditions demonstrated major changes induced by the starter feeding (Fig. 2C). As shown in Table 2, the most upregulated proteins in the M + S lambs included phosphoprotein 1, isocitrate dehydrogenase 1 (IDH1), FUN14 domain containing 2, signal sequence receptor 4, zonadhesin, and methylcrotonoyl-CoA carboxylase 1 (MCCC1). The most downregulated proteins in the M + S lambs included heat shock protein family B member 6, destrin, actin depolymerizing factor, cofilin 2 (CFL2), integrin subunit alpha 5 (ITGA5), and transgelin.

**Table 1**
Effects of starter feeding on the thickness of different stratum of rumen epithelium in lambs ($n = 10$).

| Item | M | M + S | *P*-value |
|---|---|---|---|
| Total epithelia, μm | 91.77 ± 1.86 | 110.06 ± 2.80 | <0.001 |
| Stratum corneum, μm | 13.73 ± 0.83 | 22.81 ± 0.67 | <0.001 |
| Stratum granulosum, μm | 17.00 ± 0.59 | 21.70 ± 0.66 | <0.001 |
| Stratum spinosum and basale, μm | 61.04 ± 1.96 | 65.55 ± 2.85 | 0.195 |

M: milk group; M + S: milk plus starter group.

### 3.3. Gene ontology analysis of differently expressed proteins

To better understand the function of 71 DEP above, GO analysis was used to do further analysis. In general, 57 terms were annotated with the biological process (BP), and 31 terms were significantly enriched ($P < 0.05$); 28 terms were annotated with cellular component (CC), and 27 terms were significantly enriched ($P < 0.05$); 7 terms were annotated with molecular function (MF), including 3 terms that were significantly enriched ($P < 0.05$) (Appendix Fig. 2).

As shown in Fig. 3, results of BP analysis indicated that 13.43% of the DEP were related to circulatory system development ($P = 0.003$) and cardiovascular system development ($P = 0.003$), 10.45% to vasculature development ($P = 0.005$), 8.96% to blood vessel development ($P = 0.016$), 7.46% to blood vessel morphogenesis ($P = 0.033$), and 5.97% to regulation of vasculature development ($P = 0.021$), which was associated with the development of ruminal blood vessels. These BP contained 3 upregulated proteins, including chloride intracellular channel protein (CLIC4, FC = 2.62), cystathionine gamma-lyase (CTH, FC = 2.13), and methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formyltetrahydrofolate synthetase 1 (MTHFD1, FC = 1.89), and 6 downregulated proteins, including ITGA5 (FC = 0.25), collagen type I alpha 1 chain (COL1A1, FC = 0.41), transforming growth factor beta induced (TGFBI, FC = 0.43), PDZ and LIM domain 3 (PDLIM3, FC = 0.41), Ras-related protein Rap-1A (RAP1A, FC = 0.48), and Ras-related protein R-Ras (RRAS, FC = 0.53). Results of CC analysis showed that 44.78% of the proteins were located on the extracellular region part ($P < 0.001$) and extracellular region ($P < 0.001$), 19.40% to cell-substrate adherens junction ($P < 0.001$), focal adhesion ($P < 0.001$), cell-substrate junction ($P < 0.001$) and adherens junction ($P < 0.001$). The MF of these DEP included cofactor binding ($P = 0.003$), structural molecule activity ($P = 0.012$), and primary amine oxidase activity ($P = 0.019$). The DEP contained in each GO terms are shown in Appendix Table 3.

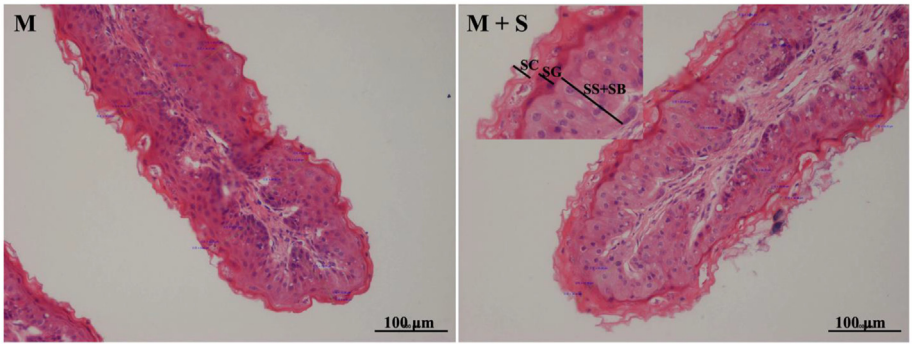


**Fig. 1.** Representative ruminal epithelial micrograph of lambs. The thickness of SC, SG, and total epithelia of rumen epithelia were higher in M + S lambs than that in M lambs. M: milk group; M + S: milk plus starter group. SC = stratum corneum; SG = stratum granulosum; SS = stratum spinosum; SB = stratum basale.

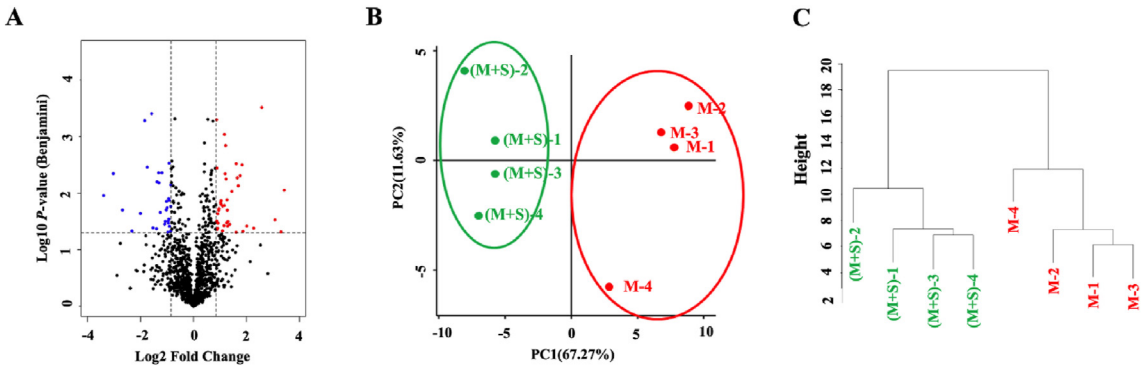


**Fig. 2.** Global proteins expression pattern of lambs. (A) Volcano map showing the $\log_{10}$ (false discovery rate) and $\log_2$ (fold change) of proteins, the red dot represents the upregulated protein, the blue dot represents the downregulated protein, the black dot indicates no significant change in protein expression. (B) Principal component analysis (PCA) plot of each sample on whole proteome. (C) Hierarchical clustering of each sample on whole proteome. M: milk group; M + S: milk plus starter group.

### 3.4. Kyoto encyclopedia of genes and genomes analysis of differentially expressed proteins

The KEGG pathway and DEP contained in each KEGG pathway are shown in Fig. 4 and Table 3. The KEGG pathway was mainly divided into 6 categories: metabolism (butanoate metabolism, synthesis and degradation of ketone bodies, metabolic pathways, glutathione metabolism, metabolism of xenobiotics by cytochrome P450, sulfur metabolism, biosynthesis of amino acids, one carbon pool by folate, 2-oxocarboxylic acid metabolism, selenocompound metabolism, terpenoid backbone biosynthesis, citrate cycle, glycine, serine and threonine metabolism, valine, leucine and isoleucine degradation, cysteine and methionine metabolism, drug metabolism - cytochrome P450, arachidonic acid metabolism, carbon metabolism, and oxidative phosphorylation), environmental information processing (Rap1 signaling pathway, Ras signaling pathway, cAMP signaling pathway, and MAPK signaling pathway), organismal systems (Long-term potentiation, pancreatic secretion, neurotrophin signaling pathway, leukocyte transendothelial migration, platelet activation, and chemokine signaling pathway), cellular processes (focal adhesion, and peroxisome), genetic information processing (protein processing in endoplasmic reticulum), and human diseases (chemical carcinogenesis, renal cell carcinoma, and dilated cardiomyopathy).

### 3.5. PPI analysis

In most cases, certain biological processes and pathways can share the same group of genes, implicating the potential interlinkage between them. The PPI of the DEP allows us to better understand the key proteins affected by starter feeding in the rumen epithelium (Fig. 5). The PPI network indicated that 24 DEP have various relationships in PPI and were recognized as key nodes: acetyl-coenzyme A synthetase (ACSS1), adenylate kinase isoenzyme 1, a-kinase anchoring protein 12, serum albumin (ALB), 3-hydroxybutyrate dehydrogenase 1 (BDH1), caveolae associated protein 1, CD44 molecule (CD44), CFL2, COL1A1, CTH, 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMGCS2), IDH1, ITGA5, lanC like 1, PDLIM3, PDLIM7, phosphoglucomutase 5, periostin, ribosomal protein S19, 40S ribosomal protein S7 (RPS7), pyr_redox_2 domain-containing protein, stomatin, synaptopodin 2 (SYNPO2), and zyxin (ZYX), especially COL1A1, PDLIM3, ALB, and CD44. Most of DEP were associated with blood vessel development and extracellular matrix, including COL1A1, PDLIM3, ALB, CD44, ITGA5, and so on. Many of DEP were associated with metabolism pathways, including HMGCS2, BDH1, CTH, and IDH1. These were in accordance with the results of pathway and GO analysis.

## 4. Discussion

### 4.1. Rumen fermentation and epithelial morphology

As reported in our previous publication with the same animal experiment, starter diet supplementation increased the concentrations of total volatile fatty acid (VFA), acetate, butyrate (Lin et al., 2019), and $NH_3$–N in the rumen. These results indicated that starter feeding increased the rumen fermentation of substrates, especially carbohydrates. Previous study has indicated that the $NH_3$–N concentration in ruminal fluid was significantly higher in lambs fed with higher crude protein diets (Yang et al., 2016).

**Table 2**
The differentially expressed proteins in the ruminal epithelia of lambs in the milk plus starter group compared with milk group lambs.

| Uniprot | Protein name | Gene name | Molecular weight, kDa | Theoretical isoelectric point (pI) | Unique peptides number | Sequence coverage, % | Mascot score | Fold change | Up or down regulation[1] |
|---|---|---|---|---|---|---|---|---|---|
| W5P359 | Stress induced phosphoprotein 1 | *STIP1* | 68.16 | 4.03 | 4 | 9.00 | 33.12 | 10.48 | Up |
| Q6XUZ5 | Isocitrate dehydrogenase [NADP] cytoplasmic | *IDH1* | 46.78 | 4.03 | 1 | 34.30 | 81.15 | 8.12 | Up |
| W5NZS7 | FUN14 domain containing 2 | *FUNDC2* | 20.52 | 4.14 | 1 | 5.80 | 8.53 | 6.40 | Up |
| W5P940 | Signal sequence receptor subunit 4 | *SSR4* | 18.89 | 4.14 | 1 | 6.40 | 19.61 | 5.00 | Up |
| W5Q358 | Zonadhesin | *ZAN* | 274.40 | 6.11 | 1 | 0.60 | 7.97 | 4.00 | Up |
| W5PW57 | Uncharacterized protein | *LOC101114663* | 30.43 | 6.05 | 2 | 39.40 | 187.87 | 3.57 | Up |
| W5QHK3 | Methylcrotonoyl-CoA carboxylase 1 | *MCCC1* | 77.35 | 4.37 | 2 | 4.20 | 13.82 | 3.34 | Up |
| W5NVR9 | Chromosome 11 open reading frame 54 | *C11orf54* | 35.19 | 4.31 | 3 | 14.60 | 21.15 | 2.99 | Up |
| W5QIR6 | Glycine amidinotransferase | *GATM* | 53.20 | 4.37 | 6 | 17.30 | 63.56 | 2.89 | Up |
| W5P8X9 | Chloride intracellular channel protein | *CLIC4* | 28.71 | 4.02 | 2 | 13.40 | 20.65 | 2.62 | Up |
| W5QBV7 | CD44 molecule | *CD44* | 76.41 | 6.74 | 1 | 1.70 | 7.76 | 2.58 | Up |
| W5PR16 | Abhydrolase domain containing 11 | *ABHD11* | 34.59 | 6.75 | 3 | 12.70 | 20.10 | 2.48 | Up |
| W5PUG3 | Uncharacterized protein | | 8.52 | 5.83 | 1 | 13.50 | 22.28 | 2.46 | Up |
| W5PPE2 | Microsomal glutathione S-transferase 3 | *MGST3* | 16.86 | 9.75 | 3 | 31.60 | 25.61 | 2.37 | Up |
| W5P1M4 | Uncharacterized protein | | 41.67 | 6.92 | 13 | 60.10 | 323.31 | 2.28 | Up |
| W5PQY2 | Cystathionine gamma-lyase | *CTH* | 44.44 | 6.00 | 2 | 5.90 | 15.27 | 2.13 | Up |
| W5PQG6 | Inositol-3-phosphate synthase 1 | *ISYNA1* | 55.65 | 6.43 | 4 | 12.50 | 71.56 | 2.09 | Up |
| W5QJ02 | Dehydrogenase/reductase 7 | *DHRS7* | 36.85 | 6.76 | 10 | 34.70 | 186.12 | 2.03 | Up |
| W5PSF3 | Diazepam binding inhibitor, acyl-CoA binding protein | *DBI* | 11.60 | 5.41 | 3 | 41.60 | 22.23 | 2.02 | Up |
| W5QD23 | LanC like 1 | *LANCL1* | 45.36 | 5.32 | 2 | 7.30 | 11.45 | 1.99 | Up |
| W5PEV6 | Ribosomal protein S19 | *RPS19* | 17.39 | 4.14 | 1 | 8.30 | 8.69 | 1.95 | Up |
| W5QGT9 | 3-hydroxy-3-methylglutaryl coenzyme A synthase | *HMGCS2* | 56.94 | 4.43 | 13 | 39.60 | 280.41 | 1.93 | Up |
| W5QEI1 | RRM domain-containing protein | *HNRNPC* | 33.37 | 8.72 | 2 | 7.60 | 15.35 | 1.93 | Up |
| W5QGP1 | 3-hydroxybutyrate dehydrogenase 1 | *BDH1* | 38.35 | 4.53 | 6 | 24.70 | 155.80 | 1.91 | Up |
| C5IWU3 | ADP-ribosylation factor 3 | *ARF3* | 20.60 | 5.84 | 2 | 39.20 | 91.48 | 1.91 | Up |
| W5QJ32 | Methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formyltetrahydrofolate synthetase 1 | *MTHFD1* | 101.27 | 4.32 | 15 | 24.20 | 205.66 | 1.89 | Up |
| W5QIQ6 | Pyr_redox_2 domain-containing protein | *SQRDL* | 49.94 | 8.75 | 14 | 41.30 | 309.13 | 1.89 | Up |
| W5PHF2 | Acyl-CoA dehydrogenase short/branched chain | *ACADSB* | 47.50 | 6.00 | 3 | 11.30 | 128.88 | 1.87 | Up |
| W5NYL0 | Amine oxidase | *MAOA* | 59.80 | 4.54 | 4 | 15.90 | 79.93 | 1.81 | Up |
| W5PRA0 | 3′(2′), 5′-bisphosphate nucleotidase 1 | *BPNT1* | 33.47 | 4.41 | 3 | 14.60 | 34.44 | 1.81 | Up |
| W5P5T8 | 40S ribosomal protein S7 | *RPS7* | 19.89 | 4.31 | 2 | 19.00 | 16.10 | 1.81 | Up |
| W5PEC3 | Annexin | *ANXA6* | 84.21 | 6.05 | 9 | 13.40 | 134.11 | 0.55 | Down |
| W5QF76 | Cannabinoid receptor interacting protein 1 | *CNRIP1* | 18.74 | 4.37 | 1 | 9.70 | 12.77 | 0.55 | Down |
| W5PY34 | Dimethylarginine dimethylaminohydrolase 1 | *DDAH1* | 36.71 | 6.05 | 2 | 8.30 | 35.82 | 0.54 | Down |
| W5NUT0 | Caveolae associated protein 1 | *CAVIN1* | 33.25 | 3.90 | 4 | 15.70 | 60.01 | 0.54 | Down |
| W5PWZ2 | Galectin | *LGALS1* | 11.97 | 5.32 | 2 | 28.70 | 47.27 | 0.54 | Down |
| W5P601 | Stomatin | *STOM* | 30.96 | 4.37 | 2 | 11.10 | 40.33 | 0.53 | Down |
| W5PK03 | Periostin | *POSTN* | 90.14 | 5.66 | 3 | 27.40 | 197.93 | 0.53 | Down |
| W5PUU2 | RAS related | *RRAS* | 23.09 | 4.21 | 5 | 28.50 | 38.61 | 0.53 | Down |
| B2LU28 | TPM1 | | 32.69 | 4.00 | 3 | 14.10 | 27.73 | 0.52 | Down |
| W5PH02 | Cation_ATPase_N domain-containing protein | | 26.81 | 4.14 | 1 | 4.90 | 7.00 | 0.51 | Down |
| W5Q8Q6 | KN motif and ankyrin repeat domains 2 | *KANK2* | 92.05 | 6.00 | 3 | 5.90 | 18.64 | 0.49 | Down |
| W5PG95 | Heat shock 70 kDa protein 1A/1B | *HSPA1A* | 70.40 | 4.37 | 4 | 23.20 | 186.93 | 0.49 | Down |
| W5QED8 | Ras-related protein Rap-1A | *RAP1A* | 20.99 | 4.37 | 2 | 14.70 | 20.91 | 0.48 | Down |
| W5NYR6 | A-kinase anchoring protein 12 | *AKAP12* | 199.04 | 4.49 | 4 | 5.10 | 35.11 | 0.47 | Down |
| B0LRM7 | 60S ribosomal protein L6 | | 20.37 | 9.75 | 3 | 24.40 | 45.56 | 0.47 | Down |
| W5PP64 | Uncharacterized protein | *FHL1* | 33.47 | 8.75 | 5 | 23.60 | 178.96 | 0.45 | Down |
| W5P1J8 | Amine oxidase | *LOC101113086* | 85.25 | 5.88 | 4 | 5.50 | 76.39 | 0.45 | Down |
| W5NUA0 | Proline and arginine rich end leucine rich repeat protein | *PRELP* | 43.65 | 4.53 | 1 | 3.10 | 9.34 | 0.44 | Down |
| W5PC25 | Uncharacterized protein | *LOC101116273* | 27.59 | 6.00 | 1 | 18.30 | 18.88 | 0.44 | Down |
| C5IJA8 | Adenylate kinase isoenzyme 1 | *AK1* | 21.65 | 6.74 | 3 | 18.60 | 18.10 | 0.43 | Down |
| W5Q0F3 | Transforming growth factor beta induced | *TGFBI* | 70.36 | 6.74 | 7 | 19.10 | 54.23 | 0.43 | Down |
| W5P5S7 | PDZ and LIM domain 7 | *PDLIM7* | 63.37 | 5.84 | 4 | 7.70 | 28.85 | 0.42 | Down |

(*continued on next page*)

**Table 2** (*continued*)

| Uniprot | Protein name | Gene name | Molecular weight, kDa | Theoretical isoelectric point (pI) | Unique peptides number | Sequence coverage, % | Mascot score | Fold change | Up or down regulation[1] |
|---|---|---|---|---|---|---|---|---|---|
| W5P481 | Collagen type I alpha 1 chain | *COL1A1* | 140.64 | 4.03 | 2 | 2.30 | 38.83 | 0.41 | Down |
| W5QH03 | LIM domain containing preferred translocation partner in lipoma | *LPP* | 65.67 | 5.50 | 12 | 32.60 | 162.17 | 0.41 | Down |
| W5PDJ3 | PDZ and LIM domain 3 | *PDLIM3* | 39.35 | 5.32 | 4 | 16.50 | 63.17 | 0.41 | Down |
| W5PUA9 | Phosphoglucomutase 5 | *PGM5* | 62.15 | 3.84 | 11 | 24.70 | 161.87 | 0.41 | Down |
| W5QC29 | Desmin | *DES* | 53.53 | 9.41 | 12 | 39.60 | 323.31 | 0.39 | Down |
| W5P3H8 | Insulin like growth factor 2 receptor | *IGF2R* | 271.69 | 4.21 | 2 | 1.00 | 11.83 | 0.37 | Down |
| W5Q5Y7 | Synaptopodin 2 | *SYNPO2* | 134.84 | 4.53 | 12 | 14.90 | 256.45 | 0.33 | Down |
| W5NT24 | Uncharacterized protein | | 445.58 | 4.21 | 8 | 4.90 | 83.24 | 0.33 | Down |
| P14639 | Serum albumin | *ALB* | 69.19 | 4.10 | 2 | 54.50 | 323.31 | 0.32 | Down |
| W5Q9H1 | Zyxin | *ZYX* | 58.03 | 11.00 | 3 | 12.10 | 47.64 | 0.31 | Down |
| W5P9I9 | Acetyl-coenzyme A synthetase | *ACSS1* | 64.06 | 4.53 | 3 | 5.70 | 16.79 | 0.30 | Down |
| W5P214 | Transgelin | *TAGLN* | 25.04 | 4.03 | 11 | 51.60 | 223.54 | 0.28 | Down |
| W5QEA9 | ATP synthase F(0) complex subunit B1 | *ATP5F1* | 28.73 | 6.74 | 3 | 12.50 | 30.77 | 0.27 | Down |
| W5Q3T7 | Integrin subunit alpha 5 | *ITGA5* | 116.64 | 3.98 | 3 | 4.00 | 26.14 | 0.25 | Down |
| W5PA65 | Cofilin 2 | *CFL2* | 18.61 | 6.00 | 2 | 17.00 | 19.88 | 0.21 | Down |
| W5NVV0 | Uncharacterized protein | | 26.20 | 4.56 | 2 | 9.90 | 12.37 | 0.17 | Down |
| W5Q831 | Destrin, actin depolymerizing factor | *DSTN* | 20.19 | 4.37 | 3 | 20.10 | 19.14 | 0.10 | Down |
| W5P4P2 | Heat shock protein family B (small) member 6 | *HSPB6* | 17.48 | 5.21 | 3 | 29.30 | 24.94 | 0.09 | Down |

[1] The differentially expressed proteins (DEP) were selected using the criteria false discovery rate < 0.05 and fold change [(M + S)/M] > 1.8 (upregulated) or < 0.56 (downregulated).

Therefore, in the present research, the increase in $NH_3$−N concentration in the rumen of the lamb may be attributed to the high protein content in the starter diet. Our research revealed an increase in the thickness of the SC, SG, and total epithelia of rumen epithelium as a result of starter feeding. This may be contributed to an enlarged rumen surface area and nutrition absorption. Previous studies indicated that VFA is a vital factor promoting the development of rumen epithelium, especially butyrate (Baldwin et al., 2004; Malhi et al., 2013). Thus, the VFA produced by starter diet fermentation is the main factor promoting the development of rumen epithelium.

### 4.2. Proteome analysis

In our study, we identified the DEP and signaling pathways relevant to the starter feeding by LC-MS/MS. A total of 71 proteins and multiple signaling pathways were significantly altered by starter feeding. Nutrients are modulators of gene and protein expression and affect the biological processes and cellular responses (Bionaz and Loor, 2012; Zeisel, 2012). Therefore, we performed GO enrichment and KEGG analysis on DEP to analyze the effect of supplementary feeding on the rumen physiological function of lambs.

### 4.3. Blood vessel development and extracellular matrix

In the present study, 7 of 31 BP terms were associated with the development of blood vessels, including circulatory system development, cardiovascular system development, vasculature development, positive regulation of vasculature development, blood vessel development, blood vessel morphogenesis, and regulation of vasculature development, which contains 3 upregulated proteins (CLIC4, CTH, and MTHFD1; FC = 2.62, 2.13, and 1.89, respectively) and 6 downregulated proteins (ITGA5, COL1A1, TGFBI, PDLIM3, RAP1A, and RRAS; FC = 0.25, 0.41, 0.43, 0.41, 0.48, and 0.53, respectively). The substantial capillary bed in the ruminal lamina propria plays an important role in nutrient transport from the lumen into the blood (Kay et al., 1969; Dobson, 1979). Previous study indicated that CLIC4 was required for vascular tubulogenesis in the new blood vessels formation (Suh et al., 2007). Cystathionine gamma-lyase has also been well studied in stimulating blood vessel growth by the formation of hydrogen sulfide (Szabo and Papapetropoulos, 2011). On the contrary, TGFBI suppressed angiogenesis (Lee and Nam, 2020). Moreover, previous study indicated that proteolytic degradation of the extracellular matrix is needed for the new blood vessels formation, and, interstitial collagenase seems to play a vital role in this biological process (Karelina et al., 1995). According to our findings, the COL1A1, encoding the collagen, was decreased with starter feeding. In addition, the proteins related to the extracellular matrix (PDLIM3, ITGA5, SYNPO2, and ZYX; FC = 0.41, 0.25, 0.33, and 0.31, respectively) were downregulated in the ruminal papillae of lambs in M + S group. Previous studies demonstrated that these proteins are closely related to the formation of focal adhesions, cytoskeleton and extracellular matrix (Petit et al., 2000; Wang et al., 2009; De Souza Viana et al., 2013). Therefore, starter feeding promoted the development of blood vessels, which is beneficial to the functional development of rumen epithelia related to nutrient absorption. The blood vessels are mainly located on the lamina propria of the rumen. Previous study indicated that the lamina propria projected within the elevations of the epithelial stratum basale, forming the nascent papillae in the rumen of prenatal sheep (Tanvi et al., 2016). Thus, these changes of GO terms may be related to the morphological changes of rumen papilla. The extracellular matrix is a dynamic structure that is

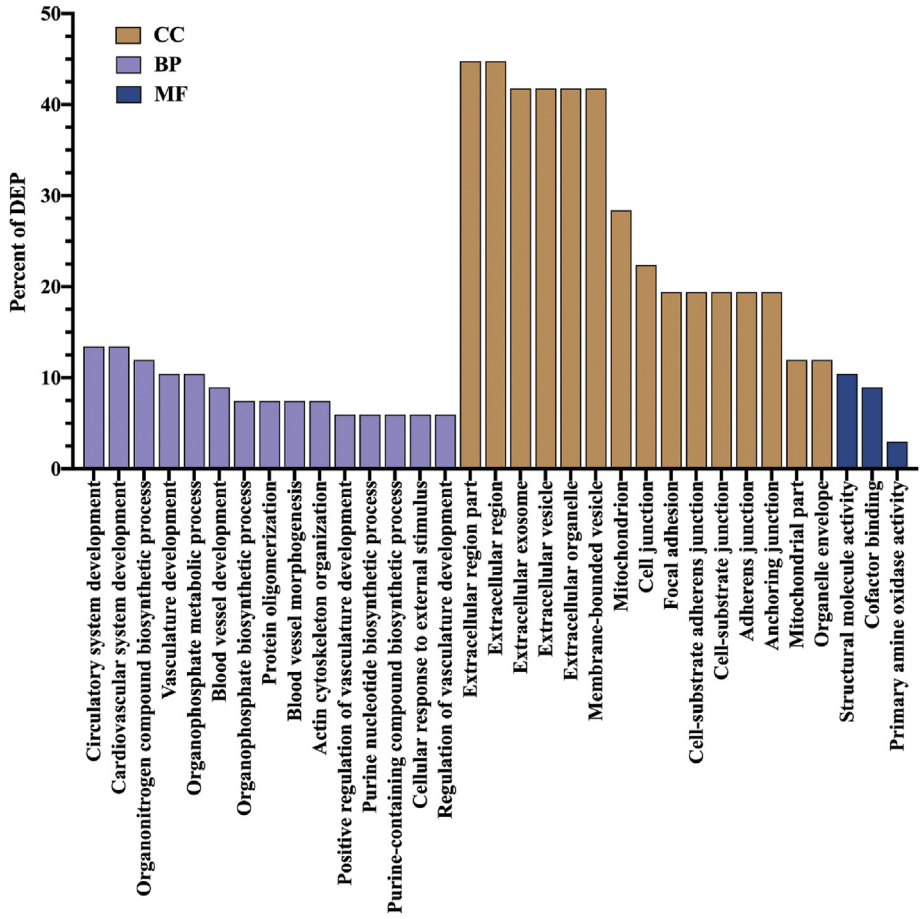


**Fig. 3.** The top 15 significant terms of the gene ontology analysis in biological process (BP), cellular component (CC), and molecular function (MF) categories. DEP = differentially expressed proteins.

constantly remodeled to control tissue homeostasis and exerts important roles in organogenesis and development of the intestine (Bonnans et al., 2014). According to our findings, the most affected CC terms were related to the extracellular matrix, including focal adhesion, adherens junction, cell-substrate junction, cell-substrate adherens junction, and the extracellular region part. Previous research about intestinal development showed that extracellular matrix remodeling is essential for intestinal development and maturation (Bonnans et al., 2014). However, the underlying molecular mechanisms are poorly understood. The research about the extracellular matrix is less investigated on the rumen and thus needs further study.

### 4.4. Metabolism

The mature rumen epithelium is the main site of butyrate metabolism (Penner et al., 2011). In our study, the most significant enriched signaling pathway belongs to the butanoate metabolism, and synthesis and degradation of ketone bodies. Previous study indicated that, with the intake of starter feed and the establishment of rumen fermentation, VFA replaces glucose as the main energy substance of the rumen epithelium (Penner et al., 2011; Baldwin and Connor, 2017). According to our findings, starter diet supplementation significantly increased the concentration of butyrate (Lin et al., 2019), which is the main reason for increasing the signaling pathway of butanoate metabolism. The formation of ketone bodies was the mainly pathway for butyrate metabolism, and exerted profound influences on energy metabolism and function development of rumen epithelia. In the present study, expression of 2 proteins (BDH1 and HMGCS2; FC = 1.91 and 1.93, respectively) involved in synthesis and degradation of ketone bodies were positively modified by starter feeding. HMGCS2 is the rate-limiting enzyme in the biosynthesis of ketone bodies in ruminal epithelia (Lane et al., 2002; Ma et al., 2017). Acetoacetic acid produces β-hydroxybutyrate under the catalysis of BDH1 (Naeem et al., 2012). Many of the products in VFA metabolism were the substrates of the energy production, including reduced flavine adenine dinucleotide 2 (FADH2), reduced nicotinamide adenine dinucleotide (NADH), and acetyl CoA, which are the co-enzymes or substrates of the citrate cycle. We found starter feeding activated the rumen epithelial citrate cycle pathway, as reflected by the 8.12 times increase in IDH1, which involved in the citrate cycle pathway. IDH1 contributes to citric acid cycle flux in rumen epithelial cells through continued production of α-ketoglutarate which, upon entry into mitochondria, is metabolized (Naeem et al., 2012). ATP, produced by ketone bodies and the citrate cycle, provides energy for the physiological activities of rumen epithelial cells, such as proliferation and differentiation. Moreover, ketogenesis is a hallmark of the metabolic development of ruminal epithelium tissue (Wang et al., 2016). Thus, the enhancement of ketogenic capacity probably indicates that starter diet supplementation promotes the development of rumen epithelial metabolism in pre-weaned lambs. However, in our

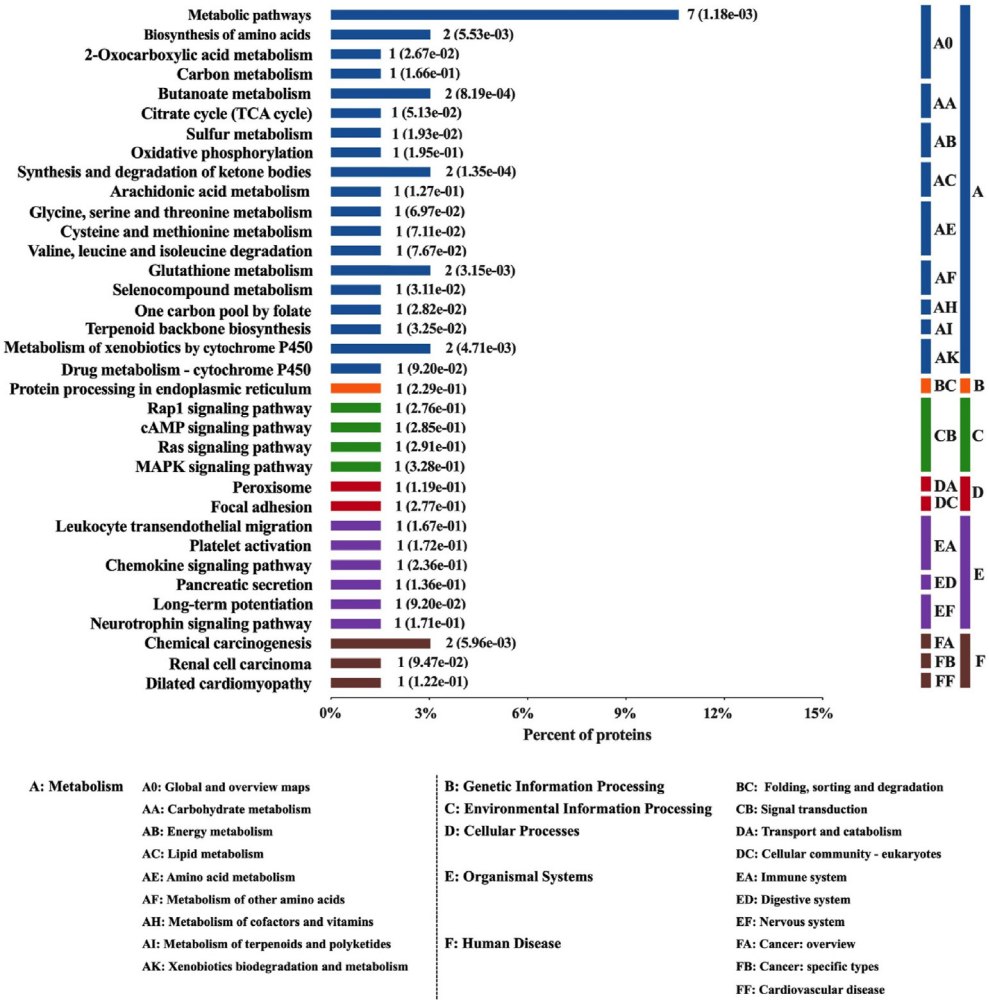


**Fig. 4.** Kyoto encyclopedia of genes and genomes (KEGG) analysis of differently expressed proteins. The values before parentheses are the number of differentially expressed proteins in each KEGG terms. The values in parentheses are the *P*- value of each KEGG terms.

study, the high concentration of butyrate inhibited the acetate activation to acetyl-CoA thioesters, reflected by ACSS1 expression being downregulated by 3.33 times, which may have facilitated the transport of acetate to other organizations for utilization.

The strong activation of biosynthesis of amino acids by starter feeding may be related to an increase in the metabolism of protein in the immature rumen epithelia. Although ewe milk contains a high percentage of protein, it enters the abomasum directly through the esophageal groove. However, the protein in the starter diet can enter the rumen directly. In the present study, the protein (IDH1 and CTH; FC = 8.12 and 2.13, respectively) expression related to the biosynthesis of amino acids pathway was positively modified by starter feeding. The IDH1 isoform catalyzes the conversion of isocitrate to α-ketoglutaric acid, which could be aminated by glutamate dehydrogenase to form glutamate (Liu et al., 2006; Hou et al., 2010). Glutamate is the main transfer carrier of ammonia in the body and transfers ammonia to the liver for detoxification (Nocek et al., 1980; Abdoun et al., 2006). The reason for this result, in the present study, may be that the absorption of ammonia was increased by the rumen epithelium, in order to combine with glutamic acid and pyruvate to form nontoxic glutamine, and then transferred to the liver, which produces urea through the urea cycle. These results are similar to Laarman et al. (2012) who found that starter diet supplementation increased the messenger ribonucleic acid (mRNA) expression of genes associated with urea cycle in the liver of calves. Taking these results together, we speculated that starter feeding promoted catabolism of proteins in the rumen and transport of ammonia in the ruminal epithelium.

Many studies indicated that, as the rumen matures, a comparatively lower amount of dietary protein escapes degradation and the resulting peptides and amino acids may be absorbed and metabolized by the rumen tissue (Quigley et al., 1985; Remond et al., 2000b; Kirat et al., 2013). Meanwhile, absorption of glycine, valine, serine and methionine by the ruminal epithelium was conformed to be higher at lower concentrations of total amino acids on the mucosal side of the ruminal epithelium (Remond et al., 2000a). In our study, the large amount of starter protein in the rumen provided more nitrogen for ruminal microbial protein synthesis, and increased the availability of amino acids to be absorbed by the ruminal epithelia to promote the rumen development and host growth. These may be the reasons for starter feeding enhancing the rumen epithelial glycine, serine and threonine metabolism pathway, valine, leucine and isoleucine degradation pathway, and cysteine and methionine metabolism pathway.

**Table 3**
Kyoto encyclopedia of genes and genomes (KEGG) analysis of differentially expressed proteins in the ruminal epithelia of lambs in the milk plus starter group compared with milk group lambs.

| Main categories | Subcategories | Pathway name | DEP | *P*-value |
|---|---|---|---|---|
| Metabolism | Global and overview maps | Metabolic pathways | IDH1, CTH, LOC101114663, ATP5F1, BDH1, HMGCS2, MTHFD1 | 0.001 |
| Metabolism | Global and overview maps | Biosynthesis of amino acids | IDH1, CTH | <0.001 |
| Metabolism | Global and overview maps | 2-Oxocarboxylic acid metabolism | IDH1 | 0.027 |
| Metabolism | Global and overview maps | Carbon metabolism | IDH1 | 0.166 |
| Metabolism | Carbohydrate metabolism | Butanoate metabolism | BDH1, HMGCS2 | <0.001 |
| Metabolism | Carbohydrate metabolism | Citrate cycle (TCA cycle) | IDH1 | 0.051 |
| Metabolism | Energy metabolism | Sulfur metabolism | SQRDL | 0.019 |
| Metabolism | Energy metabolism | Oxidative phosphorylation | ATP5F1 | 0.195 |
| Metabolism | Amino acid metabolism | Glycine, serine and threonine metabolism | CTH | 0.070 |
| Metabolism | Amino acid metabolism | Cysteine and methionine metabolism | CTH | 0.071 |
| Metabolism | Amino acid metabolism | Valine, leucine and isoleucine degradation | HMGCS2 | 0.077 |
| Metabolism | Metabolism of other amino acids | Glutathione metabolism | IDH1, MGST3 | 0.003 |
| Metabolism | Metabolism of other amino acids | Selenocompound metabolism | CTH | 0.031 |
| Metabolism | Lipid metabolism | Synthesis and degradation of ketone bodies | BDH1, HMGCS2 | <0.001 |
| Metabolism | Lipid metabolism | Arachidonic acid metabolism | LOC101114663 | 0.127 |
| Metabolism | Metabolism of cofactors and vitamins | One carbon pool by folate | MTHFD1 | 0.028 |
| Metabolism | Metabolism of terpenoids and polyketides | Terpenoid backbone biosynthesis | HMGCS2 | 0.033 |
| Metabolism | Xenobiotics biodegradation and metabolism | Metabolism of xenobiotics by cytochrome P450 | MGST3, LOC101114663 | 0.005 |
| Metabolism | Xenobiotics biodegradation and metabolism | Drug metabolism - cytochrome P450 | MGST3 | 0.092 |
| Organismal Systems | Immune system | Leukocyte transendothelial migration | RAP1A | 0.167 |
| Organismal Systems | Immune system | Platelet activation | RAP1A | 0.172 |
| Organismal Systems | Immune system | Chemokine signaling pathway | RAP1A | 0.236 |
| Organismal Systems | Nervous system | Long-term potentiation | RAP1A | 0.092 |
| Organismal Systems | Nervous system | Neurotrophin signaling pathway | RAP1A | 0.171 |
| Organismal Systems | Digestive system | Pancreatic secretion | RAP1A | 0.136 |
| Cellular Processes | Cellular community - eukaryotes | Focal adhesion | RAP1A | 0.277 |
| Cellular Processes | Transport and catabolism | Peroxisome | IDH1 | 0.119 |
| Environmental Information Processing | Signal transduction | Rap1 signaling pathway | RAP1A | 0.276 |
| Environmental Information Processing | Signal transduction | cAMP signaling pathway | RAP1A | 0.285 |
| Environmental Information Processing | Signal transduction | Ras signaling pathway | RAP1A | 0.291 |
| Environmental Information Processing | Signal transduction | MAPK signaling pathway | RAP1A | 0.328 |
| Genetic Information Processing | Folding, sorting and degradation | Protein processing in endoplasmic reticulum | SSR4 | 0.229 |
| Human Diseases | Cancer: overview | Chemical carcinogenesis | MGST3, LOC101114663 | 0.006 |
| Human Diseases | Cancer: specific types | Renal cell carcinoma | RAP1A | 0.095 |
| Human Diseases | Cardiovascular disease | Dilated cardiomyopathy | DES | 0.122 |

DEP = differentially expressed proteins; IDH1 = isocitrate dehydrogenase [NADP] cytoplasmic; CTH = cystathionine gamma-lyase; ATP5F1 = ATP synthase F(0) complex subunit B1; BDH1 = 3-hydroxybutyrate dehydrogenase 1; HMGCS2 = 3-hydroxy-3-methylglutaryl coenzyme A synthase; MTHFD1 = methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formyltetrahydrofolate synthetase 1; SQRDL = pyr_redox_2 domain-containing protein; MGST3 = microsomal glutathione S-transferase 3; RAP1A = Ras-related protein Rap-1A; SSR4 = signal sequence receptor subunit 4; DES = desmin.

Moreover, according to our study, we found that the 2 proteins expression of glycine amidino transferase, glycine amidinotransferase (FC = 2.89) and MCCC1 (FC = 3.34), playing a vital role in the catabolism of amino acid were positively modified by starter feeding. The glycine amidinotransferase gene catalyzes the conversion of arginine (Ballard and Thompson, 2013). The MCCC1 is important for the catabolism of leucine (Cao et al., 2016). The products of amino acid metabolism were the substrates of the citrate cycle, which may also be one of the reasons to accelerate the citrate cycle in the rumen epithelia.

Glutathione metabolism plays a key role in the pathogenesis of many diseases, and is related to oxidative stress (Droge and Breitkreutz, 2000; Liang et al., 2019). Thus, appropriate regulation of glutathione metabolism is necessary for host health (Wu et al., 2004). In our study, the level of 2 proteins, IDH1 (FC = 8.12) and microsomal glutathione S-transferase 3 (MGST3, FC = 2.37), involved in the glutathione metabolism pathway, were positively modified by starter feed supplementation. The IDH1 isoform catalyzes the conversion of isocitrate to α-ketoglutaric acid and nicotinamide adenine dinucleotide phosphate (NADPH) in the cytosol (Liu et al., 2006; Naeem et al., 2012). Previous study indicated that NADPH, the pivotal coenzyme involved in glutathione antioxidant systems, can convert oxidized glutathione into glutathione to promote glutathione regeneration (Zhou et al., 2016). MGST is now referred to as membrane-associated proteins in glutathione metabolism and known to be involved in butyrate-mediated defense against oxidative stress (Pool-Zobel et al., 2005). According to our study, starter diet supplementation increased the expression of MGST3, which is similar to the results of Connor et al. (2013) who found that starter diet supplementation up-regulated the mRNA expression of MGST3 in the ruminal epithelium of calves. Ma et al. (2018) indicated that sodium butyrate supplementation in high grain diets improving antioxidant stability of dairy goats, is

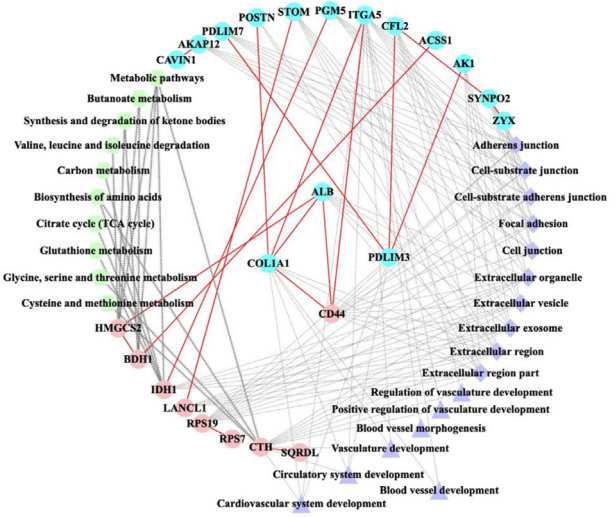


**Fig. 5.** The PPI network combined with gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) pathway enriched in differentially expressed proteins (DEP). Circle nodes for proteins, hexagon for KEGG pathway, triangle for biological process (BP) term, diamond for cellular component (CC) term. In case of fold change analysis, proteins were colored in pink (up-regulation) and light blue (down-regulation). Solid lines represent protein–protein interactions, dashed lines represent interaction between the DEP and GO terms, and contiguous arrows represent interaction between the DEP and KEGG pathways. ACSS1 = acetyl-coenzyme A synthetase; AK1 = adenylate kinase isoenzyme 1; AKAP12 = a-kinase anchoring protein 12; ALB = serum albumin; BDH1 = 3-hydroxybutyrate dehydrogenase 1; CAVIN1 = caveolae associated protein 1; CD44 = CD44 molecule; CFL2 = cofilin 2; COL1A1 = collagen type I alpha 1 chain; CTH = cystathionine gamma-lyase; HMGCS2 = 3-hydroxy-3-methylglutaryl coenzyme A synthase; IDH1 = isocitrate dehydrogenase [NADP] cytoplasmic; ITGA5 = integrin subunit alpha 5; LANCL1 = lanC like 1; PDLIM = PDZ and LIM domains; PGM5 = phosphoglucomutase 5; POSTN = periostin; RPS19 = ribosomal protein S19; RPS7 = 40S ribosomal protein S7; SQRDL = pyr_redox_2 domain-containing protein; STOM = stomatin; SYNPO2 = synaptopodin 2; ZYX = zyxin.

associated with increasing the mRNA expression of MGST3 in the liver. In the present study, the enhancement of the glutathione metabolism signaling pathway may be due to the increase in the butyrate concentration in the rumen (Lin et al., 2019). The increased anti-stress ability of the rumen epithelium helps to reduce the adverse effects of weaning stress on lambs.

### 4.5. Cellular processes, genetic and environmental information, and processing

Cell proliferation, apoptosis, and differentiation induced by signaling and protein expression regulate organ development (Naeem et al., 2014). Nutrients like VFA can act as signal molecules and activate the corresponding signaling pathway (Jewell et al., 2013; Naeem et al., 2014). In the present study, 2 upregulated DEP, inositol-3-phosphate synthase 1 (Koguchi et al., 2016) and RPS7 (Yu et al., 2014), were associated with the P53 signaling pathway that is related to regulators of the cell apoptosis of ruminal epithelia, and regulated by the effect of VFA (Gui and Shen, 2016). Moreover, in the present study, the RAP1A, whose expression was downregulated by 2.08 times with starter feeding, was enriched in the rap1 signaling pathway, cAMP signaling pathway, Ras signaling pathway, and MAPK signaling pathway. RAP1A can inhibit the activity of RAS (Azoulay-Alfaguter et al., 2015), and is regulated by butyrate (Han et al., 2016). This means that the decrease in the expression of RAP1A caused by starter feeding could activate the RAS signaling, which contributes to mitosis of rumen epithelial cells. Moreover, the cAMP signaling pathway is closely related to cell proliferation and cell cycle (Stork and Schmitt, 2002). MAPK signaling pathway has been confirmed to be related to the proliferation of ruminal epithelia (Shen et al., 2017). Thus, RAP1A may be a key target for nutritional regulation of rumen epithelial development, and the role of the 4 signaling pathways in rumen epithelial development requires further research.

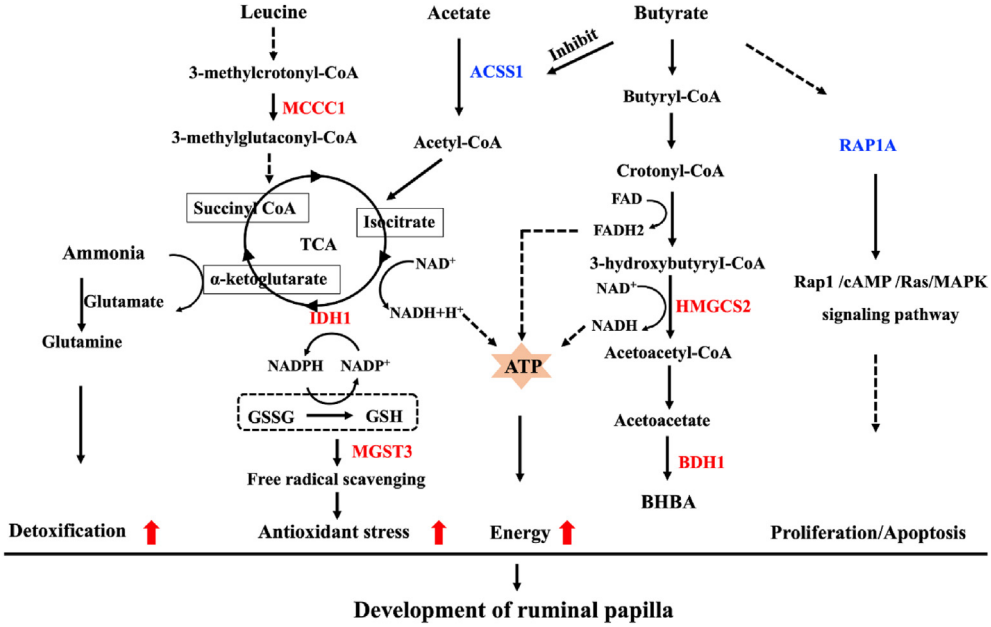


**Fig. 6.** Summary of differentially expressed proteins profiles associated with ruminal epithelial development in response to starter feed supplementation in pre-weaned lambs. The relationships depicted are from the most current information in the kyoto encyclopedia of genes and genomes (KEGG) knowledge base. Proteins were colored in red (up-regulation) and blue (down-regulation). ACSS1 = acetyl-coenzyme A synthetase; ATP = adenosine triphosphate; BDH1 = 3-hydroxybutyrate dehydrogenase 1; BHBA = β-hydroxybutyrate; FAD = flavin adenine dinucleotide; FADH2 = reduced flavine adenine dinucleotide 2; GSH = reduced glutathione; GSSG = oxidized glutathione; HMGCS2 = 3-hydroxy-3-methylglutaryl coenzyme A synthase; IDH1 = isocitrate dehydrogenase (NADP) cytoplasmic; MCCC1 = methylcrotonoyl-CoA carboxylase 1; MGST3 = microsomal glutathione S-transferase 3; NAD = nicotinamide adenine dinucleotide; NADH = reduced nicotinamide adenine dinucleotide; RAP1A = Ras-related protein Rap-1A; TCA = citrate cycle.

## 5. Conclusion

Previous studies mainly focused on the transcriptional level. The protein level is more reflective of the actual situation of the rumen epithelial physiological activity. The results showed that starter feeding regulates a variety of physiological processes in the rumen epithelium. As summarized in Fig. 6, starter feeding increases the protein expression associated with energy supply, antioxidant capacity and ammonia detoxification function, which ensure the healthy development of the rumen epithelium. Furthermore, the development of epithelial morphology promotes the absorption of nutrients, thereby enhancing the metabolism of epithelial nutrients. The metabolism of nutrients provides energy for rumen epithelial development. These findings provide a new perspective and insight into nutritional interventions to promote the development of the rumen on the protein level in young ruminants.

## Author contributions

**Daming Sun**: Data curation, Writing- Original draft preparation, Investigation. **Junhua Liu:** Conceptualization, Methodology. **Shengyong Mao**: Project administration, Formal analysis. **Weiyun Zhu**: Supervision, Resources. **Junhua Liu** and **Daming Sun:** Writing-Reviewing and Editing.

## Conflict of 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.

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## Appendix

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2021.06.014.

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