

# Early solid diet supplementation influences proteomic of rumen epithelium in goat kids

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## Research

**Keywords:** proteome, rumen epithelium, solid diets, goat; Gene Ontology; differentially expressed proteins

**Posted Date:** March 19th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-17915/v1>

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# Abstract

**Background:** Rumen is an important organ for nutrient absorption and metabolism in goats, and its constant development is a prerequisite for the healthy growth of goat kids. Previous studies have shown that the intake of solid feed in early life can significantly promote the development of rumen tissue morphology and metabolic function in young ruminants. However, the expressed proteome in rumen epithelium of goats supplemented solid diet is still limited. In this study, 24 pairs of twin goats were randomly divided into milk replacer group (MR) and milk replacer with supplemented solid diets group (SS) using proteomic analysis based on ITRAQ technique, which allow to further analyze potential biological mechanism.

**Results:** Significant increased papilla length and width, epithelium thickness as well as muscle layers thickness were observed in SS group. A total of 6003 differentially expressed proteins were detected in the SS group compared to the MR group by analyzing the rumen protein expression of six biological replicates, of which 251 proteins were up-regulated and 750 proteins were down-regulated. According to Gene Ontology, the differential expressed proteins (DEPs) were significantly enriched in functions related to cell development and biosynthesis. The networks of KEGG pathways demonstrated that most of the 30 significantly enriched pathways involved in cellular metabolism, signal transduction and immunity.

**Conclusions:** The supplementation of solid feed promoted the growth of rumen epithelial cells, improved the rumen's immune function, and activated lipid metabolism. Our findings provided new insights into the molecular mechanisms underlying rumen developmental differences in goat kids caused by solid feed.

## Background

Rumen plays a key role in ruminants' performance and production [1]. Goat kids are born with immature rumen having no physiological and metabolic functions [2]. Physical and metabolic development of the rumen is essential for its smooth transition from non-ruminant to mature ruminant state, and for improving the growth performance of young ruminants. As we know, the introduction of solid diets in early life was a vital driver for the development of rumen epithelium due to the nutrient components in solid diet [3]. Previous studies found that supplementing solid feed during pre-weaning period can effectively increase rumen weight and papillae size, enhance the physical barrier of rumen to harmful substances, and ultimately have a positive effect on the health and growth of young ruminants [4–6]. Other studies reported that early feeding starter can influence gene expression of rumen epithelium in lambs and sheep, for example, down-regulated genes including IL-6, IL-10, and IFN- $\gamma$  [7] and up-regulated genes containing MCT1, MCT4 and NHE3 [8]. Experiments with goats confirmed that high-grain diets simultaneously increased the volatile fatty acids (VFA) production and the expression of genes involved in VFA absorption and cell proliferation in rumen [9–11]. In calf studies, the implementation of similar methods significantly increased the expression of fat and muscle tissue synthesis genes [12]. Studies on the effects of diets on rumen epithelial mRNA gene expression have become increasingly rich and sophisticate. However, little studies investigate the expressed proteins in rumen epithelium affected by

diet. In sheep and dairy cattle, studies reported that the differential expression on selected proteins related to material transport and metabolism due to the changes of diet and rumen environment [13]. Therefore, it is urgent to perform systemic cognition on rumen epithelial protein expressions of goats supplemented solid diet, which can help us understand the molecular mechanism of rumen development, and further improve the feeding strategy for young ruminants. Isobaric tags for relative and absolute quantitation (iTRAQ) is used in quantitative proteomics due to high sensitivity and conveniences [14]. In this study, we applied proteomic analysis based on iTRAQ [15] to evaluate changes in protein expression of rumen epithelium in early supplementation goats compared to baseline group without solid diet. Through the exploration based on the molecular level, we will understand how solid diet drives proteome expression of rumen epithelium, which can support for the optimal breeding strategy to improve the performance and the growth potential of young ruminants.

## **Materials And Methods**

### **Animals and diets**

Twenty four pairs of twin goats with an average weight of  $4.53 \pm 0.52$  kg was separated from their dams at 20 days of age and randomly divided into two groups. One group (MR) was fed with milk replacer that was provided by Beijing Precision Animal Nutrition Research Center, China, and another group (SS) was fed with milk replacer supplemented with concentrate and alfalfa pellet. Each group had six replicates and four kids per pen as a replicate. During the trial, all goat kids had ad libitum access to water, MR, concentrate, and alfalfa pellets. Nutritional levels of MR, concentrate and alfalfa pellets are shown in table S1. On the 60 days of age, six goats (healthy and BW close to the average of the corresponding groups) were chosen from each group, and slaughtered for rumen sample collection. The rumen epithelial tissue in ventral sac was quickly harvested and snap frozen in a liquid nitrogen tank for total protein extraction.

### **Histomorphology analysis**

A 2 cm × 2 cm rumen epithelium was collected from MR and SS groups, washed with physiological saline, and fixed in a 250 mL jar containing 10% neutral formalin solution. The samples were dehydrated by different concentrations of ethanol, embedded in paraffin sections, and cut into 6 μm sections. The rumen papilla structure was observed under light microscope at a magnification of 4 × 10 times (Olympus BX-51; Olympus Corporation, Tokyo, Japan) after staining with Yihong-hematoxylin (H.E.). The image-pro express image analysis processing system (Image-Pro Plus 6.0, Media Cybernetics, Silver Spring, MD, USA) was used to observe and measure the rumen papilla length, papilla width, and stratum corneum thickness.

### **Protein extraction and iTRAQ labeling**

Proteins was extracted by using lysis buffer 3 (8 M Urea, 40 mM Tris-HCl or TEAB with 1 mM PMSF, 2 mM EDTA and 10 mM DTT, pH 8.5) and two magnetic beads (diameter 5 mm). The mixtures were placed into

a TissueLyser for 2 min at 50 Hz to release proteins. After centrifugation with 25,000 g at 4°C for 20 min, and then the supernatant was transferred into a new tube, reduced with 10 mM dithiothreitol (DTT) at 56 °C for 1 hour and alkylated by 55 mM iodoacetamide (IAM) in the dark at room temperature for 45 min. After centrifugation (25,000 g, 4°C, 20 min), the supernatant containing proteins was quantified by Bradford. Mix 15–30 µg proteins with loading buffer in centrifuge tube and heat them at 95°C for 5 minutes. Then, the supernatant was centrifuged at 25000 g for 5 minutes and loaded to sample holes in 12% polyacrylamide gel. The SDS-PAGE in constant voltage at 120V for 120 minutes was performed to detect proteins quality. Once finished, it was stained in gel with Coomassie Blue for 2 hours, then destaining solution (40% ethanol and 10% acetic acid) was added and ultimately it was put on a shaker (exchange destaining solution for 3 ~ 5 times, 30 minutes a time). The protein solution (100ug) with 8M urea was diluted 4 times with 100 mM TEAB. Then the proteins were digested at 37 °C overnight by Trypsin Gold (Promega, Madison, WI, USA) in a ratio of protein: trypsin = 40:1. After trypsin digestion, the peptides were desalted using Strata X C18 column (Phenomenex) and vacuum-dried according to the manufacturer's protocol. The peptides were dissolved in 30 ul 0.5M TEAB with vortexing. After the iTRAQ labeling reagents were recovered to ambient temperature, they were transferred and combined with proper samples. Peptide labeling was performed by iTRAQ Reagent 8-plex Kit according to the manufacturer's protocol. The labeled peptides with different reagents were combined, desalted with a Strata X C18 column (Phenomenex), and vacuum-dried according to the manufacturer's protocol.

## **Liquid chromatography–tandem mass spectrometry (LC/MS) analysis**

The separation of peptides was carried out on a Shimadzu LC-20AB HPLC Pump system coupled with a high pH RP column. The peptides were reconstituted with buffer A (5% ACN, 95% H<sub>2</sub>O, adjust pH to 9.8 with ammonia) to 2 ml and loaded onto a column containing 5-µm particles (Phenomenex). The peptides were separated at a flow rate of 1 mL/min with a gradient of 5% buffer B (5% H<sub>2</sub>O, 95% ACN, adjusted pH to 9.8 with ammonia) for 10 min, 5–35% buffer B for 40 min, 35–95% buffer B for 1 min. The system was then maintained in 95% buffer B for 3 min and decreased to 5% within 1 min before equilibrating with 5% buffer B for 10 min. Elution was monitored by measuring absorbance at 214 nm, and fractions were collected per 1 min. The eluted peptides were pooled into 20 fractions and vacuum-dried. Each fraction was resuspended in buffer A (2% ACN, 0.1%FA) and centrifuged at 20,000 g for 10 min. The supernatant was loaded onto a Thermo Scientific™ UltiMate™ 3000 UHPLC system equipped with a trap and an analytical column. The samples were loaded on a trap column at 5 µL/min for 8 min, and then eluted into the homemade nanocapillary C18 column (ID 75 µm x 25 cm, 3 µm particles) at a flow rate 300 nL/min. The gradient of buffer B (98%ACN, 0.1%FA) was increased from 5–25% in 40 min, and then increased to 35% in 5 min, followed by 2 min linear gradient to 80%, then maintenance at 80% B for 2 min, and finally return to 5% in 1 min and equilibrated for 6 min. The peptides separated from nanoHPLC were subjected into the tandem mass spectrometry Q EXACTIVE HF X (Thermo Fisher Scientific, San Jose, CA) for DDA (data-dependent acquisition) detection by nano-electrospray ionization. The parameters for MS analysis were listed as following: electrospray voltage: 2.0 kV; precursor scan range: 350–1500 m/z at a resolution of 60,000 in Orbitrap; MS/MS fragment scan range: >100 m/z at a resolution of 15,000 in HCD

mode; normalized collision energy setting: 30%; dynamic Exclusion time: 30 s; Automatic gain control (AGC) for full MS target and MS2 target: 3e6 and 1e5, respectively. The MS/MS scan numbers following one MS scan: 20 most abundant precursor ions above a threshold ion count of 10,000.

## Protein quantification and data analysis

The raw MS/MS data was converted into MGF format, and the MGF files were searched by the local Mascot server against the database. Besides, quality control was performed to determine if a reanalysis step was needed. An automated software, called IQuant, was applied to the quantification of proteins. All proteins with a false discovery rate (FDR) less than 1% will proceed with downstream analysis including GO, COG/KOG and Pathway. Further, we also performed deep analysis based on differentially expressed proteins, including Gene Ontology (GO) enrichment analysis, KEGG pathway enrichment analysis, COG/KOG function annotation, cluster analysis, protein interaction analysis and subcellular localization analysis.

## Analysis of gene expression by RT-PCR

In the research, several genes of rumen tissues (three samples per group) were selected to detect expression levels by RT-PCR. The primers designed with primer 5.0 were produced by Nanjing Biomarker Co., Ltd. China (Table S2) The status of DNA extracted from Grinding fluid were determined using a Nano-Drop 2000 spec-trophotometer. The cDNA library was constructed under the effect of reverse transcriptase. Illumina2500 sequencer was used to read amplified segments of these specific genes. Data were calculated using the  $2^{-\Delta\Delta Ct}$  method.

## Results

### Effect of supplemented solid feed on rumen epithelial morphology

We used tissue sections to observe the morphological changes of rumen epithelium between the two groups. Compared with the lambs fed milk replacer (MR), the rumen morphology of the lambs supplemented solid fed (SS) on the basis of MR was more developed (Fig. 1A), including a significant increased papilla length ( $P < 0.001$ ), papilla width ( $P < 0.001$ ), muscle layers thickness ( $P < 0.001$ ) and epithelium thickness ( $P < 0.05$ ) (Fig. 1B-E).

Figure 1 Comparison of epithelial morphology between milk replacer (MR) and solid feed (SS). The changes of (A) rumen epithelium morphology, (B) Papilla width, (C) Papilla length, (D) Muscle layers thickness, and (E) Epithelium thickness between MR and SS. Horizontal lines with a star symbol between Bar charts represent a significant difference based on one-way ANOVA analysis; \* $0.01 < P < 0.05$ ; \*\* $0.001 < P < 0.01$ ; \*\*\* $P < 0.001$ .

# Protein Identification

Based on analysis of iTRAQ data, totally 114,401 (94,287 unique) spectra were identified. Of the 26,793 peptides, 24,609 peptides were unique (Fig. S1). A total of 6003 proteins were identified with 1% FDR during the process of analysis (Fig. S1), and more than half of the proteins contained at least two peptides (Fig. S1). Sequence coverage of most identified protein species was less than 20%, approximately 60% of the protein species were between 10 and 70 kDa (Fig. S1). All proteins identified by LC-E-SI-MS/MS from the peptides were listed in Supplementary Information Table S3.

For experimental design with more than one replicate, proteins with 1.2 fold change (mean value of all comparison groups) and p-value (t-test of all comparison groups) less than 0.05 were defined as DEPs (Fig. S2). In this experiment, compared with the MR group, 1001 DEPs were identified in the SS group, of which 251 were up-regulated and 750 were down-regulated (Table S4).

## The analyses of Gene Ontology enrichment

To further explore the specific functional characteristics of the DEPs, we performed the GO enrichment analysis of the DEPs using David Bioinformatics Resources [16, 17]. The results indicated that some parts of functions were remarkably enriched in the DEPs (Fig. 2A-C). In cellular components, the most significant GO terms of the DEPs mainly related to the assembly of DNA, the support of cell structure and the synthesis of proteins. These notable changes are mainly concentrated on extracellular matrix, nucleosome, DNA packaging complex, stress fiber and complex of collagen trimers. Molecular functions mainly involved the interaction and binding of proteins, the integrity of the cellular framework and the promotion of cell growth, which included platelet-derived growth factor binding, protein complex binding, actin binding, growth factor binding and extracellular matrix structural constituent. The biological process annotation showed that the DEPs mostly contributed to the synthesis of extracellular matrix and the assembly of chromosomes such as supramolecular fiber organization, extracellular matrix organization, extracellular structure organization, nucleosome organization and chromatin assembly.

Figure 2 Gene ontology (GO) classification of differentially expressed proteins. The classification including (A) cellular component, (B) molecular function and (C) biological process.

## The analyses of KEGG pathway enrichment

To understand the effects of the DEPs on related pathways after the introduction of solid feed, we performed KEGG pathway enrichment analysis of the DEPs. As shown in Table 1, we found 30 significantly enriched pathways which are closely related to composition of extracellular matrix (such as "Focal adhesion", and "ECM-receptor interaction", among others.), disease and immunity (such as "Amoebiasis Staphylococcus aureus infection" and "Complement and coagulation cascades", among others.), material metabolism (such as "Glycosphingolipid biosynthesis - globo and isoglobo series",

“Glycosaminoglycan degradation”, “Arachidonic acid metabolism” and “Steroid hormone biosynthesis”, among others). We next sort to perform protein interaction analysis using the ten most significantly enriched pathways and presented them in the form of a network map by Cytoscape [18] (Fig. 3). From Fig. 3, the focal adhesion pathway gained the most favor of the DEPs. Seven of remaining pathways were related to disease and immunity and the almost DEPs involving in these ten pathways were down regulated. Moreover, the protein interaction network related to metabolic-related pathways were emphasized since they accounted a large proportion among top pathways (Fig. 4), which promotes us to comprehend the inner link between proteins and functions more intuitively. Figure 4 was focused on material metabolism and signal transmission. Obviously, lipid metabolism (Fig. 4A) was most active due to the most up-regulated protein enriched.

Table 1  
 Pathway enrichment analysis of differentially expressed proteins (DEPs).

Pathway name	Regulated Proteins	Up-regulated proteins	Down-regulated proteins	P value	Pathway ID
Focal adhesion	52	3	49	0.000	ko04510
ECM-receptor interaction	24	0	24	0.000	ko04512
Amoebiasis	30	4	26	0.000	ko05146
Systemic lupus erythematosus	23	2	21	0.000	ko05322
Protein digestion and absorption	19	0	19	0.000	ko04974
Hypertrophic cardiomyopathy (HCM)	19	0	19	0.001	ko05410
Staphylococcus aureus infection	17	2	15	0.001	ko05150
Viral myocarditis	21	2	19	0.002	ko05416
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	17	0	17	0.003	ko05412
Complement and coagulation cascades	20	4	16	0.003	ko04610
Human papillomavirus infection	47	4	43	0.004	ko05165
Dilated cardiomyopathy (DCM)	20	0	20	0.004	ko05414
Glycosphingolipid biosynthesis - globo and isoglobo series	5	0	5	0.005	ko00603
Lysosome	24	3	21	0.006	ko04142
Glycosaminoglycan degradation	6	0	6	0.008	ko00531
Small cell lung cancer	19	2	17	0.010	ko05222
Carbohydrate digestion and absorption	7	1	6	0.010	ko04973
Graft-versus-host disease	9	1	8	0.012	ko05332
Leukocyte transendothelial migration	21	3	18	0.013	ko04670
Glycosphingolipid biosynthesis - ganglio series	4	1	3	0.018	ko00604
Arachidonic acid metabolism	27	16	11	0.018	ko00590
Proteoglycans in cancer	31	4	27	0.022	ko05205
Fluid shear stress and atherosclerosis	23	8	15	0.023	ko05418

Pathway name	Regulated Proteins	Up-regulated proteins	Down-regulated proteins	P value	Pathway ID
Steroid hormone biosynthesis	15	13	2	0.023	ko00140
Relaxin signaling pathway	19	2	17	0.024	ko04926
Folate biosynthesis	12	11	1	0.026	ko00790
PI3K-Akt signaling pathway	40	5	35	0.026	ko04151
Type I diabetes mellitus	9	1	8	0.026	ko04940
Vitamin B6 metabolism	4	0	4	0.048	ko00750
Ovarian steroidogenesis	11	8	3	0.050	ko04913

Figure 3 The top ten significantly enriched pathway relationship networks. Purple circles represent the different pathways, red and blue circle represent up-regulated and down-regulated proteins separately. Edges with different colors represent classes of KEGG pathway.

Figure 4 Relationship networks of metabolic pathways with significant enrichments. Purple circles represent the different pathways, red and blue circles represent up-regulated and down-regulated proteins separately. Edges with different colors represent classes of KEGG pathway. (A) lipid metabolism (B) carbohydrate metabolism (C) signal transduction.

Figure 5 The expression results of the six genes encoding DEPs. Fold change represents the ratio of relative expression of genes or proteins between SS and MR. An up-regulated expression means that the fold change was above 1 and vice versa.

## Expression Analysis of Genes Corresponding to DEPs

The expression of protein is adjusted and controlled by its corresponding mRNA transcription level. In this study, based on proteomic results, we selected 6 genes related to lipid synthesis, material transport, signal transduction and cell division, and then performed qRT-PCR to explore the relationship between mRNA transcription and protein expression. From Fig. 5, the genes of HMGCS2, BDH1, SLC16A1 and HMGCL shows the same rising trend at both protein level and mRNA level. However, the opposite trend of protein and mRNA expression occurred in genes of PIK3B and POLE.

## Discussion

With the growth of young ruminants from birth to adulthood, their rumen morphology and functions undergone huge changes, which also results in a remarkable shift in nutrient digestion patterns. Our

previous study and others confirmed that feeding solid feed, including concentrate (high grain diet) and forages, has a beneficial effect on rumen development [19, 20]. With alteration of rumen microbiome and increased concentration of volatile fatty acids (VFA) by solid diet intervention [21], the growth of the rumen epithelium and its ability of nutrition transport and metabolism was improved [22]. In the current study, greater rumen epithelial morphology in solid diet groups was identified. Using the proteomic analysis, our results suggested that the DEPs of rumen epithelium driven by solid diet were mostly associated with cell division, immunity, metabolism and signal transduction, which indicates rumen in goats consumed solid diet had a more mature function.

## **Rumen morphological changes**

Rumen is a significant digestive organ in ruminants [23]. The papillae on the rumen wall can promote the contact area of the rumen wall with the digesta, and enhance the ability to absorb nutrients. The length, width and thickness of the rumen papilla are professional indicators for evaluating rumen development. In this study, significant improvement of rumen tissue morphology in solid diet group, especially the rumen papilla and stratum corneum, were observed. It was supposed that solid diet can improve rumen development and functions. Many studies [24–27] have shown that the rumen tissue morphology has undergone tremendous changes with the feeding of solid feed, including increased rumen papilla width, appropriate stratum corneum thickness and better integrity. Therefore, it is not surprising that our proteomic results revealed the DEPs between treatment. Additionally, increased function including muscle protein synthesis and cell framework construction, were observed in solid diet rumen which corresponded to the results of tissue sections.

## **Cell synthesis, protein construction and immune**

Solid feed can stimulate rumen growth through increasing rumen VFA concentration [3, 28]. In this research, due to the feeding of solid feed, the processes of cell synthesis and protein binding had more active performance, including extracellular matrix, DNA packaging complex, extracellular matrix organization, extracellular structure organization, nucleosome organization, protein complex binding, actin binding, growth factor binding, and actin filament binding. Notably, in previous studies, the increases of rumen VFA concentration can synchronously rise the rate of cell mitosis [29], which was exactly the first step in cell synthesis. In addition, 6 up-regulated proteins participated in cell differentiation, gene expression and immune regulation were enriched in the pathway of focal adhesion, and complement and coagulation cascades. In contrast, disease pathways were enriched by down-regulated proteins, which may imply that the intake of solid feed can improve the immune ability and disease resistance of the body. In fact, body's health influenced by diet has been reported in many studies [30–32]. Recently, researchers discovered that the timely introduction of solid foods for infants can effectively reduce the incidence of allergic diseases [33], which could be due to the characteristics of dietary metabolites that shape and improve the intestinal immune system of young animals [34]. The

results of our analysis also support this view. Surprisingly, the proteins enriched in the pathway of protein digestion and absorption were all down-regulated, this phenomenon might be related to the stress response caused by the introduction of solid feed. To sum up, we can confirm that supplementing solid feed accelerated the proliferation of rumen epithelial cells and increased the resistance of the gut to disease.

## Rumen metabolism and signal transduction

With the changes of rumen tissue morphology, the material metabolism functions of rumen also undergo essential shifts. The energy metabolism is a critical metabolic pathway that has a decisive effect on the growth and development of organisms. However, the rumen is a unique organ of ruminants, and the energy consumed for growth does not depend on sugars, ketone bodies or glutamines, but on the utilization of microbial terminal fermentation products, e.g. VFA [35–37]. Thus, lipid metabolism is more prominent during the process of rumen epithelial growth. VFA can also be used as signaling molecules and intermediate mediators to indirectly regulate the expression levels of some genes and proteins in the rumen epithelium. For example, butyrate in VFA can effectively regulate histone deacetylation, affect the expression of associated genes, and mediate multiple ion transport channels.

Our data suggested that many proteins associated with lipid metabolism are mostly up-regulated by solid diet. In our analysis, we can also recognize over half of the active upregulated proteins gathered by pathways of arachidonic acid metabolism, steroid hormone biosynthesis and folate biosynthesis. Among them, arachidonic acid is an indispensable polyunsaturated fatty acid for the development of animals, especially for young animals. In fact, arachidonic acid can be involved in cell membrane synthesis as a structural lipid in combination with phospholipids. It also acts as a direct precursor of prostaglandin E2 (PGE2), prostacyclin (PGI2), thromboxane A2 (TXA2) and leukotrienes and C4 (LTC4), which have important regulatory effects on the biological metabolism. Notably, in the steroid hormone biosynthesis pathway, the synthesis process of glucuronosyltransferase and 3 $\alpha$ -Hydroxy-Steroid Dehydrogenase were enriched by most upregulated proteins. Coincidentally, both enzymes can effectively promote the conversion of multiple steroid substances into bile [38, 39], thereby improving the ability of rumen to absorb fat-soluble vitamins and fatty acids, including arachidonic acid [40]. Recent studies also showed that dietary and microbial factors can control the synthesis of intestinal bile and its metabolites and further regulate the growth and function of related cells [41]. In another our study, the introduction of solid feed caused dual changes in the rumen microbiota and its digestion end products. Obviously, the metabolism of bile acids was also affected by the complex changes, and its mechanism deserves further study. Folic acid, converted by reductase into physiologically active tetrahydrofolate (THFA or FH4), is involved in base synthesis and plays a critical role in protein synthesis and cell division.

In addition to lipid metabolism-related pathways, the lysosome and PI3K-Akt signaling pathways were also enriched with the DEPs. Lysosomes have a strong substance degradation function. In the case of tissue nutrient deficiency, cells initiate autophagy, and macromolecules such as proteins in the cytoplasm

are moved into lysosomes to degrade into small molecules, thus maintaining the internal environment of the cells [42]. In this study, the way of feeding solid feed provided abundant nutrients for the rumen, which might have some inhibitory effect on lysosome activity. The PI3K-Akt (protein kinase B) signaling pathway can be mediated by a variety of signaling molecules, the lipid molecule PIP3 is involved in the activation of Akt as a signaling molecule. Once Akt is activated, it can regulate critical cellular responses such as apoptosis, protein synthesis, and metabolism through substrate phosphorylation. Unsurprisingly, A0A452FAR7 regulated by PIK3CB gene was significantly up-regulated in many other pathways, such as cellular processes, transport and catabolism, carbohydrate metabolism. In previous study, PIK3CB was confirmed to be closely related to the development of cells [43]. In this study, the introduction of solid feed activated the expression of PIK3CB and, as previously reported [44], PIK3CB was involved in multiple pathways related to the growth of rumen epithelium. The above analysis may provide some insights into the underlying causes of rumen development.

## Conclusions

Using proteomic technology, we measured the differentially expressed proteins and their related pathways which were caused by solid diet supplementation. Some biological functions related to cell proliferation, protein binding and frame construction were significantly enriched in solid diet group, which well explains the improved rumen morphology by solid diet. Moreover, based on the analysis of network, we confirmed that VFAs could participate more extensively in the biological metabolism, and regulate the secretion of related lipids including vitamins and steroid hormones in the form of signal molecules, which further influenced the rumen epithelial biological functions. The proteomics dataset from this research provides a new perspective for studying the molecular mechanism how solid feed stimulates epithelial proteome and rumen development. Additionally, our study broaden theoretical knowledge of the intrinsic mechanisms of rumen development in goats.

## Declarations

**Abbreviations:** MR: milk replacer group; SS: milk replacer with supplemented solid diets group; DEPs: differential expressed proteins; FDR: false discovery rate; GO: Gene Ontology; VFA: volatile fatty acids.

**Ethics approval and consent to participate:** The study was conducted at the Green Sheep Valley Farm in Haimen City, Jiangsu Province. Procedures for breeding and slaughtering were implemented in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals promulgated by the Ministry of Science and Technology, China, revised version, March 2017. The trial was also reviewed and approved by the Animal Ethics Committee of the Chinese Academy of Agricultural Sciences (AEC-CAAS-FRI-CAAS20180305).

**Consent for publication:** Not applicable.

**Availability of data and materials:** All data generated or analysed during this study are included in this published article and its additional files.

**Competing interests:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

**Funding:** This research was funded by the National Natural Science Foundation of China, grant number 31872385, the National Key R&D Program of China, grant number 2018YFD0501902 and Earmarked Fund for China Agriculture Research System, grant number CARS-39.

**Author Contributions:** Conceptualization, N.Z. and Q.D.; methodology, Y.Z. and W.H.; software, K.C.; validation, K.C. and N.Z.; formal analysis, Y.Z. and W.H.; resources, N.Z.; data curation, Q.D.; writing—original draft preparation, Y.Z.; writing—review and editing, K.C. and Y.H.; visualization, Y.Z.; supervision, N.Z.; project administration, Q.D. and N.Z.; funding acquisition, N.Z.

**Acknowledgement:** We appreciate the Beijing Genomics Institute (BGI) for assistance in protein sequencing.

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## Supplementary Files Legend

### Additional files

Additional file1: Table S1. Nutritional components of milk replacer, concentrate, and alfalfa particles. (XLS 21kb)

Additional file4: Table S2. The qPCR primers designed for verification of the differential expressed genes. (XLS 29kb)

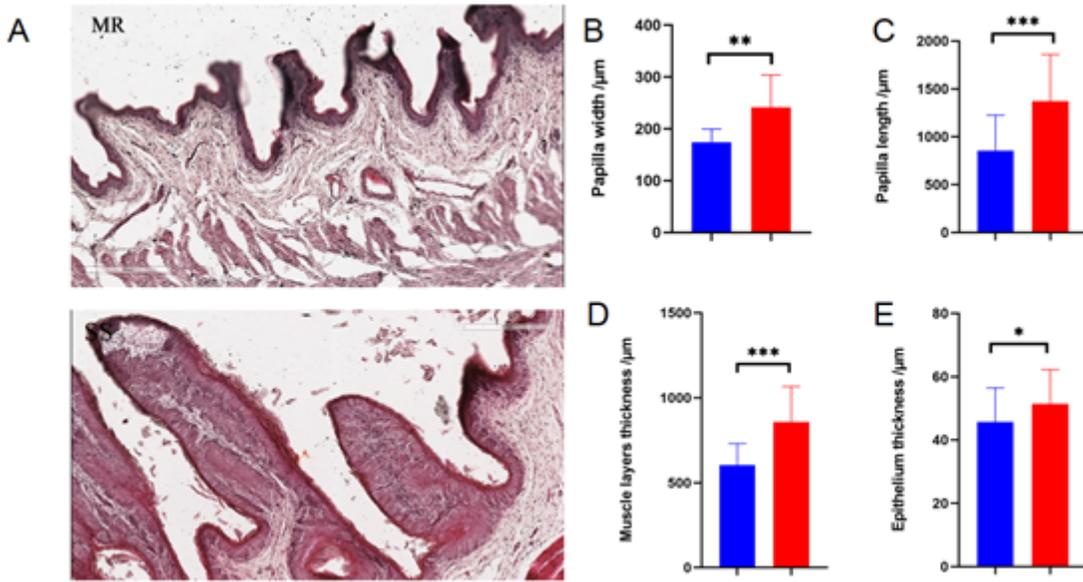
Additional file2: Figure S1. Generation of iTRAQ data. (DOXC 100kb)

Additional file3: Figure S2. Repeatability analysis of biological replicates. (DOCX 141kb)

Additional file5: Table S3. The identified proteins of the study. (XLS 9021kb)

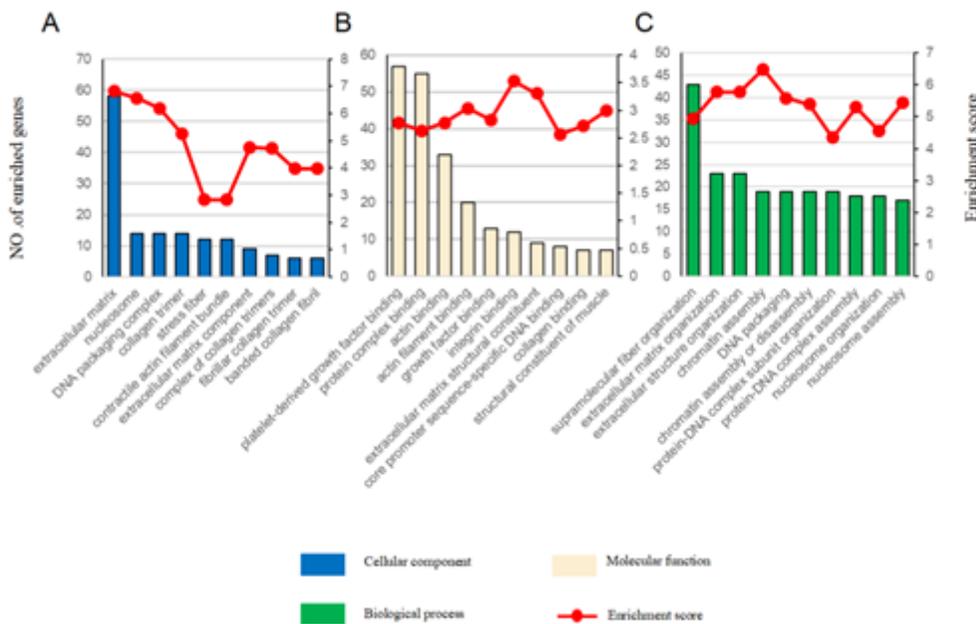
Additional file6: Table S4. The different expressed proteins of the study. (XLS 3114kb)

## Figures



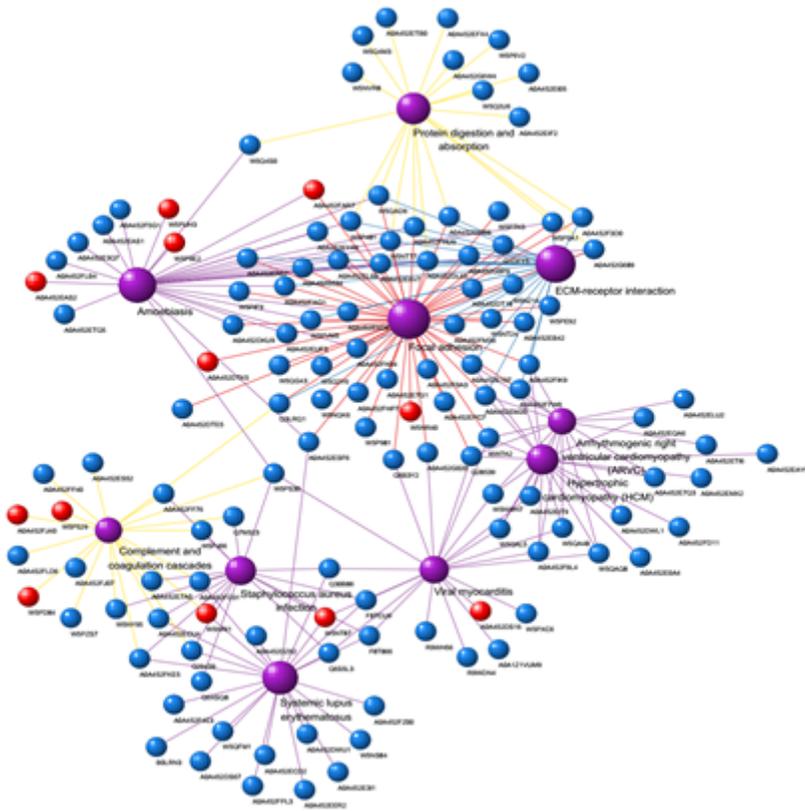
**Figure 1**

Comparison of epithelial morphology between milk replacer (MR) and solid feed (SS). The changes of (A) rumen epithelium morphology, (B) Papilla width, (C) Papilla length, (D) Muscle layers thickness, and (E) Epithelium thickness between MR and SS. Horizontal lines with a star symbol between Bar charts represent a significant difference based on one-way ANOVA analysis; \* $0.01 < P < 0.05$ ; \*\* $0.001 < P < 0.01$ ; \*\*\* $P < 0.001$ .



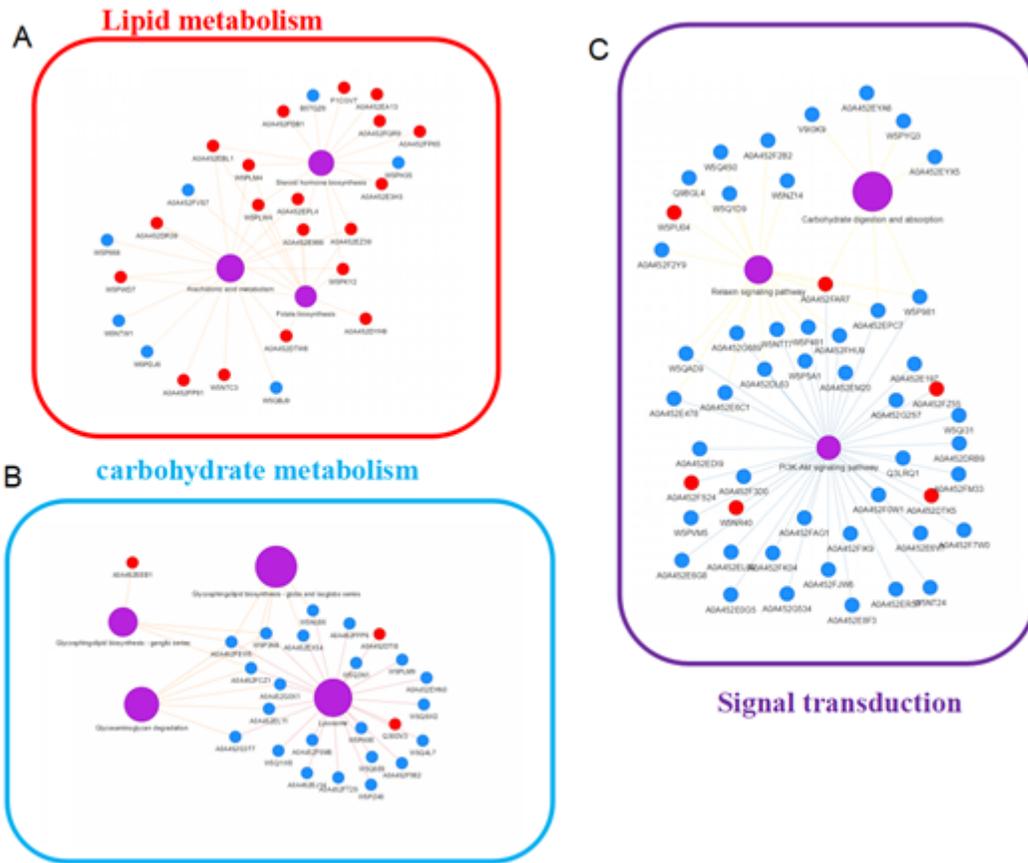
**Figure 2**

Gene ontology (GO) classification of differentially expressed proteins. The classification including (A) cellular component, (B) molecular function and (C) biological process.



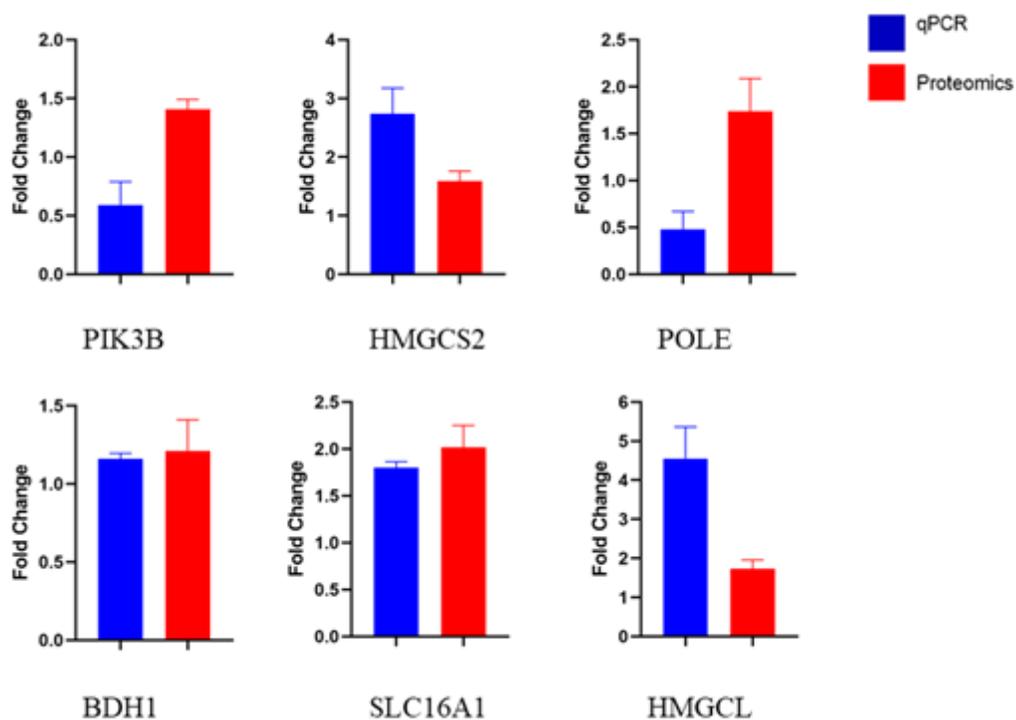
**Figure 3**

The top ten significantly enriched pathway relationship networks. Purple circles represent the different pathways, red and blue circle represent up-regulated and down-regulated proteins separately. Edges with different colors represent classes of KEGG pathway.



**Figure 4**

Relationship networks of metabolic pathways with significant enrichments. Purple circles represent the different pathways, red and blue circles represent up-regulated and down-regulated proteins separately. Edges with different colors represent classes of KEGG pathway. (A) lipid metabolism (B) carbohydrate metabolism (C) signal transduction.



**Figure 5**

The expression results of the six genes encoding DEPs. Fold change represents the ratio of relative expression of genes or proteins between SS and MR. An up-regulated expression means that the fold change was above 1 and vice versa.

## Supplementary Files

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